

STUDIES ON THE BIOSYNTHESIS OF AROMATIC COMPOUNDS
IN NEUROSPORA CRASSA

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ABSTRACT

Mutant strains of *Neurospora* have been isolated which exhibit nutritional requirements for aromatic compounds. Some of these mutants require a single nutrient in order to grow, for example, tryptophan, phenylalanine, or tyrosine. Others have been found to require all three of these amino acids, and in addition, *p*-aminobenzoic acid. It was desired to ascertain the number of gene changes from wild type which are responsible for the observed nutritional requirements. For this purpose, a number of the new strains were subjected to genetic analysis.

There was reason to believe that certain double and triple mutants would be found to accumulate compounds of interest which fail to accumulate in culture filtrates of strains containing only one altered gene. Therefore, these double and triple mutants were prepared by appropriate crosses, and were found to accumulate a number of compounds of biological interest. In addition, double and triple mutants have been of value in determining the order in which some of these genetic blocks occur in a biosynthetic sequence.

Culture filtrates of the mutant strains have been examined for the presence of accumulated materials. Evidence is presented that the following compounds occur in filtrates of various mutants: protocatechuic acid, shikimic acid, 5-dehydroshikimic acid, vanillic acid, *p*-hydroxyphenylacetic acid, phenylpyruvic acid, anthranilic acid, prephenic acid,

and N^α-acetylkynurenine.

Shikimic acid, although found to be accumulated in culture filtrates of one mutant, does not relieve the multiple requirement for aromatic compounds in other strains. This suggests that the biosynthesis of aromatic compounds in *Neurospora* may involve intermediates different from those elaborated by *E. coli*. In the latter organism, 5-dehydroshikimic acid has been identified by Davis and co-workers as an "early" precursor of aromatic compounds, whereas in *Neurospora*, it appears that 5-dehydroshikimic acid may be a "late" precursor, possibly the last compound that is a common precursor of all three aromatic amino acids.

It was felt that a method for the detection of vicinal diol compounds on paper chromatograms would aid the identification of such compounds as shikimic acid in culture filtrates of the mutants. Therefore, a method based on periodate oxidation was developed and found to be of use in the detection of various compounds of biological importance.

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I INTRODUCTION

- A. Summary of the classes of aromatic compounds found in nature.

Living organisms have been found to produce an immense variety of compounds containing the benzenoid ring. In stating the problem of how these rings are formed, it is perhaps appropriate to list those compounds or classes of compounds which have actually been detected in natural products. Unfortunately, a complete list would be prohibitively long. The compounds or groups of compounds enumerated below represent only those which seem to be of unusual interest.

1. tryptophan
2. phenylalanine
3. tyrosine
4. p-aminobenzoic acid
5. riboflavin
6. folic acid
7. vitamin B₁₂
8. vitamin K
9. indole acetic acid
10. serotonin
11. adrenalin
12. thyroxin

13. anthocyanins and related compounds
14. lignin
15. tannins
16. alkaloids
17. estrogenic hormones
18. chitin

Of these, only the first five have definitely been implicated in the metabolism of *Neurospora*. The scope of this thesis will be limited to a discussion of the biosynthesis and metabolism of tryptophan, phenylalanine, tyrosine, and p-aminobenzoic acid. With the exception of the latter four compounds, very little is known about the origin of the benzene ring.

- B. Discussion of the historical background and of the recent work of others in this field.

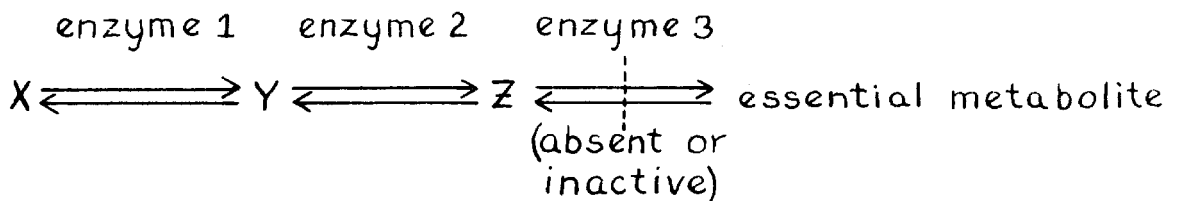
It has long been realized that mammals have only a very limited capacity to synthesize aromatic compounds de novo from non-aromatic sources. It is clear that the aromatic amino acids can be catabolized with the production of energy, that phenylalanine can be converted to tyrosine, that tyrosine can be transformed into important products such as adrenalin, thyroxin, and melanin, and that numerous other transformations can occur. These, however, do not constitute de novo synthesis. Some cyclic, non-aromatic com-

pounds such as quinic acid (1) and cyclohexane carboxylic acid (2) have been found to be aromatized by mammals and subsequently excreted as hippuric acid. A number of other cyclohexane and cyclohexene derivatives are likewise aromatized before excretion (3). These transformations appear to be detoxification mechanisms which are probably of rather slight metabolic importance to a normal animal. With the possible exception of the estrogenic hormones, there is perhaps no case in which mammals are able to form benzenoid rings from acyclic precursors. The inability to synthesize tryptophan, phenylalanine, riboflavin, and folic acid is reflected in a clear-cut dietary requirement for these compounds.

The above considerations dictate that aromatic biosynthesis be studied in organisms in which it is known to occur, i.e., higher plants, certain fungi, and bacteria.

The Use of Mutants

The work of Beadle and Tatum (4) showed that mutant strains of microorganisms may be obtained which lack the ability to carry out particular metabolic conversions, perhaps due to the absence or inactivity of certain enzymes. This situation is illustrated diagrammatically below:



Under these circumstances, the "essential metabolite" at the end of the reaction sequence becomes a nutritional requirement for the strain, and compound Z, which would probably escape detection in a normal organism, may accumulate in large amounts.

This idealized situation is often complicated by one or a number of the following considerations:

A. Compound Z may be highly unstable, may polymerize, or be otherwise non-enzymatically altered.

B. Compound Z may be at the "branch point" in the biosynthetic sequence of two or more essential metabolites, and may be drained off into production of the other metabolites.

C. The abnormally large concentration of Z may call adaptive detoxification mechanisms into play.

D. The equilibria of the reactions shown above may favor accumulation of Y, or even of X.

E. The unusual concentration of Z may inhibit other enzymes and thus induce requirements for essential metabolites not directly related to Z.

In spite of these complications, the use of mutant organisms has been of the greatest value in elucidating the biosynthesis of aromatic compounds.

A mutant strain of *Neurospora*, designated as C-86, was described by Gordon, Haskins, and Mitchell (5). This strain will grow if supplied with any one of the following com-

pounds: tryptophan, indole, anthranilic acid, kynurenine, nicotinic acid, phenylalanine, tyrosine, or quinic acid. Of these, all are aromatic except for the cyclic polyhydroxy compound, quinic acid.* It is to be noted that nicotinic acid contains a pyridine ring rather than a benzene ring. The fact that any one of the above compounds would promote growth of the strain suggested that quinic acid is a precursor of aromatic compounds in Neurospora, and also introduces the possibility that aromatic compounds may be interconvertible.

A strain showing markedly different nutritional requirements was isolated by Tatum (6). This mutant of Neurospora requires four aromatic compounds (tryptophan, phenylalanine, tyrosine, and p-aminobenzoic acid), in order to grow. It is unable to grow if furnished with only three of these. It was found that shikimic acid,* which differs from quinic acid only by the elements of water, would relieve all four of the requirements. Quinic acid was found to be inactive for growth of this strain. It should be mentioned that shikimic acid is inactive for growth of C-86. These findings, therefore, are in sharp contrast.

Davis (7), working with Escherichia coli, succeeded in isolating a large number of mutant strains which require various aromatic compounds. Of these, none was found to use quinic acid as a substitute for aromatic amino acids, and none was found to make alternative use of the three

*Structural formula is shown on p. 20.

aromatic amino acids. However, numerous strains were found to require all four of the compounds required by Tatum's *Neurospora* mutant, and, like the latter, to use shikimic acid as a substitute for these. In addition to the quadruple requirement mentioned above, a fifth factor is required for rapid growth of a number of strains. This material has been found to be p-hydroxybenzoic acid (8). When such strains are grown in medium of rather high pH (7.5), a requirement for a sixth growth factor may be demonstrated (9). This factor, which is present in culture filtrates of wild-type E. coli, has not as yet been identified. Both p-hydroxybenzoic acid and the "sixth factor" are readily replaced by shikimic acid. On the basis of these nutritional requirements, it was suggested by Davis (10) that shikimic acid is a normal precursor of these six nutrients.

A slight complication has been introduced by the finding of strains which require only:

- A. phenylalanine + tyrosine
- B. tryptophan + phenylalanine + tyrosine
- C. tryptophan + phenylalanine + tyrosine + p-aminobenzoic acid.

Two explanations of this phenomenon may be given.

(1) The differences in nutritional requirements might reflect a series of mutant genes. It would then be predicted that p-hydroxybenzoic acid branches off the main biosynthetic sequence first, and that mutants of class C are

blocked just distal to this particular branch point. Similarly, p-aminobenzoic acid, and then tryptophan could be expected to branch off the main sequence, explaining the existence of classes B and A, respectively.

(2) The differences in nutritional requirements could be due to different degrees of completeness of the genetic block. Strains which are completely unable to synthesize aromatic compounds would then be expected to require all six compounds. Those in which a small amount of the common precursor of these six compounds can be formed might use it preferentially as a substitute for p-hydroxybenzoic acid, causing these strains to be classified in C of the previous page. Those in which a somewhat larger amount of the common precursor is formed are pictured as being able to satisfy the above requirement plus the requirement for p-aminobenzoic acid, thus explaining the existence of Class B. Strains which are able to synthesize a considerable amount of the common precursor, but not enough to satisfy their requirements for phenylalanine and tyrosine, fall into Class A. Thus it is conceivable that strains which are allelic with respect to the metabolic lesion might nevertheless show qualitatively different nutritional requirements due to a quantitative difference in the phenotypic expression of the alleles.

The latter explanation is the one favored by Davis (9), and a variety of evidence for its correctness has been

obtained.

Another anomaly is the inability of some strains to utilize shikimic acid as a substitute for phenylalanine and tyrosine. It has been found (10) that the conversion of shikimic acid into these two metabolites is antagonized by the presence of a precursor of shikimic acid, 5-dehydroshikimic acid.

Evidence for the presence of this precursor of shikimic acid was obtained by Davis in 1951 (7). Culture filtrates of a number of mutants were subjected to chromatography, and the chromatograms inspected by bioassay with a strain which was known to utilize shikimic acid for growth. Some of these filtrates were thus shown to contain two biologically active compounds. One of these corresponded to shikimic acid, and the other did not correspond to any known compound. Other filtrates were seen to contain only the unknown compound. This compound was isolated and identified as 5-dehydroshikimic acid (11). It is highly unstable in alkaline solution, but is stable in weakly acidic solution. Treatment with strong mineral acid and heat converts 5-dehydroshikimic acid into protocatechuic acid (3,4 dihydroxybenzoic acid), which is at the same oxidation level. The latter compound does not possess activity for growth of any known strain. An enzyme capable of converting 5-dehydroshikimic acid to shikimic acid has been found by Yaniv and Gilvarg (12, 13). This enzyme has been purified about tenfold, and has been found to be

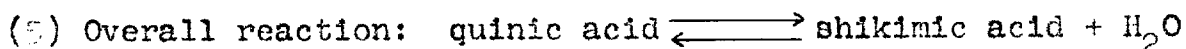
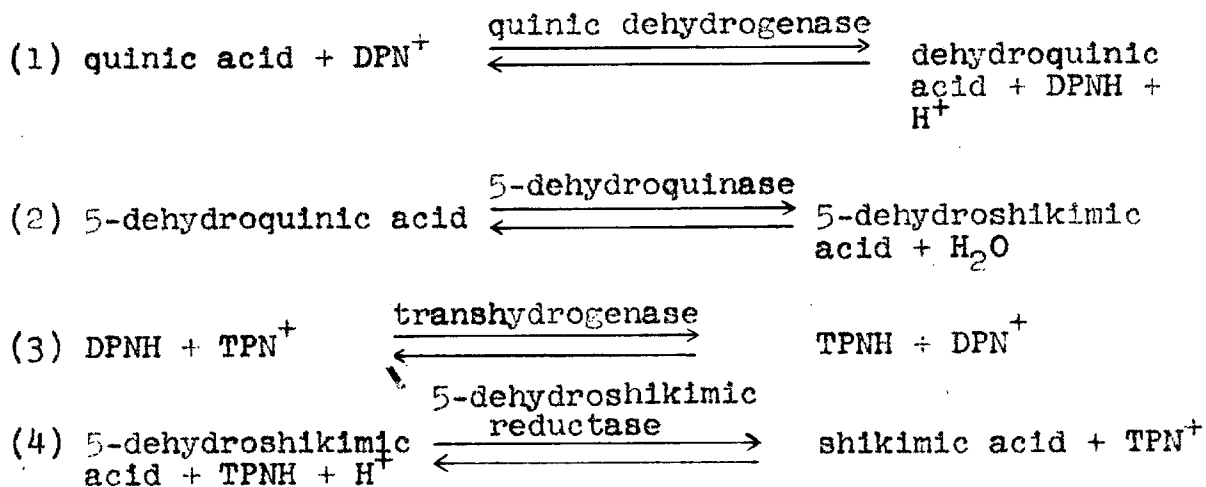
specific for TPN as sole cofactor for the reduction of 5-dehydroshikimic acid. The reaction has been shown to be reversible by coupling oxidation of shikimic acid with reduction of oxidized glutathione.

It seemed logical that strains which respond to both shikimic acid and 5-dehydroshikimic acid might accumulate precursors of the latter compound. When such strains were tested for crossfeeding, it became evident that some strains accumulated materials which fed other strains weakly. This crossfeeding behavior, however, was so slight as to be of only limited value for purposes of bioassay. A large number of cells of a strain showing slight response to the accumulated precursor were grown in its presence, and a secondary mutant was obtained which responded well to the unknown material. This strain was then used as an assay organism during the course of purification of the precursor of 5-dehydroshikimic acid. The material was isolated and characterized as 5-dehydroquinic acid (14). It is extremely labile in basic solution. When heated in acid solution, it is partly dehydrated to 5-dehydroshikimic acid. Catalytic reduction has been shown to yield quinic acid. Mitsuhashi and Davis (15) have studied an enzyme found in wild-type E. coli which is capable of dehydrating 5-dehydroquinic acid to 5-dehydroshikimic acid. This enzyme has been purified about tenfold. It has been shown to be incapable of dehydrating quinic acid to shikimic acid, and is likewise unable to

dehydrate malic acid, citric acid, or isocitric acid. No cofactor requirement has been demonstrated. The enzyme has been named 5-dehydroquinase, and has been detected in *Aerobacter*, in an alga, in green plants, and in yeast. It could not be detected in guinea pig liver. It can be seen that the distribution of this enzyme is correlated with the ability to synthesize aromatic compounds.

Certain mutants of *Aerobacter aerogenes* have been found by Davis and Weiss (16) to respond to quinic acid. These mutants, however, differ in their metabolism from the quinic acid mutant of *Neurospora* (see p.4). Unlike the *Neurospora* mutant, they are reminiscent of the mutants of *E. coli*, in that they require a plurality of aromatic compounds in the absence of the appropriate hydroaromatic compounds. In addition, they respond to shikimic acid, to 5-dehydroshikimic acid, and to 5-dehydroquinic acid. The *Neurospora* mutant does not respond to shikimic acid. No mutants of *E. coli* have been found which will utilize quinic acid or accumulate it. Davis and co-workers have also searched in vain for a mutant of *Aerobacter aerogenes* which will utilize 5-dehydroquinic acid but not quinic acid. For these reasons, Davis (13) has suggested that quinic acid is not an obligatory intermediate in aromatic biosynthesis, but that *A. aerogenes* has an "adventitious enzyme," quinic dehydrogenase, which can introduce quinic acid into the pathway. This enzyme has been studied by Mitsuhashi and Davis (17) and found to be

specific for DPN as the carrier of hydrogen. The dehydrogenation of quinic acid may be coupled with the reduction of 5-dehydroshikimic acid to give the following series of reactions:



Quinic dehydrogenase has thus far been found only in A. aerogenes. It could not be detected in a number of other organisms which are known to synthesize aromatic compounds. This is considered added evidence that quinic acid is not an obligatory intermediate in biosynthesis of the benzenoid ring.

In an attempt to detect precursors of 5-dehydroquinic acid, the earliest known compound in this sequence, Davis and co-workers have looked for crossfeeding behavior between mutants known to be blocked before 5-dehydroquinic acid. No such behavior has been found. This could be due to allelism of all such mutants, to one or more of the complications

enumerated on p. 4 of this thesis, or to inability of accumulated intermediates to penetrate the cell membrane of assay strains. In an attempt to resolve this question, Kalan (13) prepared extracts of a mutant which accumulates 5-dehydroshikimic acid. These extracts were incubated with a culture filtrate of a mutant blocked before 5-dehydroquinic acid. Considerable amounts of 5-dehydroshikimic acid were formed, and this has been identified by bioassay with the appropriate strains and by chromatography. It is clear, then, that materials are accumulated by mutants blocked before 5-dehydroquinic acid, and that these materials, though not detectable directly by bioassay, may be enzymatically converted to known precursors of aromatic compounds. The compound immediately preceding 5-dehydroquinic acid in the sequence has been designated as V.

The success of this enzymatic experiment introduced the possibility that these extracts might effect the conversion of carbohydrates to 5-dehydroshikimic acid. Kalan and Sreenivasan (18) incubated such extracts with various substrates and examined the incubation mixtures for the presence of 5-dehydroshikimic acid. The following substrates were tried: glucose, glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, hexosediphosphate, ribose-5-phosphate, 6-phosphogluconic acid, sedoheptulose-7-phosphate, and sedoheptulose-1,7-diphosphate. All except 6-phosphogluconic acid gave rise to measurable amounts of 5-dehydroshikimic

acid, but sedoheptulose-1,7-diphosphate was by far the most efficient of these. In the presence of added DPN, this sugar has been converted into 5-dehydroshikimic acid by crude extracts in a yield of as high as 60%.

Sedoheptulose is an acyclic, straight chain sugar containing seven carbon atoms. Dehydroshikimic acid is a cyclic, seven-carbon acid. It is tempting to assume that sedoheptulose-1,7-diphosphate undergoes direct cyclization, followed by a series of oxidations and dehydrations to dehydroquinic acid and thence to dehydroshikimic acid. Ironically, evidence has been obtained (19) which indicates that differentially labelled sedoheptulose-1,7-diphosphate is incorporated into the ring of 5-dehydroshikimic acid only after undergoing a cleavage between carbon atoms 3 and 4, and that the fragments are recombined and the order of the carbon atoms changed before cyclization occurs.

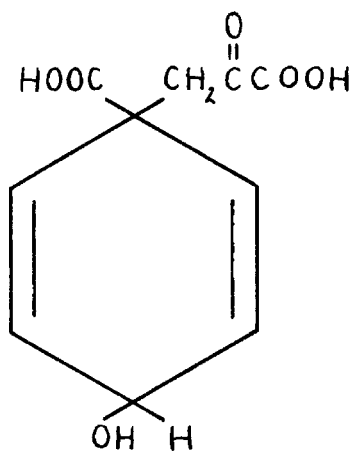
The investigations of Davis and co-workers have shed considerable light on the process by which shikimic acid is converted into aromatic compounds. It was found that a number of mutants which accumulate shikimic acid also accumulate a substance designated as Z_1 . This material is in itself inactive for the growth of all strains tested, but on heating with acid, it is converted to an active material which has been identified as shikimic acid (20). Chromatographic evidence indicates that Z_1 is considerably less polar than shikimic acid. Since a number of mutants have

been shown to accumulate large quantities of Z_1 and only traces of shikimic acid, it has been suggested (13) that Z_1 is the compound following shikimic acid in the biosynthetic pathway.

A second compound which yields shikimic acid on acid hydrolysis has been detected in the culture filtrates of all mutants that accumulate shikimic acid, and is pictured as being a side product rather than a true intermediate (13). This compound has been identified as 5-phosphoshikimic acid.

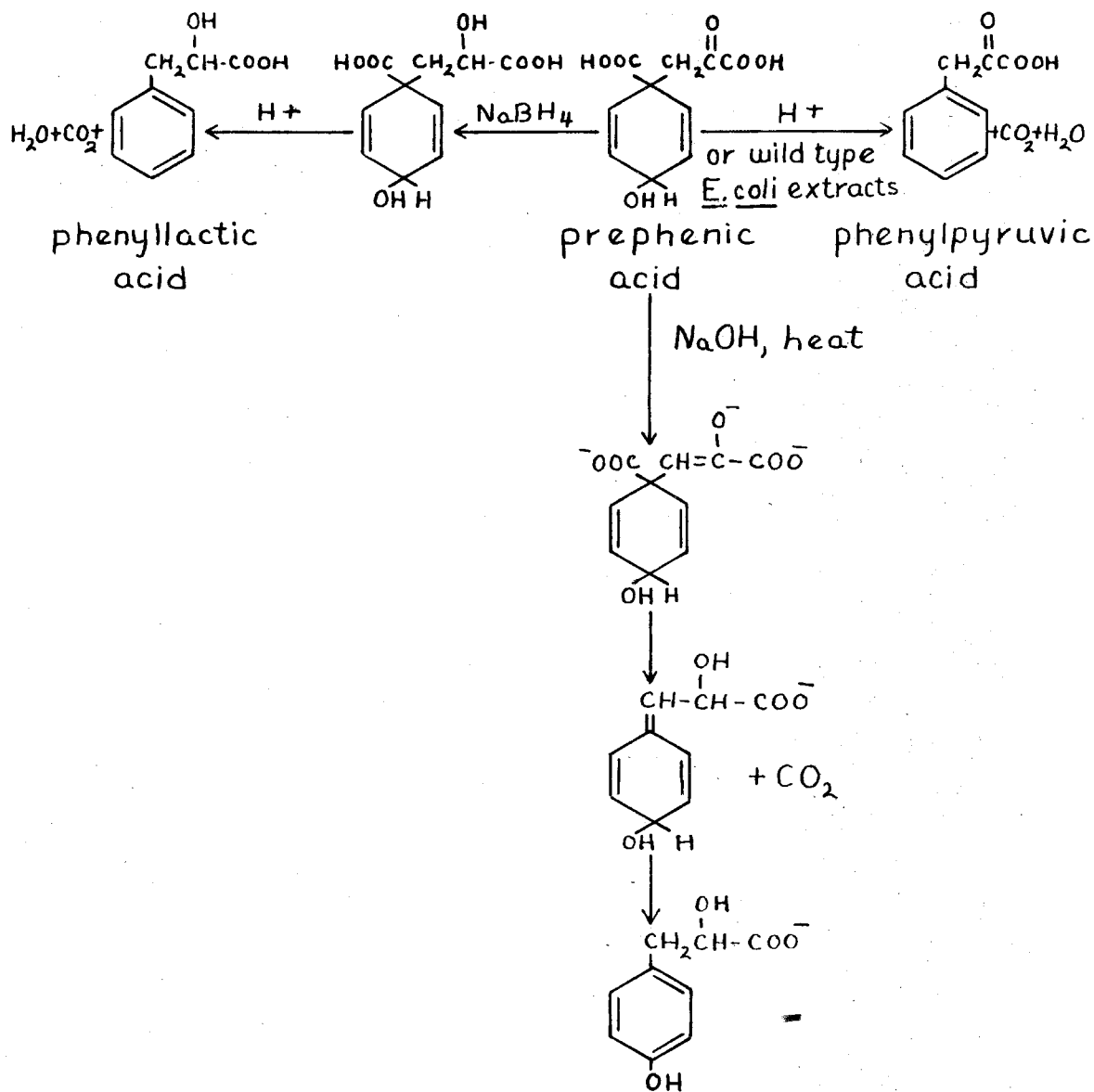
Another compound which is in itself not utilized for growth of mutants has turned out to be of the utmost interest. This compound is accumulated by a number of mutants which require phenylalanine or tyrosine. It was observed by Katagiri and Sato (21) and by Davis (22) that these mutants accumulate a substance which can be converted by mild acidity or by autoclaving into a substance which will replace the phenylalanine requirement of the mutant which accumulates the material. The derived substance which replaces phenylalanine was found to be phenylpyruvic acid (22). Weiss et al. (23) found that an enzyme present in wild type E. coli would effect the conversion at room temperature and at neutral pH, but that this enzyme is absent in extracts of mutants which accumulate the inactive material. Therefore it has been postulated that the inactivity is due to a permeability barrier, and that this compound is a true intermediate in the biosynthesis of phenylalanine. The compound has been isolated

by Weiss et al. (23) as the barium salt. Elementary analysis suggests the empirical formula $C_{10}H_{10}O_7Ba$. In the presence of acids, the material yields an equivalent of phenylpyruvic acid and one equivalent of CO_2 . This transformation takes place in the absence of oxygen or other electron acceptors; therefore it is evident that the material is at the same oxidation level as phenylpyruvic acid. The compound takes up between three and four molar equivalents of hydrogen in the presence of platinum, indicating the absence of an aromatic structure. The infrared spectrum is also incompatible with the notion that the compound contains an aromatic ring. The substance shows strong end-absorption in the ultraviolet. On reduction with sodium borohydride, which acts on keto groups but not on double bonds, this end absorption is decreased to a negligible value. This suggests that the end absorption is due to the keto group rather than to a system of conjugated double bonds. The product of reduction is unstable to acid, as is the parent material. The product of acid decarboxylation of the borohydride-reduced material has been identified as phenyllactic acid. Heating the native material with strong alkali converts it quantitatively into *p*-hydroxyphenyllactic acid (24). On the basis of these observations, the following structure of the intermediate has been suggested, and the material has been named "pre-phenic acid."



prephenic acid

The various conversions discussed above are pictured as follows:



p-hydroxyphenylacetate

It is evident that the structure suggested for prephenic acid differs from that of the empirical formula by the elements of water. There is some evidence that this discrepancy is due to the presence of a molecule of water of crystallization in the barium salt.

In the opinion of this author, the aspects of the proposed structure which are least certain are

- (1) the position of the hydroxyl group, and
- (2) the attachment of the carboxyl group and the pyruvyl side chain to the same carbon atom of the ring.

Evidence for the placement of the hydroxyl group as shown rests on the absence of optical activity, and the base catalyzed conversion to p-hydroxyphenyllactic acid. The latter criterion may be questioned, as it has been shown by Bamberger (25) that a number of compounds of this general structure undergo a great multiplicity of different rearrangements, often involving shifts of sidechains to new positions on the ring. The work of Schepartz and Gurin (26) has indicated that a similar reaction occurs in the catabolism of tyrosine. Evidence for the attachment of the labile carboxyl group and the pyruvyl sidechain to the same carbon atom rests on labelling data which, in the opinion of the present author, are rather unclear and are subject to a number of interpretations.

It can be seen from the structure proposed for pre-

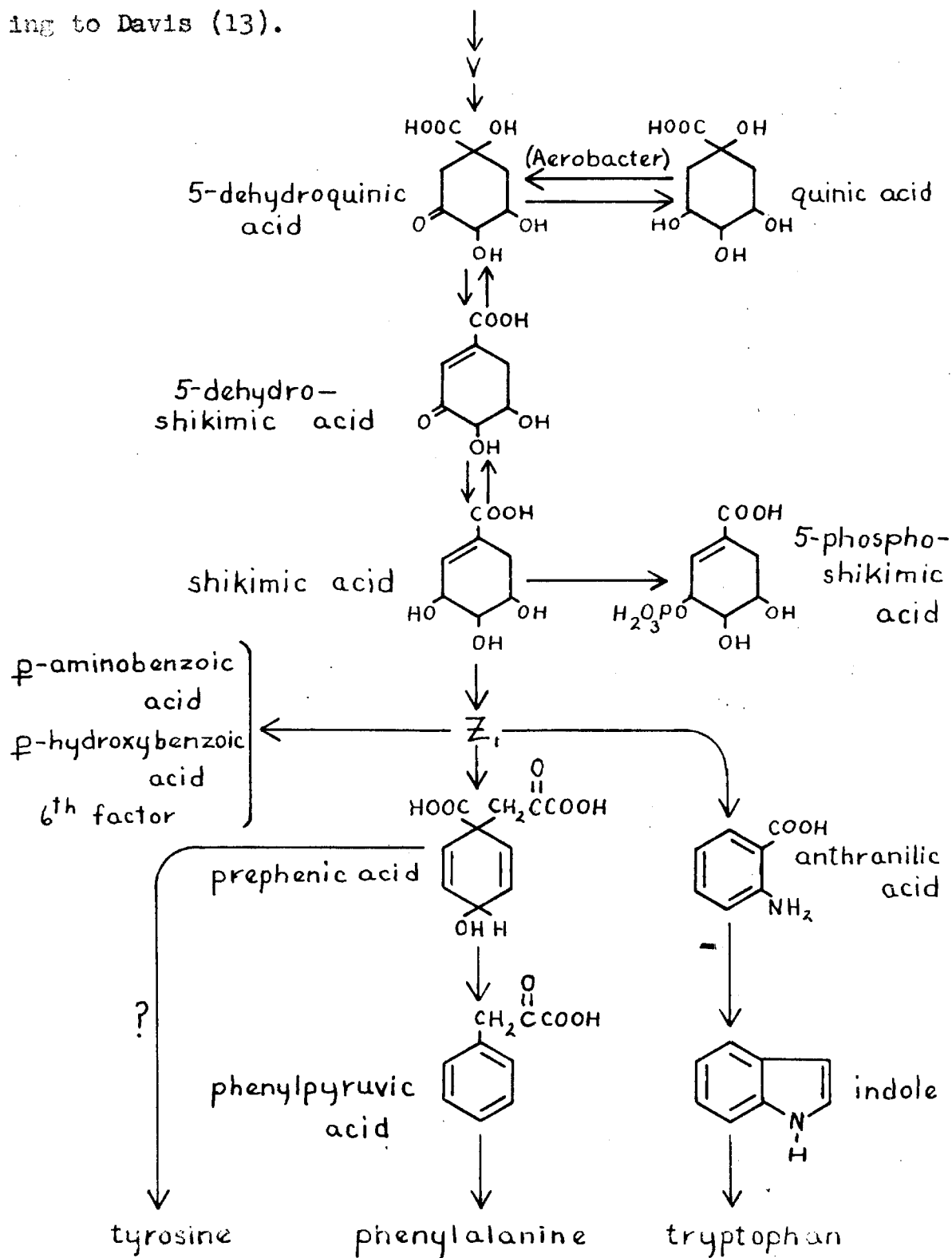
phenic acid that it is a reasonable precursor of tyrosine as well as of phenylalanine. It is also to be noted that some tyrosine-requiring strains accumulate this compound. As yet, no enzymes have been found to convert prephenic acid into tyrosine-replacing materials, but Davis has reported that work along these lines is in progress.

The use of mutants has also been of value in studying the anabolism and catabolism of tryptophan. The investigations of Tatum, Bonner, and Beadle (27), Haskins and Mitchell (28), and others have given evidence that anthranilic acid is converted, via indole, into tryptophan. The organism used in most of these investigations was *Neurospora*. The compounds mentioned as precursors of tryptophan have since been found by Davis (7) to replace tryptophan in tryptophan-requiring mutants of *E. coli*.

The conversion of tryptophan to nicotinic acid has also been studied in *Neurospora*. The work of Beadle, Mitchell, and Nyc (29), Bonner (30), and others has established kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and possibly quinolinic acid as intermediates in this conversion. The work of Nyc et al. (31) has indicated that the carboxyl group of anthranilic acid is lost in the process of conversion to tryptophan and nicotinic acid.

The biosynthesis of tryptophan and of nicotinic acid has been thoroughly discussed in the Doctoral thesis of Haskins (32) and in a review of Dalglish (33).

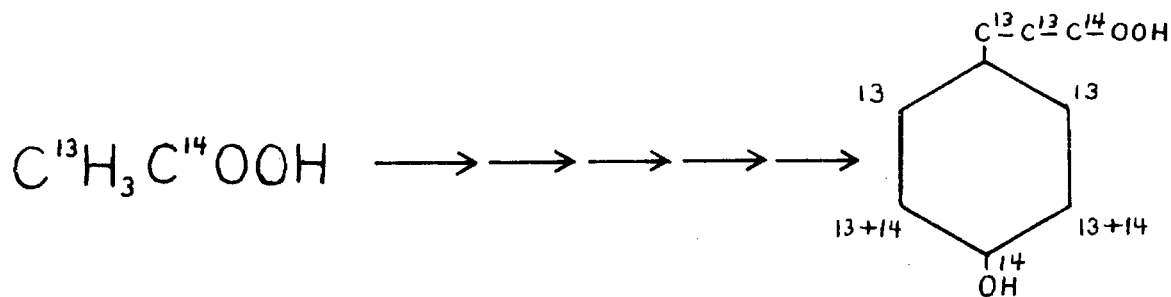
It seems appropriate to append a diagram showing the pathway by which aromatic compounds are synthesized according to Davis (13).



The Use of Isotopic Tracers

Studies by several groups of workers have resulted in a considerable body of isotopic data concerning origin of the carbon atoms of the aromatic amino acids. These data, while not in themselves indicative of a pathway of biosynthesis of aromatic amino acids, do exclude certain possibilities from consideration, and must be brought into harmony with any complete theory of the origin of aromatic compounds in nature.

Baddiley et al. (34) have investigated the origin of tyrosine in the yeast Torulopsis utilis. The strain used was adapted to acetate as the sole carbon source. The acetate was labelled with C¹⁴ in the carboxyl carbon and C¹³ in the methyl carbon. After growth of the yeast on this differentially labelled carbon source, the proteins were hydrolyzed and tyrosine isolated. By use of very elegant degradative procedures, the contribution of the two carbon atoms of acetate to each of the carbon atoms of tyrosine was determined. The isotopic distribution is essentially as pictured below.

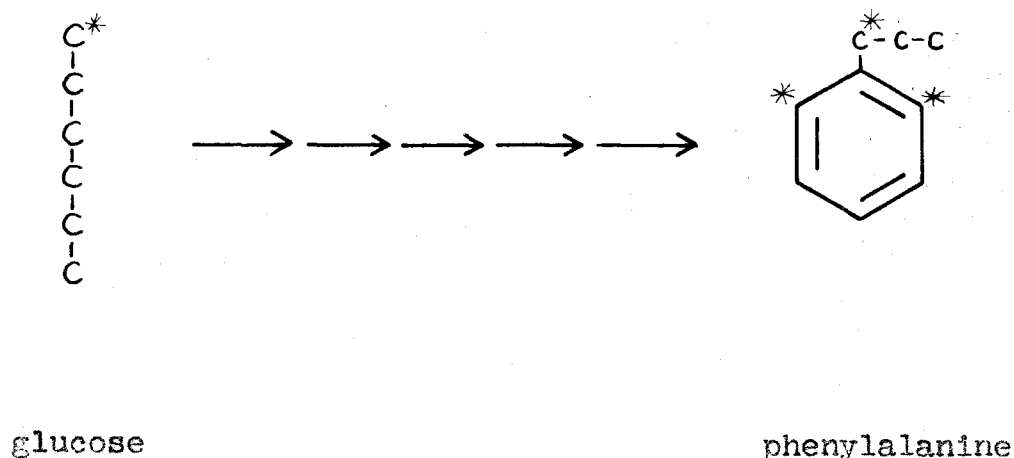


It is to be noted that carbon atoms 3 and 5 of the ring are of mixed origin. Due to the symmetry of the ring, these two carbon atoms are necessarily isolated together and are indistinguishable. Therefore, it is quite possible that one of these indistinguishable carbon atoms is derived entirely from the carboxyl group of acetate, and the other from the methyl group of acetate.

On the basis of these findings, some suggestions of possible mechanisms for formation of tyrosine were advanced. These mechanisms left some of the data unexplained, and required the postulation of a number of rather improbable reactions. Gilvarg and Bloch (35) have suggested a reinterpretation of the data of Baddiley et al. This alternate interpretation, which will be discussed below, has met with the approval of the latter authors.

Investigations of Gilvarg and Bloch (35) on the synthesis of phenylalanine and tyrosine in Saccharomyces cerevisiae have permitted a number of conclusions to be drawn. It was found that when this yeast was grown on a medium containing both acetate and glucose, the aromatic amino acids were derived almost entirely from glucose. It is clear that this phenomenon is not due to failure of the organism to utilize acetate, as the fatty acids and a number of acyclic amino acids were found to derive a substantial portion of their carbon from added acetate. Glucose labelled with C^{14} in the 1 position was administered to the yeast. The phenylalanine

and tyrosine were isolated and degraded to give data on the individual carbon atoms. The distribution of radioactivity was essentially as shown below (36).



Here it must be noted that carbon atoms 2 and 6 of the ring are indistinguishable, and therefore it is possible that almost all of the C^{14} is at one of these two positions. As the 1, 3, 4, and 5 positions of the ring were found to contain little radioactivity, it seems clear that the ring is not formed by condensation of equilibrated two-carbon units.

If, on the other hand, the ring were formed by condensation of two equilibrated triose units, the labelled carbon atoms would be found either ortho or para to one another. The observed labelling excludes this possibility. Early work of

Gilvarg and Bloch (35) suggested the possibility that hexose is cyclized directly to form the benzene rings of phenylalanine and tyrosine, and that the sidechain is derived from triose. This involves the assumption that the radioactivity is asymmetrically distributed between carbon atoms 2 and 6 of the ring, and appears to be present in both positions only because of the symmetry of the molecule. The hypothesis of direct cyclization of a hexose unit is in good agreement with the data of Baddiley et al. (34), if it is assumed that acetate is converted, via the tricarboxylic acid cycle, into pyruvate, and the latter is transformed to hexose by a reversal of glycolysis. A more recent publication by Gilvarg and Bloch (36) has emphasized that the mechanism of direct cyclization of hexose fits the observed labelling of the ring qualitatively but not quantitatively. In all cases, the specific activities of the rings of phenylalanine and tyrosine were found to be significantly lower than that of the glucose administered to the yeast. Therefore, it is inferred that one or more of the unlabelled carbons of the administered glucose makes a disproportionately large contribution in the synthesis of the ring.

The biosynthesis of tyrosine in yeast has been further investigated by Thomas et al. (37). These investigators administered pyruvate labelled with C^{14} in the carbonyl carbon, isolated the tyrosine, and subjected it to the usual degradations. In agreement with previous work, it was con-

cluded that the sidechain is derived from an intact three-carbon fragment. The ring was found to be labelled predominantly in carbon atoms 1, 3, and 5. These data exclude direct cyclization of hexose formed by reversal of glycolysis. Thomas et al. have suggested that condensation of two asymmetric four-carbon fragments could give the observed distribution of radioactivity in the carbons of the ring. These four-carbon fragments are pictured as arising from pyruvate by a $C_3 - C_1$ condensation.

Ory and Lyman (38) have studied a strain of Lactobacillus arabinosus which is able to synthesize tyrosine and phenylalanine if pyridoxine is included in the medium and CO_2 is present in the gas phase over the medium. In the absence of CO_2 , these two amino acids are required for growth of the organism. Experiments in which $C^{13}O_2$ was included in the gas phase have demonstrated that the latter does not contribute to the carbon skeleton of tyrosine. The authors have suggested that possibly CO_2 is the origin of that carboxyl group of prephenic acid which is lost during the aromatization step. It was also reported that shikimic acid will replace phenylalanine and tyrosine for growth of this organism in the absence of CO_2 , an observation which is consistent with the above interpretation.

The origin of shikimic acid from differentially labelled glucose has been investigated by Shigeura et al. (39). The method consisted of administering radioactive glucose to a

mutant strain of E. coli which accumulates shikimic acid. The shikimic acid was isolated and degraded in order to determine the distribution of radioactivity in the various carbon atoms. It has since been found by these authors that the procedures for degradation of shikimic acid were unsatisfactory and that the radioactivity of individual carbon atoms could not be accurately estimated. It has been reported in a personal communication that these authors have since developed a highly satisfactory method of degrading shikimic acid, and that data concerning the origin of its various carbon atoms from glucose are forthcoming.

Isotopic tracers have also been used by two groups of workers to obtain evidence of the conversion of shikimic acid to aromatic amino acids. Shigeura and Sprinson (40) administered radioactive shikimic acid to a culture of growing wild-type E. coli and found that 18% of the tyrosine formed was derived from added shikimic acid. Tatum et al. (41) grew a shikimic acid-utilizing mutant of *Neurospora* on a medium containing a radioactive carbon source and unlabelled shikimic acid. Tyrosine was isolated and its specific activity was found to be significantly lowered by the added shikimic acid. Non-aromatic amino acids were found to have the expected high specific activity. These data, then, add to the evidence that shikimic acid can be converted by microorganisms to aromatic amino acids.

The Use of Metabolic Inhibitors

It has been found by Beerstecher and Shive (42) that growth of Escherichia coli is inhibited by β -thienylalanine, and that this inhibition is relieved by tyrosine. Another metabolic inhibitor, β -phenylserine, was observed to act in a different manner. Inhibition by this latter compound was found to be relieved by phenylalanine, but not by tyrosine. Phenylpyruvic acid was completely ineffective in relieving inhibition by either compound. Beerstecher and Shive concluded that phenylalanine is converted to tyrosine by direct oxidation, and that the conversion is inhibited by β -thienylalanine. It was postulated that β -phenylserine inhibits conversion of a precursor of phenylalanine to phenylalanine. This is termed the "straight pathway" of synthesis of aromatic compounds, as opposed to the "branched pathway" advanced by Davis and co-workers.

Bergmann et al. (43,44) have studied inhibition of growth of E. coli by a considerable number of substituted phenylalanines. These workers have interpreted their findings as favoring the "straight pathway," and have suggested that tryptophan is converted into phenylalanine, which, in turn, is converted into tyrosine.

It is felt by this writer that the data obtained from inhibition analysis are subject to a number of interpretations, and that the evidence in favor of the "branched pathway" is of a more convincing sort.

II EARLY EXPERIMENTAL APPROACHES USED IN THIS INVESTIGATION

The Possibility that Seven-carbon Sugars are Related to the Biosynthesis of Aromatic Compounds

Wild-type Neurospora crassa is able to grow on a chemically defined medium containing sucrose as the carbon source. It is clear that the proteins of Neurospora contain aromatic amino acids. Obviously, then, sucrose can serve as a "precursor" of the aromatic amino acids, as well as "precursor" of all other organic cellular constituents (other than biotin).

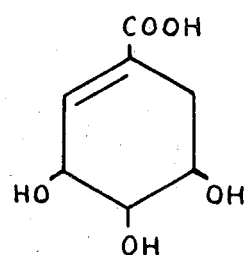
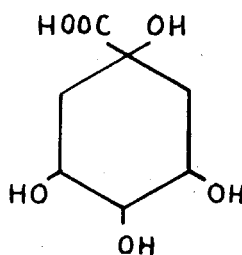
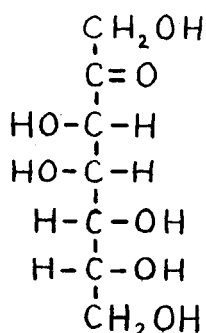
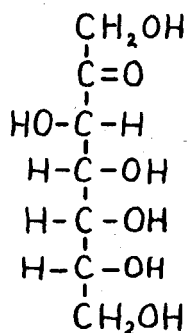
It seems reasonable, then, that early precursors of aromatic compounds might be acyclic polyhydric compounds closely related structurally to the carbon source. Somewhat later in the biosynthetic sequence, a cyclic polyhydric compound might be expected. Elimination of water would then result in the formation of a benzenoid ring.

Evidence for the correctness of these general predictions was furnished by the work of Gordon, Haskins, and Mitchell (5); these workers found that the aromatic requirement of C-86, a mutant strain of Neurospora, could be relieved by a seven-carbon polyhydric acid known as quinic acid. In addition, Davis (7) found that some mutants strains of Escherichia coli would use the seven-carbon polyhydric compound shikimic acid

for this purpose. (See section on Historical Background). On the basis of these observations, it was suggested by Dr. H. K. Mitchell that the naturally-occurring seven-carbon sugars might serve as acyclic precursors for the cyclic compounds quinic acid and shikimic acid, and thus for the aromatic amino acids.

Two seven-carbon sugars are known to occur in nature. These are sedoheptulose, which is found in quantity in the leaves of the genus *Sedum* (45), and mannoheptulose, which occurs in the leaves, fruit, and seeds of avocados (46).

Structural formulas of these sugars and of quinic acid and shikimic acid are given below:



D-althroheptulose
(sedoheptulose)

D-mannoheptulose

quinic acid

shikimic acid

A number of species of the genus *Sedum* were obtained through the courtesy of the Curator of the Huntington Library Botanical Gardens. Sedoheptulose was detected in juice of the leaves by paper chromatography as described on p. 64 . *S. rubritinctum* was found to be a particularly rich source. The juice of the leaves of this species (1.0 ml./flask) was introduced into 125 ml. Erlenmeyer flasks containing 20 ml. of Fries minimal medium. Flasks of this supplemented medium were inoculated with wild-type 5297a and with C-86A. After a week's growth, the media were subjected to chromatographic analysis. It was noted that in both cases, the sucrose had been completely metabolized, but sedoheptulose appeared to be undiminished in the medium.

It was desired to try similar tests with mannoheptulose. Unfortunately, this sugar is somewhat less abundant in avocados than is sedoheptulose in *Sedum*. Attempts to make a purified preparation were not successful.

These investigations were carried out very early in the training of the author; the difficulties encountered in the isolation of rare sugars from natural materials, and the failure of sedoheptulose to be metabolized by wild type *Neurospora* discouraged further work on this project.

Since this work, the phosphate esters of sedoheptulose have become known (47), and Kalan et al. (18) have found evidence that sedoheptulose-1,7-diphosphate can be converted by extracts of *E. coli* into compounds known to be precursors of

aromatic compounds. For discussion of this, see Introduction, p. 12 .

It seems quite possible that the same pathway might exist in *Neurospora*, and that the failure of free sedoheptulose to be metabolized could be due to the absence of the requisite kinases for converting it to the phosphorylated sugar.

The Possibility of Finding Metabolic Inhibitors Which will Cause the Accumulation of Precursors to Aromatic Compounds

In a number of instances, metabolic inhibitors, or "anti-metabolites" have proved to be of great value in elucidating the steps in a biosynthetic pathway. A few examples are the use of malonate to cause accumulation of succinate in respiring systems (48), the use of fluoroacetate or fluoro-citrate to cause accumulation of citrate (49), and the use of sulfonamides to cause accumulation of purine precursors (50). It seemed reasonable to try an analogous approach to the problem of synthesis of aromatic compounds in *Neurospora*.

Seventeen aromatic compounds which were immediately available to us were tested for ability to inhibit growth of wild-type 5297a. In the case of each compound, a variety of concentrations was employed. The basal growth medium used was Fries minimal. Growth was allowed to proceed for 96 hours at 25^o. At the end of this time, the mycelial pads were dried and weighed. Those media in which 50% inhibition of growth was found to occur were saved.

Of the compounds tested, chloramphenicol, β -phenylserine, *p*-hydroxyphenylacetic acid, α -acetamidocinnamic acid, α -phenylglycine, and mandelic acid did not inhibit detectably even at concentrations of 1000 $\mu\text{g./ml.}$ Hydrocaffeic acid did not inhibit growth at the highest concentration employed (500 $\mu\text{g./ml.}$). *p*-Hydroxybenzaldehyde appeared to inhibit very slightly at this concentration.

The remaining compounds were found to inhibit growth at various concentrations. The concentrations required to give 50% inhibition of growth are shown below.

<u>Compound tested</u>	<u>Concentration for 50% inhibition of growth</u>
<u>o</u> -aminocinnamic acid hydrochloride	350 $\mu\text{g./ml.}$
benzoic acid	200 $\mu\text{g./ml.}$
<u>o</u> -nitrocinnamic acid	100 $\mu\text{g./ml.}$
<u>trans</u> -cinnamic acid	100 $\mu\text{g./ml.}$
N-methyl anthranilic acid	100 $\mu\text{g./ml.}$
<u>o</u> -coumaric acid	50 $\mu\text{g./ml.}$
<u>o</u> -fluorophenylalanine	2.5 $\mu\text{g./ml.}$
<u>p</u> -fluorophenylalanine	about 1.5 $\mu\text{g./ml.}$
<u>m</u> -fluorophenylalanine	0.5 $\mu\text{g./ml.}$

Media in which wild type had been allowed to grow under conditions of 50% inhibition were tested for accumulation of aromatic precursors as described on p. 60 . The assay strain used was C-86A, which is known to respond to a wide variety of aromatic compounds of biological interest. Medium to be tested (2.0 ml.) was introduced into 18 ml. of minimal medium, thereby causing a tenfold dilution of both the inhibitor and of any metabolites which might be present. o-Aminocinnamic acid hydrochloride, benzoic acid, o-nitrocinnamic acid, trans-cinnamic acid, o-coumaric acid, o-fluorophenylalanine, p-fluorophenylalanine and m-fluorophenylalanine were not found to cause accumulation of any materials having biological activity for C-86A. However, medium from wild type which had been inhibited by N-methyl anthranilic acid was found to have appreciable growth promoting properties for the assay strain.

It seemed possible that N-methyl anthranilic acid might in itself be utilized by the assay strain at moderate concentrations, though inhibitory at higher concentrations. Accordingly C-86A was tested at various concentrations of this compound, and was indeed found to exhibit this type of behavior. The concentration of N-methyl anthranilic acid which gave best growth was 50 $\mu\text{g./ml.}$, or about half the concentration required to give 50% inhibition of wild type.

As this compound was a synthetic product, it seemed possible that it could be contaminated with anthranilic acid, which is known to promote growth of C-86A. Both compounds

are highly fluorescent, and it was possible to detect extremely small amounts of impurities on paper chromatograms. A solvent containing three parts *t*-butanol and one part 1 M NH_4OH was found to separate anthranilic acid and N-methyl anthranilic acid (R_F values were 0.55 and 0.80 respectively). No impurity of anthranilic acid nor of any other fluorescent compound could be detected in the sample of N-methyl anthranilic acid. Therefore it is concluded that N-methyl anthranilic acid will support the growth of C-86A. Later it was found to relieve the tryptophan requirement of a mutant which requires four aromatic compounds (See p. 96).

The observation that chloramphenicol and β -phenylserine do not inhibit the growth of *Neurospora* is surprising, in that they have been reported to be strongly inhibitory for a number of other organisms (42, 51). This anomaly could be due to important differences in internal metabolism, or could be due to failure of the compounds to penetrate to the site of action.

In summary, no inhibitor was found to cause active accumulation of compounds with biological activity for growth of C-86A, although the activity of N-methyl anthranilic acid was observed by this most circuitous route.

III ISOLATION OF MUTANT STRAINS OF NEUROSPORA CRASSA

As has been observed in the Introduction of this thesis, mutant strains of microorganisms have been of great importance in investigating the biosynthesis of aromatic compounds. The discovery by Tatum (6) and by Dubes (52) that mutants of *Neurospora* may be obtained which require four aromatic compounds encouraged the hope that other mutants might be found which would be useful in the present study. There were already available a large number of strains which require tryptophan for growth, one strain (5212) which requires phenylalanine, and a strain (described in the Introduction of this thesis) which makes alternate use of a number of aromatic compounds. The only mutant available to this investigation which requires more than one aromatic amino acid was that furnished by Dr. George Dubes. It was desired to obtain additional mutants of *Neurospora* which require aromatic compounds.

The new mutants were isolated by two techniques.

(1) Use was made of the technique of Lein, Mitchell, and Houlihan (53) in which irradiated conidia are used to fertilize wild type protoperithecia of the opposite mating type. Ascospores are collected, germinated on plates, and poorly-growing cultures transferred to supplemented medium and subsequently tested for nutritional requirements. A slight

modification was used, which consisted of the following: ordinarily the ascospores are collected on plates of minimal medium, germinated by heat treatment, and the poorly-growing cultures selected visually. This results in the isolation of mutants with a wide variety of nutritional requirements, only a small proportion of which can be expected to have lesions in the synthesis of aromatic compounds. To eliminate selection of some of these "undesired" mutants, the spores were germinated on a "complete" agar medium which had previously been boiled with generous amounts of Norite A to remove aromatic compounds. This medium was then "minimal" with respect to aromatic amino acids, but "complete" with respect to a number of other compounds.

(2) A modification of the technique of Woodward et al. (54,55) was used. This technique consists of suspending irradiated conidia in minimal medium, incubating, and filtering at intervals for a number of days through sterile cheesecloth. Wild-type conidia germinate and put out long hyphae which become tangled in the cheesecloth; but those conidia which, because of a nutritional requirement are unable to grow, pass through and become relatively enriched in the filtrate. After four days of this procedure, the filtrate contains only nutritionally mutant conidia and slow-growing "nutritionally wild-type" conidia. At this point the filtrate is plated on medium supplemented with the nutrients for which mutants are desired. This medium contains sorbose to induce

colonial growth (56).

As macroconidia are known to be multinucleate, it is clear that, in this procedure, it is necessary to irradiate the conidia heavily enough so that a proportion of them will have only one functional nucleus remaining.

The reason for using a modification of the published technique in this case was that, at the time of our first use of this method, it had been published only in abstract form (54). The details as worked out in the present study turned out to be rather different from those which were later published (55), and therefore will be described below. The three experiments that were carried out using this procedure differed only in small details and in scale of operation, so the second experiment (described below) typifies all three of them.

Conidia of wild-type 5297a from eight slants were suspended in 320 ml. of Fries minimal medium and filtered through four layers of cheesecloth to remove bits of mycelium. A small sample was removed for plate counts and will be called 0' irradiated sample. The remainder of the conidial suspension was divided between four sterile beakers (250 ml.). These were irradiated 3', 6', 9', and 15' with agitation at a distance of about 10 cm. from an Americanaire Sterilamp. Small samples were then removed from each beaker for serial dilutions and plate counts. All plating was done on the sorbose medium described by Tatum et al. (56). Counts showed

the following concentrations of conidia having at least one functional nucleus:

minutes irradiated	conidia/ml.	% survival	number of mutants found
0	$3.3(10)^6$	(100)	-
3	$1.3(10)^6$	40	5
6	$3.5(10)^5$	11	3
9	$1.2(10)^5$	3.6	2
15	$3.2(10)^4$	1.0	3

Streptomycin and penicillin G were added to each beaker to give final concentrations of 100 µg./ml. and 30 µg./ml. respectively; this was done to reduce the danger of bacterial contamination during subsequent manipulations. The four conidial suspensions were incubated four days at 25° in flasks without agitation. During this time, the suspensions were filtered through sterile cheesecloth every twelve hours and the adhering hyphae discarded. After the last filtration, 1 ml. samples were plated without dilution on medium containing sorbose and the following: DL-tryptophan, 100 µg./ml.; DL-phenylalanine, 100 µg./ml.; L-tyrosine, 100 µg./ml.; p-aminobenzoic acid, 0.5 µg./ml. Plates from each of the four samples averaged about two colonies per plate. From

these plates, 125 colonies were picked up and grown in tubes of Fries medium supplemented as above. The resulting cultures were tested for growth on Fries minimal medium as compared with the above supplemented medium. Thirteen of them did not grow on the minimal medium, and were classified as aromatic-requiring mutants.

The last search for mutants differed from the one described above in the following details:

(A) Strain C-102A, which is indistinguishable from wild type at 25°, but grows colonially at 35°, was used. Plating was therefore done on a non-sorbose containing medium and the plates incubated at 35°.

(B) Mutants with nutritional requirements other than the aromatic compounds were selected for use by other workers. Accordingly, the plates were supplemented not only with the four compounds used in previous searches, but also with DL-lysine, diaminopimelic acid, riboflavin, α -lipoic acid, guanosine, pyridoxine, and fructosylaspartic acid.

(C) Of 580 cultures obtained from this experiment, 106 were clearcut nutritional mutants.

SUMMARY OF MUTANTS FOUND IN THESE STUDIES

Experiment #	Selection technique	Number of mutants found
1	(1)	3
2	(2)	4
3	(2)	13
4	(2)	106

Total: 126

CLASSIFICATION OF THE MUTANTS

(Pooled data from the four experiments)

Nutritional requirement	Number of mutants found
tryptophan	29
phenylalanine	2
tyrosine	4
p-aminobenzoic acid	1
multiple aromatic	26
"C-86 class" (see p. 98)	1
lysine	39
riboflavin	1*
guanosine	2
pyridoxine	1
α -lipoic acid	0
fructosyl aspartic acid	0
<hr/>	
	Total: 105 ⁺

*The sole riboflavin mutant was found as a double mutant in combination with a multiple aromatic-requiring mutant. (See p. 49).

⁺Twenty-one strains have been found which will grow a trace on one or a combination of the above supplements, but will not grow at all on minimal medium. To date, these mutants have not been successfully classified.

Use of Crassin, a Fungicidal Antibiotic, for Selection of Mutants of Neurospora crassa

A substance designated as "crassin" was found by Dr. H. K. Mitchell as a fungicidal secretion of a Bacillus, and has been purified to a powder of high potency. At his suggestion, we undertook to develop a technique for isolation of Neurospora mutants analogous to the technique which employs penicillin for the selection of bacterial mutants (57).

Conidia of the temperature colonial mutant C-102 were suspended in Fries minimal medium, filtered through glass wool to remove bits of mycelium, and irradiated as previously described. The conidia were washed by centrifugation and resuspension in fresh medium in order to remove growth factors which might have been leached out of the killed conidia. Crassin was added to give a concentration of 200 µg./ml. The suspension was incubated with agitation and samples withdrawn at intervals, subjected to serial dilutions, and plated on a "complete" medium. Colonies which grew from surviving conidia were picked up and grown in tubes. The resulting cultures were tested on minimal and on "complete" media. Counts of the colonies indicated the following decrease in titer of viable conidia as a result of incubation with crassin.

Minutes incubation with crassin	Viable conidia/ml.	% survival
0	3.1(10) ⁶	(100)
5	4.0(10) ⁶	130
15	3.5(10) ⁶	110
30	2.0(10) ⁶	65
50	1.6(10) ⁶	50
80	6.5(10) ⁵	20
120	3.5(10) ³	0.1
160	1.2(10) ³	0.04
200	1.2(10) ²	0.004
240	4. (10) ¹	0.001

Pooled results from a number of experiments indicated that about 3% of the conidia surviving crassin treatment were nutritional mutants. This is probably significantly higher than could be found in the absence of selection, but is not high enough to recommend it as an alternate to the methods previously discussed.

Varying the concentration of crassin, the physiological age of the conidia used in the work, and the conditions of incubation did not seem to improve the yield of mutants.

The mutants obtained during these experiments were discarded.

Genetic Studies of the New Mutants

An examination of the genetic behavior of the new mutants was desirable for the following reasons:

(1) In order to demonstrate a true metabolic relationship between the aromatic amino acids and the vitamin p-aminobenzoic acid in *Neurospora*, it seemed important to show whenever possible that an observed biochemical lesion was the result of a single gene alteration from wild type. Even in cases where only one requirement was observed, evidence of this sort was considered to be of value.

(2) Evidence for the allelism of various strains could eliminate considerable duplication of work in subsequent biochemical studies.

(3) The work of Mitchell and Houlahan (58) showed that double mutants may frequently be used to establish the order of a series of mutant genes in a biosynthetic pathway. In the present study, this was the most useful method available, since the multiple aromatic mutants in *Neurospora* do not appear to cross-feed.

(4) In some cases, double and triple mutants might be expected to secrete qualitatively or quantitatively different substances than are found in any of the component strains.

Because of the rather large number of mutants involved, it was necessary to limit genetic studies to those which seemed most likely to give useful biochemical data.

All crosses were made on slants of Westergaard-Mitchell medium (59) supplemented with DL-tryptophan (100 $\mu\text{g./ml.}$), DL-phenylalanine (100 $\mu\text{g./ml.}$), L-tyrosine (100 $\mu\text{g./ml.}$), and p-aminobenzoic acid (0.5 $\mu\text{g./ml.}$). The crosses were incubated until ripe, and asci were dissected and germinated by heat treatment on plates of minimal agar (60). In almost all cases, the germinated mutant spores could easily be distinguished from the wild types by the length of the hyphae after twelve hours' growth. This will be called "the visual criterion."

CROSSES OF THE NEW MUTANTS TO WILD TYPE OF THE OPPOSITE MATING
 TYPE: EVIDENCE THAT THE OBSERVED NUTRITIONAL REQUIREMENTS
 OF VARIOUS STRAINS ARE DUE TO A SINGLE GENE ALTERATION
 FROM WILD TYPE

Strains (grouped as probable alleles See p. 52)	Total number of asci	Number of asci contain- ing more than two mutant spore pairs	Number of asci show- ing 2nd division segregation	comment
13-II-1A	29	0	5	
13-II-2a	27	0	6	
76R9A	2	0	1	Fertility low
6D6a	14	0	1	
6-IV-3a	7	0	0	See p. 47
15-IV-1a	1	0	1	See p. 48
44A	9	0	3	
13-II-3a	10	0	5	See p. 48
3-IV-3a	26	0	20	
25A	21	0	6	

Evidence that the Observed Nutritional Requirements of 6-IV-3a are Due to a Single Gene Alteration from Wild Type

Strain 6-IV-3a was classified as a multiple-requiring aromatic mutant, and was found to accumulate protocatechuic acid. It was crossed to 5256A. The visual criterion for spores was tested by classifying twenty random germinated spores on plates; these spores were then isolated, grown on slants, and tested on liquid media to determine the nutritional requirements. In each case, the visual criterion was confirmed.

Fertility was low, and only nine asci showed the required amount of germination. Of these, seven were visually classified as compatible with the interpretation that a single gene alteration was responsible for the observed requirements. Two asci appeared to contain three mutant spore pairs and one wild type spore pair, suggesting that two genes were segregating.

Each spore from the latter two asci was transferred to a slant of supplemented Fries agar. It was found that in each of these asci, a pair of "mutant" spores failed to grow. Therefore it cannot be said that there is any evidence that two mutant genes are involved. A possible explanation is that in each of these asci a wild-type spore pair started to germinate before heat treatment, and that these spores were then killed by the treatment that was intended to induce their

germination. Thus, dead wild type hyphal buds might not have been visually distinguished from live mutants.

"Sterile" Mutants 13-II-3a and 15-IV-1a

Crosses of these two mutants to wild type of the opposite mating type were completely sterile. Attempts to overcome this by using very rich media, and by making crosses using the mutant strains as protoperithecial or as conidial parent were likewise unsuccessful. 13-II-3a requires phenylalanine, and is the only strain not found to use phenylpyruvic acid as a substitute. 15-IV-1a was found to resemble C-86 (5) in its growth requirements.

When conidia of these two strains were centrifuged together and incubated a wild-type heterocaryon was formed. This heterocaryon crossed reasonably well with 5256A. Eleven usable asci were obtained, and all spores from each ascus picked up and cultured. Each spore culture was tested on three liquid media: (1) minimal; (2) minimal + nicotinamide; (3) minimal + aromatic supplement. 13-II-3 will grow only on the aromatic supplement; 15-IV-1 will grow on aromatic supplement or on nicotinamide; wild type will grow on all three media.

On the basis of these tests, ten of the asci were found to segregate for the 13-II-3 gene, and one for the 15-IV-1 gene. Of the ten asci in the former class, five showed segregation of the gene in the second division. The sole

ascus containing the 15-IV-1 gene showed second division segregation. In no case was any ascus found to contain more than two mutant spore pairs.

Reisolates of these mutants were only slightly fertile with wild type.

Proof that the Observed Nutritional Requirements of 76A are Due to the Action of Two or More Gene Alterations from Wild Type

This mutant requires the usual four aromatic compounds, and, in addition, the aromatic vitamin riboflavin. At first it was believed that study of this strain would demonstrate a metabolic relationship between riboflavin and the aromatic amino acids. However, genetic study revealed that two separable genes were responsible for the nutritional requirements.

The strain was crossed to 5297a. Fertility was moderate, but the ascus walls deteriorated in all cases before the spores ripened. Therefore it was necessary to plate random spores and make spore counts.

Germination was good under these conditions. Of 406 spores which were observed, 311 were seen to be mutant, and 95 to be wild type. If two unlinked genes are segregating, the expected ratio is 3/1. Assuming this ratio, the standard error is 0.0215. The actual deviation is 0.0150, or 0.7 times the standard error. This deviation or more would be expected in about 50% of all cases examined. It is clear that the probability of one gene difference giving rise to

this ratio of mutant to wild-type progeny is vanishingly small.

Random spore cultures were made, and the two mutant components examined. Cultures requiring only riboflavin were given to other workers. Reisolates which required the three aromatic amino acids plus p-aminobenzoic acid were saved for future study.

The above data exemplify the importance of genetic analysis of mutants which are to be used in biochemical studies. On structural grounds, it is tempting to assume that the aromatic rings of riboflavin and of the other compounds under discussion are formed by a common pathway. In the absence of genetic analysis, isolation of a mutant requiring all five of these compounds would have been taken as very strong evidence of their common origin. Clearly, this conclusion would have been unwarranted.

Examination of the Mutant Strains for Allelism

In a number of cases, two or more mutant strains were found to exhibit the same nutritional requirements and to accumulate the same compounds. Some of these were tested for allelism (See p. 52).

The test consisted of crossing the strains in question as previously described, allowing the cross to ripen, and collecting random spores on plates of minimal agar. The spores were induced to germinate, and after twelve hours'

growth were examined. The presence or absence of wild-type recombinants was determined visually. In crosses which gave no obvious wild-type spores, it was the custom to pick up between ten and forty spores showing the least stunted hyphae, and to test the resulting cultures for biochemical requirements on liquid media. In no case was any wild type found as a result of the latter procedure.

It must be stated that an absolute proof of the allelism of two strains is impossible. If they are non-allelic, there is a certain probability that any given spore examined will be a wild-type recombinant. If the genes are closely linked, this probability becomes smaller. For any given map distance between two genes, the probability of detecting non-allelism increases with the number of spores examined. However, this probability never reaches 1.0 even if the genes are unlinked and an extremely large number of spores are examined.

Failure of two strains to form a wild-type heterocaryon cannot be used as evidence for allelism, as it is known (61) that many pairs of strains which are obviously non-allelic fail to form a wild-type heterocaryon.

CROSSES BETWEEN STRAINS WHICH WERE SUSPECTED OF BEING ALLELES

Strains crossed	Number of random spores observed	Number of wild-type spores	Comment
76R3a x 13-II-1A	-	-	Sterile.
76R4A x 13-II-2a	-	-	"
76R9A x 15-IV-3a	-	-	"
13-II-1A x 13-II-2a	-	-	Sterile. Reisolates likewise sterile.
13-II-1A x 15-IV-3a	-	-	Sterile.
C-120A x 6D6a	406	0	Probably alleles
6D6A x 6-IV-3a	-	-	Sterile. Reisolates likewise sterile.
C-86A x 15-IV-1a	-	-	Sterile.
3-IV-3a x 16A	346	0	Probably alleles.
3-IV-3a x 73A	697	0	Probably alleles.
3-IV-3a x 74A	345	0	Probably alleles.
25R2a x 65A	1527	0	Probably alleles.

Proof of Non-allelism of 13-II-1A and 6D6a: Isolation of the Double Mutant

Protoperithecia of 6D6a were fertilized with conidia of 13-II-1A. Three asci gave good germination. One of these was seen by the visual criterion to contain four mutant spores and four wild-type spores. The mutant spores would all be expected to be the double mutant 13-II-1/6D6. The phenotypes of these eight cultures were confirmed by testing on liquid media.

One of the presumptive double mutant cultures was examined genetically to remove all doubt about its constitution. It was crossed to wild-type 5256A and asci dissected. Seven asci showed two mutant and two wild-type spore pairs, and three asci showed three mutant and one wild-type spore pair. The phenotypic constitution of one of the latter class of asci was confirmed by tests on liquid media. Therefore it is clear that the presumed double mutant is indeed a double mutant.

Proof of Non-allelism of 15-IV-3a and C-120A. Isolation of the Double Mutant

Protoperithecia of 15-IV-3a were fertilized with conidia of C-120A. Of the eight asci which showed satisfactory germination, five showed a wild-type spore pair.

The three mutant spore pairs of one of these asci were studied by crossing to wild type. One did not appear to be fertile. Of the other two, one yielded asci containing more than two mutant spore pairs. These phenotypes were confirmed by

tests in liquid medium. Clearly this is the double mutant. The remaining culture was crossed to wild type. Dissection of asci from this cross yielded 27 asci showing germination in all spore pairs. Each of the asci contained two mutant spore pairs and two wild-type spore pairs.

Proof of the Non-allelism of 44R3a and 5212A.
Isolation of the Double Mutant

These two phenylalanine-requiring strains were crossed and asci dissected. Six asci contained enough germinated spores to be usable. Of these, one ascus was seen to contain two mutant spore pairs and two wild-type spore pairs. The spores from this latter ascus were picked up, and the resulting cultures tested for nutritional requirements on liquid media. The phenotypes as revealed by these tests confirmed the visual classification. All the mutant cultures from this ascus would be expected to have the double mutant genotype 44/5212. One of the latter was saved for further study.

This presumed double mutant was crossed to wild-type 5256A. Asci were dissected, caused to germinate, and visually examined. Numerous asci could be seen to contain more than two mutant spore pairs. Spores from one of the asci which was classified as containing three mutant spore pairs and one wild-type spore pair were transferred to individual tubes of supplemented media. The resulting cultures were tested for nutritional requirements on liquid media, and the phenotypes confirmed.

Isolation of the Double Mutant 6D6/C-86

6D6A and C-86a were crossed by simultaneous inoculation. Asci were dissected, and the spores germinated on minimal plates. An ascus which was seen to contain two mutant and two wild-type spore pairs was picked up. The visual criterion was confirmed by appropriate tests.

All of the non-wild-type spores in this ascus would be expected to be double mutants. To verify this, one of the presumed double mutants was outcrossed to wild-type 5297a. Only one complete ascus was obtained, but it could be seen to contain four mutant spore pairs. These phenotypes were confirmed by tests on liquid media. The presumed double mutant used in this confirmatory cross will now be referred to as 6D6/C-86.

Isolation of a Tryptophan-tyrosine Double Mutant, 75001/3-IV-3

Protoperithecia of the tryptophan mutant 75001R1A were fertilized with conidia of the tyrosine mutant 3-IV-3a. Asci were dissected and germinated. Six were found to contain a wild-type spore pair. Spores from three of these asci were tested on minimal, minimal + tryptophan, minimal + tyrosine, and minimal + tryptophan + tyrosine media. In each case, one spore pair was found to be wild type, one required tryptophan, one required tyrosine, and one required tryptophan plus tyrosine. Cultures of the latter class were saved for further work.

Isolation of a Phenylalanine-tyrosine Double Mutant,
44/3-IV-3

44A and 3-IV-3a were crossed by simultaneous inoculation. Of twelve asci, nine showed wild-type spores. One ascus had two mutant spores, two ungerminated spores, and four wild-type spores. The two mutant spores would be expected to be the desired double mutant. The visual classification was confirmed by tests on liquid media, and the two mutant spores found to require both phenylalanine and tyrosine. This presumed double mutant was found to be of mating type A.

To confirm the genetic constitution, conidia of this strain were applied to protoperithecia of 5297a. Of thirty asci, twenty-one showed three mutant spore pairs and one wild-type spore pair; two asci showed only mutant spores. It is clear, then, that the strain is the desired double mutant.

Isolation of a Phenylalanine-tyrosine Double Mutant,
5212/3-IV-3

5212A and 3-IV-3a were crossed by simultaneous inoculation. One ascus which showed complete germination was seen to contain a pair of wild-type spores. Each of the spores was picked up. The eight cultures were tested in flasks on the following media: minimal, minimal + tyrosine, minimal + phenylalanine, minimal + tyrosine + phenylalanine.

As expected, one pair was found to be wild type, one pair required tyrosine, one pair required phenylalanine, and a pair required both tyrosine and phenylalanine. A culture of the latter was designated as the double mutant.

Isolation of a Tryptophan-phenylalanine Double Mutant, 75001/44; Isolation of a tryptophan-phenylalanine-tyrosine Triple Mutant, 75001/44/3-IV-3

Protoperithecia of 44A were fertilized with conidia of 75001/3-IV-3R3a. The one ascus which was found to give germination in all spore pairs contained one wild-type spore pair. The phenotypes were determined by testing in flasks of liquid media. Nutritional requirements of cultures from the four spore pairs were as follows:

- (1) none
- (2) phenylalanine (or tyrosine) + tryptophan
- (3) tyrosine
- (4) phenylalanine + tryptophan + tyrosine

Mutant 44 is known to have a tendency to grow on medium supplemented with tyrosine, so the ambiguous behavior of the second spore pair is not too surprising. It is clear that the absolute requirement of (3) and (4) for tyrosine is due to the presence of the 3-IV-3 gene. Similarly, the presence of the 75001 gene in (2) and (4) is indicated by the absolute requirement for tryptophan. Since (3) will not grow on phenylalanine, and (1) is wild, a process of elimination further suggests that the 44 gene is present in

(2) and (4). However, this remains to be established by genetic reisolation of strain 44 from these cultures.

Isolation of a Tryptophan-phenylalanine Double Mutant, 75001/5212; Isolation of a Tryptophan-phenylalanine-tyrosine Triple Mutant, 75001/5212/3-IV-3

75001/3-IV-3R3a and 5212A were crossed by simultaneous inoculation. Of five asci showing good germination, one contained a wild-type spore pair. The ascus was classified as before on liquid culture. The nutritional requirements of cultures obtained from the spore pairs were as follows:

- (1) tryptophan + phenylalanine
- (2) tyrosine
- (3) none
- (4) tryptophan + phenylalanine + tyrosine

Cultures of (1) and (4) were saved for biochemical studies.

IV METHODS USED FOR DETECTING ACCUMULATION OF COMPOUNDS BY THE MUTANT STRAINS

Four methods were used for detection of accumulated compounds in the media and in the mycelia of mutants.

A. Crossfeeding behavior was studied in order to detect accumulated materials possessing biological activity. Those mutants of *Neurospora* which require the three aromatic amino acids plus *p*-aminobenzoic acid provide a convenient bioassay for substances accumulated by mutants which have lesions later in the biosynthetic pathway. Activities for the individual metabolites were detected by use of media which were supplemented with three of the four required compounds. The concentrations used were 25 $\mu\text{g./ml.}$ for amino acids, and 0.5 $\mu\text{g./ml.}$ for *p*-aminobenzoic acid. These media, from which one of the four compounds had been omitted, were autoclaved. One or two milliliters of the raw culture filtrate to be tested for the presence of biologically active materials were pipetted into each of the hot flasks immediately after autoclaving. This procedure was found to result very rarely in contamination of the flasks. It was considered a valuable compromise between autoclaving, which would destroy any labile compounds, and sterilization by Seitz filtration, which is very laborious when a large number of samples are to be assayed, and has the added disadvantage of causing re-

removal of certain aromatic compounds by adsorption. This technique for sterilization of labile compounds has been discussed by Haskins (32).

After the flasks had been allowed to cool, they were inoculated with a drop of a conidial suspension of one of the mutants which require four aromatic compounds. Of these mutants, 13-II-1A, 13-II-2a, and 6D6a were variously used as assay organisms with no significant difference in results. The latter strain responds to lower concentrations of metabolites than do the previous two, and is therefore capable of detecting smaller quantities of accumulated materials. However, the low requirements are accompanied by effects of inoculum size and therefore result in less reproducibility in quantitative assays. For this reason, 13-II-2a was more frequently used in these assays. In a few cases, strain 76A was used. As has been noted, this double mutant requires riboflavin in addition to the four compounds in question. Therefore, riboflavin (1.0 µg./ml.) was included in all assay media when this strain was employed. One other strain, C-86A, was used for assays in some cases. As this mutant will grow on any one of a number of compounds, the material to be assayed was added to hot minimal medium instead of to partially supplemented medium.

The assay strain was allowed to grow for 96 hours at 25°. Then the mycelial pads, if any, were squeezed between filter paper and dried overnight at 90-110°. Weight of the

pads could be compared to the weight given by growth in the presence of known amounts of the metabolite in question. The reproducibility of these assays indicated that with care an accuracy of $\pm 10\%$ could be achieved.

Mutant strains of bacteria were also used for the detection of biologically active materials in the media of *Neurospora* mutants. These strains were made available to this study through the generosity of Dr. Bernard Davis. One strain of *E. coli*, 156-53M2, will utilize only shikimic acid as a substitute for all of the required aromatic compounds. Another strain, 83-1, will use either shikimic acid or 5-dehydroshikimic acid for this purpose.

A mutant of *Aerobacter aerogenes*, A170-143-S1, which will use either of the compounds mentioned above, and will respond to quinic acid and 5-dehydroquinic acid, was also furnished by Dr. Davis. The specificities of these mutants allowed classification of any accumulated materials.

The medium used in these tests was that described by Davis (62). Minimal medium (5 ml.) in six inch test tubes was supplemented with 0.5 ml. of the *Neurospora* culture filtrate to be tested. The tubes were pasteurized at 70° for fifteen minutes and inoculated with the bacterial test strain. After 24 hours' incubation at 35° , growth was noted by inspection. Examination of the growth of the strains with various concentrations of authentic shikimic acid revealed that the procedure used would detect $10 \mu\text{g./ml.}$ of this com-

pound in culture filtrates of Neurospora.

B. A number of the mutants accumulate materials which impart an intense blue fluorescence to the medium. These materials, many of which are not detected by any of the biological tests described above, are easily observed on chromatograms by inspection with a Keese Ultraviolet Lamp ($\lambda_{\text{max}} = 360 \text{ m}\mu$.).

C. Compounds containing the benzene ring exhibit absorption maxima in the ultraviolet which are characteristic of the compound. In numerous cases, accumulation of such compounds is large enough so that even the raw culture filtrates have to be diluted considerably to permit inspection in the Beckman Model DU Spectrophotometer.

The case of "prephenic acid" deserves special note. This compound exhibits only end-absorption in the ultraviolet, and is therefore not detected as such by this technique. However, as has been previously discussed, it is rapidly converted by mild acid treatment into phenylpyruvic acid, which absorbs very strongly at $320 \text{ m}\mu$. in alkaline solution. Prephenic acid was, therefore, detected and quantitatively assayed by the following procedure. A sample of the material to be assayed was quantitatively diluted so as to contain $30\text{-}300 \text{ }\mu\text{g./ml.}$ of "prephenic acid." An aliquot (1.0 ml.) was pipetted into each of two six-inch test tubes. One of the tubes was stored in a refrigerator. To the other was added 1N HCl (1.0 ml.). The latter tube was incubated at

35° for a period of two hours. At the end of this time, 1N NaOH (8.0 ml.) was added to the tube which had been incubated. Similarly, 1N NaOH (8.0 ml.) and 1N HCl (1.0 ml.) were mixed and then added to the tube which had been stored in the refrigerator. Both tubes were allowed to reach room temperature, and were then read at 320 m μ . The difference between the two readings indicated the amount of phenylpyruvic acid formed by incubation of the "prephenate" with acid, and was compared with the absorption given by a known amount of pure phenylpyruvic acid. It was found that longer periods of incubation did not increase the amount of phenylpyruvic acid which could be produced from a given sample of prephenic acid.

Aromatic compounds frequently exhibit different absorption spectra in acid and in basic solvents. Therefore it was considered more informative to examine the spectra of media and of isolated compounds under both conditions. In addition, 0.1 M KH_2PO_4 (pH = 3.5) was sometimes used as a solvent in these studies.

D. A number of materials could be detected on paper chromatograms by spraying with various reagents. The reagents which were found to be most useful for this problem were:

1. Ferric chloride for the detection of protocatechuic acid and phenylpyruvic acid (63).
2. Diazotized sulfanilic acid for the detection of phenolic compounds (64).

3. Orcinol + trichloroacetic acid in water-saturated n-butanol for the detection of ketoses, and in particular, ketoheptoses (65).
4. Ninhydrin in n-butanol or pyridine for the detection of amino acids (63).
5. Nitrous acid followed by naphthyl-ethylenediamine for detection of aromatic amines (63).
6. Molybdate spray followed by ultraviolet light for detection of phosphate esters in extracts of mycelia (66).
7. Periodate for the detection of vicinal diols (67).
See reprint, p. 65.

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Detection of Periodate-oxidizable Compounds on Paper Chromatograms¹

BY R. L. METZENBERG AND H. K. MITCHELL

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Buchanan, Dekker and Long² have reported a method, based on periodate oxidation, for the detection of various glycols and nucleosides on chromatograms. The procedure outlined below is less specific, but is simpler and, in our experience, more sensitive.

Experimental

Preparation of the Chromatogram.—Whatman #1 Filter Paper was used. In some solvent systems, soluble periodate-reacting materials in the paper migrate just behind the solvent front and interfere with the detection of compounds of high R_f value. This problem can be eliminated by washing the paper with the solvent to be used before applying the spots.

Solvent Systems.—Use of phenolic solvents interferes with the sensitivity of the method, as does use of buffered solvents.

Detection of Periodate-oxidizable Compounds.—The chromatogram is dried thoroughly to remove any acid or base remaining after development. It is then sprayed very lightly and evenly with aqueous KIO_4 (0.01 M), air-dried at room temperature for 8–10 minutes, and then sprayed with a solution of 35% saturated sodium tetraborate containing 0.8% KI, 0.9% boric acid and 3% soluble starch, boiled to bring the starch into solution.

On areas of the chromatogram not having any periodate-oxidizable substances, periodate reacts with iodide ion to liberate iodine, which in turn gives a blue color with the starch; but wherever periodate has been reduced to iodate ion, iodine is not liberated, and no color is observed. Therefore, white spots are seen on a blue background.

Optimum contrast is usually observed about 10 minutes after the second spraying. The spots are not permanent, but may readily be photographed by interposing the wet chromatogram between two sheets of glass, placing it over a sheet of high contrast printing paper, exposing to light,

and developing the print. This has the added advantages of increasing the contrast somewhat, and giving dark spots on a light background.

Discussion.—The specificity seems to be essentially that of the well known periodate reaction.³ Of compounds tested, tartaric acid, xylose, glucose, sucrose, mannitol, inositol, quinic acid, chlorogenic acid, shikimic acid, glucuronolactone, ascorbic acid, serine, threonine, methionine, cystine, tryptophan, riboflavin, adenosine, guanosine, inosine, xanthosine, cytidine, uridine, catechol, phloroglucinol, gallic acid, β -phenylserine, anthranilic acid and protocatechuic acid show periodate-reducing power. Very weak reactions were given by histidine, tyrosine, aspartic acid, *trans*-cinnamic acid, benzoic acid and *o*-coumaric acid. Compounds which gave no detectable reaction were arginine, ornithine, proline, leucine, isoleucine, lysine, asparagine, glycine, alanine, glutamic acid, $N\alpha$ -acetylkynurenine, phenylalanine, glutathione, chloromycetin, guanine, hypoxanthine, xanthine, cytosine, uracil, thymine, thymidine, uric acid, mandelic acid, *p*-hydroxybenzoic acid and vanillin.

Anthranilic acid, gallic acid, chlorogenic acid and cystine were observed to give transient yellow or orange colors after the periodate reagent. Malic acid, citric acid and fumaric acid gave dark spots, probably by exceeding the buffer capacity of the second reagent and causing iodate to react with iodide.

Under conditions of optimum sensitivity, the method will give a barely detectable spot with 0.25 $\mu g.$ of inositol, or its equivalent in periodate reducing power. The limiting factor appears to be the reaction of the periodate with the paper itself.

(3) E. L. Jackson, "Organic Reactions," Vol. 2, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 341.

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(2) J. G. Buchanan, C. A. Dekker and A. G. Long, *J. Chem. Soc.*, 3162 (1950).

V ISOLATION AND IDENTIFICATION OF ACCUMULATED SUBSTANCES

A. Isolation and identification of protocatechuic acid (3,4 dihydroxybenzoic acid) from culture filtrates of strain 6D6a.

The ultraviolet absorption spectrum of medium in which this mutant had grown showed strong peaks at 255 m μ . and 290 m μ . and the medium gave a strong green color when treated with ferric chloride. Accumulation of this material was found to be poor in carboys grown under forced aeration. Therefore, 1100 Erlenmeyer flasks (125 ml.) were charged with 20 ml. each of Fries medium containing DL-tryptophan, DL-phenylalanine, and L-tyrosine, each at a concentration of 5 μ g./ml., and p-aminobenzoic acid at a concentration of 0.1 μ g./ml. The flasks were autoclaved and inoculated.

After five days' growth, the culture filtrates were pooled and agitated with Norite A (21 g.), which completely removed the characteristic ultraviolet absorption peaks. The material was eluted in a yield of about 40% by boiling for ten minutes with 95% ethanol under nitrogen. The ethanolic eluate was evaporated in vacuo. The residue was taken up in a little water, and this solution was extracted with petroleum ether. Evaporation of the petroleum ether gave white crystals.

The crystalline material was sublimed onto a dry ice "cold finger" in vacuo. Repetition of this gave a product which was pure enough for chemical investigation.

The material failed to react with 2,4-dinitrophenylhydrazine, and was judged not to contain a carbonyl group. The Zeisl alkoxyl test was negative. The material reduced periodate rapidly. It was found not to contain nitrogen. Elementary analysis of the material gave the following results.*

<u>Found</u>	<u>Calculated for protocatechuic acid</u>
C = 54.86%	C = 54.55%
H = 4.32%	H = 3.92%

The ultraviolet absorption spectrum of this material in 0.1 M KH_2PO_4 was found to be identical with that of an authentic sample of protocatechuic acid. Chromatographic comparison of these in three solvent systems and detection by the ferric chloride reagent and by diazotized sulfanilic acid revealed no differences.

A sample of the isolated material was recrystallized from water.

* The author is indebted to Mr. G. A. Swinehart of the California Institute of Technology for this analysis.

Melting point of isolated material:	201-202° (uncorr.)
Melting point of authentic protocatechuic acid:	201-202° (uncorr.)
Mixed melting point:	201-202° (uncorr.)
Melting point given in literature:	200-201°

To obtain further evidence as to the identity of this compound, a sample was treated with ethereal diazomethane. The reaction mixture was kept in an icebath for thirty minutes, then was maintained at 35° for ten hours. The ether and excess diazomethane were evaporated. To the oily residue, 1N NaOH was added, and the mixture maintained at 35° over a period of two days to hydrolyze the ester. At the end of this time, it was heated to 80°, cooled, and acidified with HCl. A white precipitate came out of solution. This was purified by sublimation and recrystallization from aqueous dioxane, and was presumed to be veratric acid (3,4-dimethoxybenzoic acid).

For purposes of comparison, veratric acid was synthesized by methylation of vanillin with ethereal diazomethane, as described above, and oxidation of the reaction product with alkaline hypobromite as described by Kostanecki et al. (68). Unreacted veratraldehyde was removed by extraction with ether, and the aqueous phase was acidified. The crystals which formed were recrystallized from aqueous dioxane with a little Norite A.

Melting point of isolated material after methylation and hydrolysis:	180° (corr.)
Melting point of synthetic veratric acid:	179° (corr.)
Mixed melting point:	180° (corr.)
Melting point given in literature:	180-181°

On the basis of the above evidence, it seems clear that the material isolated from culture filtrates of 6D6a is proto-catecholic acid.

B. Evidence that culture filtrates of strain 6D6/C-86 contain shikimic acid.

Flasks were charged with Fries medium supplemented with DL-tryptophan (25 µg./ml.), DL-phenylalanine (25 µg./ml.), L-tyrosine (25 µg./ml.), and p-aminobenzoic acid (0.5 µg./ml.). The flasks were inoculated with 6D6/C-86 and allowed to grow at 25° for five days. The mycelia were removed, and culture filtrate (50 ml.) was evaporated in vacuo to dryness and re-dissolved in water (5.0 ml.). Methanol (50 ml.) was added, and the precipitate of inorganic salts removed by centrifugation. The supernatant liquid was evaporated and the residue was redissolved in water (1.0 ml.).

This extract was chromatographically compared with an authentic sample of shikimic acid. The chromatograms were cut horizontally into ten equal sections. Each section was eluted with 2.0 ml. of the medium described by Davis (62), and supplemented with DL-phenylalanine (25 µg./ml.) and

L-tyrosine (25 µg./ml.) to increase the sensitivity of the assay. The tubes were pasteurized at 70° for fifteen minutes and inoculated with Aerobacter aerogenes A170-143-S1. The tubes were incubated for one day at 35° and examined for growth. By use of this technique, it was found that the main body of active material accumulated by 6D6/C-86 is chromatographically identical with shikimic acid in four solvent systems. In one of these systems, which consisted of three parts t-butanol and one part 1N acetic acid, a small amount of active material was observed (R_F ca. 0.15) to be widely separated from the main body of active material, which corresponds to shikimic acid (R_F ca. 0.50). This minor component was also clearly separated from 5-dehydroshikimic acid (R_F ca. 0.05). The preparation of concentrates containing the latter is described below.

C. Evidence that culture filtrates of 75001/44/3-IV-3 contain 5-dehydroshikimic acid.

A culture filtrate of this mutant was obtained from the carboy from which protocatechuic acid was isolated (see p.71). A sample of this filtrate was prepared for chromatography as described for filtrates of strain 6D6/C-86.

For purposes of comparison, a concentrate containing 5-dehydroshikimic acid was prepared from culture filtrates of E. coli strain 83-2, which is known to accumulate the latter compound. This strain was generously furnished by

Dr. Bernard D. Davis. Erlenmeyer flasks (125 ml.) were charged with 40 ml. of the medium recommended by Salamon and Davis (11). The flasks were inoculated with strain 83-2 and incubated at 35° with gentle agitation for two days. The cells were removed by centrifugation, and the supernatant medium prepared for chromatography in the same manner as were the other samples. Chromatographic comparison of culture filtrates of 75001/44/3-IV-3 and of 83-2 in four solvent systems, and detection of the active material on chromatograms as previously described, revealed no differences in mobility. These activities were widely separated from shikimic acid in three of the four solvent systems chosen. As will be pointed out on p.109, culture filtrates of 75001/44/3-IV-3 contain material which is nutritionally identical with 5-dehydroshikimic acid. Evidence is presented above that the material is also chromatographically identical with 5-dehydroshikimic acid in four solvent systems.

D. Isolation and identification of protocatechuic acid from culture filtrates of 75001/44/3-IV-3.

The strain was grown in fifteen liters of Westergaard-Mitchell medium (59) containing 25 µg./ml. each of DL-tryptophan, DL-phenylalanine, and L-tyrosine. After 3½ days of forced aeration, growth appeared to have ceased. The carboy was re-supplemented to the above levels with sterile amino acids, and growth resumed for 1½ days.

At the end of this time, the mycelium was filtered off,

and the ultraviolet absorption spectrum of the culture filtrate was examined in 0.1 N HCl, 0.1 N NaOH, and 0.1 M KH_2PO_4 . In all three cases, the spectrum could be seen to bear a close resemblance to that of protocatechuic acid. The culture filtrate also reacted with ferric chloride to give a brilliant blue-green color.

The filtrate was agitated with Norite A (45 g.) for several hours. Examination of the ultraviolet absorption spectrum at the end of this time revealed that all of the material in question had been adsorbed.

The Norite A was recovered by filtration and washed thoroughly with water. About one-third of it was extracted with methyl formate in a Soxhlet extractor for six hours.

When the ester was evaporated, crystals were deposited. These were recrystallized twice from dilute acetic acid with a little Norite A to remove colored impurities.

Melting point of the isolated material:	199° (uncorr.)
Melting point of authentic protocatechuic acid:	200° (uncorr.)
Mixed melting point:	200° (uncorr.)

Chromatographic comparison of the isolated material with authentic protocatechuic acid in three solvent systems and detection by the ferric chloride reagent revealed no differences in behavior.

E. Isolation and identification of vanillic acid from culture filtrates of 75001/44/3-IV-3.

Mother liquors from isolation and recrystallization of protocatechuic acid were examined chromatographically for presence of other compounds. In basic solvent systems, two compounds could be seen to migrate more rapidly than protocatechuic acid. The compounds could be detected on paper chromatograms by ultraviolet absorption and by diazotized sulfanilic acid. The material of intermediate mobility gave an orange color with the latter reagent, whereas that of greatest mobility gave a red color.

The compound which gave an orange color with diazotized sulfanilic acid was isolated by preparative paper chromatography using Schleicher and Schuell #470 paper. The solvent system used consisted of three parts t-butanol and one part 1M NH_4OH . Guide strips were cut and the position of the desired material determined by spraying with diazotized sulfanilic acid. The remainder of the material was eluted and the eluate evaporated to dryness. The material was further purified by vacuum sublimation in a temperature gradient tube. The properties of the compound did not appear to be changed by this treatment.

The resulting gummy crystals were recrystallized six times from dilute t-butanol and once from ligroin-propanol.

The neutralization equivalent was found to be 172. The

spectrum was identical with that of protocatechuic acid in acidic solution, but sharply different in basic solution. It was postulated that the material was vanillic acid (3-methoxy -4-hydroxybenzoic acid). The neutralization equivalent of vanillic acid is 168, in reasonably good agreement with that observed.

Vanillic acid was synthesized by the method recommended by Pearl (69). The synthetic material was recrystallized in the same manner as was the isolated material.

The unknown material was found to migrate identically with the synthetic vanillic acid in five solvent systems. The color reaction with diazotized sulfanilic acid was the same. The spectra of vanillic acid in acid and in base were found to be identical with those of the isolated material.

Melting point of the isolated material:	206° (uncorr.)
Melting point of synthetic vanillic acid:	208° (uncorr.)
Mixed melting point:	207° (uncorr.)
Melting point given in literature:	207°

It seems clear that the isolated material is vanillic acid. The amount of vanillic acid present in the raw medium is not easy to estimate due to losses during isolation, but it is considerably less than the amount of protocatechuic acid.

- F. Evidence that culture filtrates of strain 75001/44/3-IV-3 contain p-hydroxyphenylacetic acid.

It was noted on p.73 that, in addition to protocatechuic acid and vanillic acid, a third compound could be observed in mother liquors from the isolation of protocatechuic acid. This material, which gave a red color with diazotized sulfanilic acid, was isolated from chromatograms in the same manner as was vanillic acid. The material was further purified by chromatography in a second solvent system.

The ultraviolet absorption spectrum of this material resembled that of authentic p-hydroxyphenylacetic acid very closely. Chromatographic comparison of the isolated material with the synthetic material in five solvent systems revealed no differences in mobility nor in color reaction with diazotized sulfanilic acid. The amount of material isolated was too small to permit chemical investigation, but it seems clear that it is truly p-hydroxyphenylacetic acid. Evidence will be presented on p. 110 that this material is probably a catabolite of the added tyrosine, and does not represent an accumulation of material behind a genetic block.

- G. Isolation and identification of phenylpyruvic acid from culture filtrates of strain 3-IV-3a.

The medium of this mutant was found to have phenylalanine-replacing activity and tryptophan-replacing activity for mu-

tants which required the four aromatic compounds under study. Evidence will be presented below that the former activity is due completely or in part to the presence of phenylpyruvic acid in the culture filtrates.

The strain was grown in a carboy containing fifteen liters of Fries medium of half the usual concentration, supplemented with L-tyrosine (5 $\mu\text{g./ml.}$). After 108 hours' growth under forced aeration, the mycelium was filtered off and discarded. The culture filtrate was agitated for three hours with Norite A (15 g.). The Norite A was filtered off and extracted for four hours in a Soxhlet extractor with methyl formate in an atmosphere of nitrogen. Evaporation of the ester yielded a crop of yellow crystals which gave a blue-green color when treated with ferric chloride. The material was purified by sublimation followed by recrystallization from dilute acetic acid and from carbon tetrachloride.

A neutralization equivalent on a small sample gave a value of about 175. Calculated for phenylpyruvic acid: 164. The small amounts of material available demanded that the titration be run on a semimicro scale, so that these values are regarded as being within experimental error.

The material did not contain nitrogen. Analysis for carbon and hydrogen showed the following results.*

*The author wishes to express his gratitude to Mr. G. A. Swinehart for this analysis.

<u>Found</u>	<u>Calculated for phenylpyruvic acid</u>
C = 65.89%	C = 65.85%
H = 5.01%	H = 4.94%

For purposes of comparison, phenylpyruvic acid was synthesized by the method of Dakin (70).

Chromatography of synthetic phenylpyruvic acid and of the isolated material in five solvents and detection by spraying with 1% FeCl₃ revealed no differences.

The isolated material relieved the phenylalanine requirement of 6D6a, as did synthetic phenylpyruvic acid.

Melting points were determined on a melting point block, and both the synthetic material and the isolated material showed a very wide variation depending on rate of heating. When the rate of heating was 1°/minute, the following melting points were obtained:

Melting point of isolated material:	129° with decomposition
Melting point of synthetic phenylpyruvic acid:	127° with decomposition
Mixed melting point:	127° with decomposition

By faster heating, much higher melting points could be obtained, but in no case did the mixed melting point show any depression. Values given in the literature are considerably higher, but show a wide spread.

Early determinations of the ultraviolet absorption spectra in 0.1 M KH₂PO₄ revealed a serious discrepancy be-

tween the isolated material and synthetic phenylpyruvic acid. Later it came to the attention of the author that the spectrum of phenylpyruvic acid in acid solution and at neutrality is sensitive to time as well as to pH (13, 71), but that the spectrum in alkali develops immediately and is stable. Since then, the apparent discrepancy in absorption spectra has been resolved in favor of their identity.

H. Evidence that culture filtrates of strain 3-IV-3a contain anthranilic acid.

As has already been mentioned, these culture filtrates contain tryptophan-replacing activity. They are, in addition, highly fluorescent. Chromatography revealed that this fluorescence is due to at least four compounds, of which only a minor amount is contributed by anthranilic acid.

During the isolation of phenylpyruvic acid from medium of this mutant, a few crystals could be seen to separate during evaporation of the methyl formate eluate before the relatively massive precipitate of phenylpyruvic acid formed. These crystals were mechanically separated and washed quickly with ethanol, which dissolved the adhering syrup much faster than it did the crystals.

The amount of crystalline material isolated was so small that it could be studied only chromatographically.

Chromatographic comparison with authentic anthranilic acid in four solvents revealed no differences except for a slight fluorescent impurity in the isolated material.

I. Isolation and identification of anthranilic acid
from culture filtrates of the double mutant
5212/3-IV-3

Crossfeeding experiments revealed that this strain accumulates materials which replace tryptophan and phenylalanine. The culture filtrates could be seen to be intensely fluorescent. Chromatography revealed that, as with 3-IV-3a, a number of compounds contribute to this fluorescence.

Two carboys, each containing fifteen liters of Fries salts at half the usual concentration, sucrose (15 g./liter), DL-phenylalanine (25 µg./ml.) and L-tyrosine (25 µg./ml.) were inoculated with this strain and grown six days under forced aeration.

The culture filtrate was stirred with Norite A (60 g.). This was then recovered by filtration and eluted by stirring with 10% aqueous aniline at room temperature for two hours. The pH of the eluate was adjusted to 8.5, and the eluate extracted several times with ether to remove the aniline. The aqueous layer was evaporated in vacuo to a dark glassy residue.

This material was dissolved in water, made slightly alkaline with NH_4OH , and poured into the top of a column of well-washed Dowex-1 in the chloride form. The column was eluted with water, then with 4M NaCl. Sixty-three fractions of 100 drops each were collected.

The fractions so obtained were assayed for phenylalanine

and tryptophan-replacing activities by use of strain 13-II-2a. An aliquot of 0.1 ml. from each fraction was pipetted into each assay flask.

Almost all of the phenylalanine-replacing activity was found in fractions 3 and 4 of the sodium chloride eluate. Tryptophan-replacing activity began to appear in fraction 14, reached a maximum in fraction 16, and trailed slowly, reaching zero in fraction 33.

Fractions 14-32, containing all of the tryptophan-replacing material, were pooled, acidified, and extracted with ether. The ethereal extract was evaporated. The reddish residue was sublimed in vacuo onto a "cold finger." The white crystalline sublimate was highly fluorescent.

A small sample was resublimed in a temperature gradient tube in vacuo. The band of crystals was scraped out of the tube and melting points determined:

Melting point of isolated material:	143° (uncorr.)
Melting point of authentic anthranilic acid:	145° (uncorr.)
Mixed melting point:	144° (uncorr.)
Melting point given in literature:	145°

The ultraviolet absorption spectrum of the isolated material was almost identical with that of authentic anthranilic acid when compared in 0.1 N HCl, 0.1 N NaOH, and 0.1 M KH_2PO_4 .

Chromatographic comparison of the isolated material with authentic anthranilic acid in eight solvent systems showed no differences.

J. Isolation and identification of prephenic acid (as the barium salt) from culture filtrates of strain 75001/5212/3-IV-3.

Westergaard-Mitchell medium (15 liters) was supplemented with DL-tryptophan (25 µg./ml.), DL-phenylalanine (25 µg./ml.), and L-tyrosine (25 µg./ml.). Brom-thymol blue (ca. 100 mg.) was added to the medium to allow subsequent adjustment of the pH. After autoclaving, the carboy of medium was inoculated with conidia from six 3-day old slants of 75001/5212/3-IV-3. The culture was grown under forced aeration at 25° for one day without adjustment of the pH. After this initial growth period, sterile NaOH (1.0 N) was added in an amount sufficient to turn the indicator a bright green (pH = 6.8 - 7.2). Addition of alkali was repeated as often as necessary to maintain the pH in this range. As the mass of the mycelium increased, it was found that more frequent adjustments of the pH were required. During the fourth day of growth, it was necessary to add alkali at intervals of three hours. It was found that use of the "constant pH medium" described on p. 91 gave considerable relief from this task, but had the disadvantage of increasing the difficulty of subsequent isolation procedures. For this reason, the special medium was not used

for isolation of prephenate. It is to be noted that even temporary failure to maintain the pH in the range designated resulted in serious losses of prephenate. Adjustment of the pH to values greater than 7.5 did not appear to result in loss of accumulated prephenate, but caused cessation of growth of the mold. Excessively high pH was found to result in death of the culture.

After four days' growth, the mycelium was removed by filtration and discarded. The culture filtrate was flash-evaporated at a temperature of about 45° to a volume of 600 ml. The concentrated material was filtered to remove precipitated salts. To the filtrate, 2M barium acetate (125 ml.) was added, with care being taken to maintain the pH at 7.0 or higher. The precipitated material was removed by filtration and discarded. To the filtrate was added two volumes of methanol and three volumes of n-propanol, and the precipitated material was allowed to settle overnight at 0°. The precipitate, which was found to contain most of the prephenate (as the barium salt) was recovered by decantation of most of the supernatant liquid and removal of the remaining supernatant by filtration. The precipitate was thoroughly homogenized with water (200 ml.). The resulting suspension was filtered and the insoluble residue discarded. The deep yellow filtrate was lyophilized. Assay of this material by conversion of a weighed sample to phenylpyruvic acid as previously described indicated a purity of about 35-40%. It

should be noted that control of the pH during all stages of isolation of this material is of extreme importance.

Attempts to purify the material further by dissolving in water and reprecipitation with various alcohols, acetone, or pyridine were only moderately successful. It was found that the material which came out of solution first under these conditions was least pure, and that the last material to precipitate was found to have a purity of about 50%. The most pure material from this fractional precipitation was fractionated two more times in the same fashion. The resulting material, which was obtained in very small amounts, was only 80% pure. Clearly, fractional precipitation of barium prephenate is an unsatisfactory method of purifying this material from culture filtrates of *Neurospora*. Isolation of the prephenate as the lead salt or as the silver salt was likewise found to be unsatisfactory.

The barium salt (80% pure) was observed to be amorphous, and attempts to crystallize it were without success. It was hoped that some other salt of prephenic acid might show a greater tendency to crystallize and thus allow complete purification by low-temperature recrystallization. Various salts were prepared by allowing barium prephenate to react with a stoichiometric amount of the sulfate or carbonate of the appropriate cations, removing the precipitates by centrifugation, and evaporating the supernatant solutions in vacuo. Salts of the following cations were obtained as oils, or as

amorphous solids: sodium, lithium, ammonium, cyclohexylamine, brucine, and cobalt hexammine. The suggestion was offered by Dr. Hugh S. Forrest that amidine salts of carboxylic acids are often relatively insoluble in water and are readily crystallized. Accordingly, guanidine prephenate was prepared and obtained as an oil which, when scratched, crystallized extremely slowly as light yellow needles. This process of crystallization required about two weeks' storage in the refrigerator, and even then left some thick syrup adhering to the crystals. Furthermore, all attempts to recrystallize this material were without success. It was suggested that benzamidine prephenate might possess more favorable properties. Benzamidine hydrochloride dihydrate was prepared as described in Organic Syntheses (72). The hydrochloride was converted to the sulfate by passage through a column of Dowex-1 in the sulfate form. The benzamidine sulfate obtained in this manner was recrystallized twice from *t*-butanol. Benzamidine prephenate was prepared, and, like the guanidine salt, found to crystallize very slowly. Attempts to recrystallize benzamidine prephenate met with failure.

It was felt that esterification of prephenic acid might result in stabilization of the molecule which would greatly facilitate purification. Attempts to methylate the material with diazomethane in ether and in chloroform were without success because prephenic acid is stable only as a salt, and could not be dissolved in these organic solvents. Therefore

the reaction had to be tried in a two-phase system. Under these conditions, esterification was found to be slow or non-existent. Synthesis of the ester was attempted with methyl iodide and sodium prephenate, in methanol. Because of the instability of prephenate, elevated temperatures were not feasible, and at 35° the reaction was found to proceed too slowly to be useful.

The following procedure was utilized to obtain barium prephenate in the crystalline state and in a high degree of purity. Barium prephenate was isolated in the usual fashion in a purity of 40%. Nine grams of this impure material were dissolved in water (100 ml.) and the pH adjusted to 9.5 with concentrated ammonium hydroxide. An insoluble residue was removed by filtration and discarded. The filtrate was decanted into a column of thoroughly washed Dowex-1 in the chloride form. After the material had been allowed to sink into the resin, the column was again thoroughly washed with water (pH = 7.0). Assay of the wash water indicated that no prephenate was eluted by this treatment. This was followed by gradient elution. The mixer was charged with water (170 ml.), equipped with a magnetic stirring device, and a siphon leading to the reservoir. The reservoir contained ammonium chloride (1.0 M) adjusted to pH 7.7 with ammonium hydroxide. A moderate hydrostatic pressure was applied to give a flow rate of about five drops/minute. This relatively high rate was desirable to minimize the decomposition of the prephenate.

The latter began to appear in the eluate after 160 ml. had been collected, and continued to be eluted in the next 90 ml. The recovery of prephenate from the column appeared to be almost quantitative. The fractions containing prephenate were pooled, and 2 M barium acetate (7.0 ml.) was added. The solution was cooled in an icebath, and two volumes of methanol followed by three volumes of n-propanol were added from a buret, with adequate stirring. The precipitated material was recovered by centrifugation, redissolved in water, and reprecipitated in the same manner. The resulting material was dissolved in water, the slight insoluble residue removed by centrifugation, and the supernatant solution evaporated to dryness in vacuo. The resulting preparation consisted of white crystals. Assay of this material by conversion of a weighed sample to phenylpyruvic acid indicated a purity of $95 \pm 2\%$. A sample of this was redissolved in water and 1.5 volumes of methanol added. The precipitate, which consisted of impure prephenate, was removed. To the supernatant solution was added the remaining half volume of methanol and three volumes of n-propanol. The precipitated material was collected and crystallized as before. Assay of this material indicated a purity of $97 \pm 2\%$. Analysis of this material gave the following values.*

*The author is much indebted to Dr. Adalbert Elek of Elek Microanalytical Laboratories, Los Angeles, for this analysis.

<u>Found</u>	<u>Calculated for barium prephenate</u>
C = 31.20%	C = 31.64%
H = 2.82%	H = 2.66%
Ba = 36.02%	Ba = 36.19%

Dr. Elek reported that the material was strongly hygroscopic, and lost about 4.9% of its weight during drying to constant weight. It is therefore probable that the material was in truth nearly 100% pure. It was also predicted by Dr. Elek that, due to the hygroscopic properties of the material and the humid weather on the day of the analysis, the hydrogen would be slightly high at the expense of barium and carbon.

A sample of barium prephenate of 95 ± 2 % purity was hydrogenated in water over a mixture of Adams' platinum black catalyst and 2% palladinized calcium carbonate, prepared according to the directions of Busch and Stoeve (73). This mixed catalyst had been found to induce much more rapid uptake of hydrogen than did either component separately. The catalyst was reduced before introduction of the barium prephenate. Under these conditions, 3.03 mols of hydrogen were consumed per mol of prephenate added. This presumably corresponds to hydrogenation of the two double bonds and the keto group of the pyruvyl sidechain. The product of this hydrogenation was found not to react with 2,4 dinitrophenylhydrazine.

The stability of barium prephenate was studied at various pH values by periodic examination in the spectrophotometer for production of phenylpyruvic acid. At 0° , the half-life of

prephenate was found to be as follows.

<u>pH</u>	<u>Half-life (approximate)</u>
6.8	No losses detected
5.1	24 hours
4.0	7 hours
2.1	5 hours
1.0	1 hour

The evidence given appears to leave little doubt that the material isolated is the same material designated by Davis as barium prephenate.

K. Isolation and identification of N^α-acetylkynurenine from culture filtrates of 5029a.

Haskins (32) succeeded in isolating kynurenic acid from culture filtrates of this mutant. The ultraviolet absorption spectrum in 0.1 M KH₂PO₄ was observed by Haskins to have absorption maxima at 235 mμ. and 245 mμ. The presence of kynurenic acid accounted for the former only, and it was clear that the latter was due to the presence of an unidentified compound.

Accumulation of this unidentified material was far superior when the mutant was grown in flasks to when it was grown in a carboy under forced aeration. It was found that accumulation of this substance was optimal when the strain was grown at a concentration of nicotinamide of 0.6 μg./ml.

Pooled culture filtrate from about 800 flasks (125 ml. Erlenmeyers charged with 20 ml. of medium) was treated with Norite A (33 g.) with agitation for two hours. The Norite A was removed by filtration and boiled for five minutes with aqueous aniline (10%). The eluate was extracted three times with ether to remove the aniline. The volume was reduced in vacuo to 30 ml. The pH was adjusted to 3.2, which resulted in the precipitation of a considerable amount of material.

The precipitate was dissolved in water, the pH adjusted to 3.9, and the solution continuously extracted with ether for several hours. At the end of this time, yellow crystals had deposited on the walls of the flask containing the ether. Continued extraction resulted in the recovery of 500 mg. of these crystals.

Recrystallization from water with a little Norite gave large clear yellow needles. The spectrum of purified material agreed with that reported by Bonner et al. (74) for N^α-acetylkynurenine, as did the melting point:

Melting point of isolated material: 189-192°

Melting point reported by Bonner: 190-195°

Carbon, hydrogen, and nitrogen analyses gave the following values: *

*The author wishes to thank Mr. G. A. Swinehart for this analysis.

<u>Found</u>	<u>Calculated for N^α-acetylkynurenine</u>
C = 57.40%	C = 57.59%
H = 5.79%	H = 5.64%
N = 9.75%	N = 11.20%

Some of the material was hydrolyzed with dilute sulfuric acid as described by Bonner et al. The kynurenine sulfate obtained was recrystallized from ethanol to give colorless rosettes.

The material before and after hydrolysis was studied chromatographically. Both the native material and its hydrolysate reacted with nitrous acid followed by naphthyl-ethylenediamine to give red spots, proving the presence of a diazotizable amino group in both compounds. However, a spot with ninhydrin appeared only after hydrolysis, consistent with the view than an α -amino acid had been produced. The solvent system used for this chromatographic examination contained three parts n-propanol, one part pyridine, one part water, and one part benzene.

VI METABOLIC CHARACTERISTICS OF THE MUTANTS

Unless otherwise stated, the basal medium used for nutritional studies was that described by Fries (75, 76). This medium contains sucrose, the carbon source, inorganic salts, and a small amount of the vitamin biotin, which is an essential nutrient for all known strains of *Neurospora*. This medium will be referred to as "minimal."

In some cases, it was desirable to use a medium which could readily be maintained at pH 7.0. When Fries medium is used, the pH drops rapidly during growth, reaching values as low as 4.0 after a few days. It was found that a modification of the minimal medium of Westergaard and Mitchell (59) allows good growth and automatically maintains the pH near 7.0. The composition of this medium is as follows.

KNO ₃	-----	1.0 g.
KH ₂ PO ₄	-----	0.5 g.
K ₂ HPO ₄	-----	0.5 g.
MgSO ₄ ·7H ₂ O	-----	0.5 g.
NaCl	-----	0.15 g.
CaCl ₂	-----	0.15 g.
biotin	-----	5.0 µg.
trace elements solution	---	1.0 ml.
sucrose	-----	12.0 g.
water	-----	1.0 liters
ammonium acetate	-----	4.3 g.

The ammonium acetate is not autoclaved with the other nutrients, but is sterilized by adding it as the solid or in solution to the medium immediately after autoclaving. It is also advisable to add a small amount of brom-thymol blue indicator to the medium. This may be autoclaved with the medium prior to addition of the ammonium acetate. Brom-thymol blue is green at pH 7.0.

In a few cases, the pH was observed to drop slightly during growth even when this special medium was employed. This situation was readily corrected by immersing the flasks for a few minutes in a jar of ammonia vapor until the indicator turned green.

Unless otherwise noted, all growth tests were run in 125 ml. Erlenmeyer flasks charged with 20 ml. of medium.

When a strain is said to "grow on Compound A," it is meant that Fries minimal medium supplemented with Compound A will support growth of that strain. It is not meant to imply that the compound will act as a sole source of carbon.

A group of sixteen mutants, which will be referred to as the 13-II-1 class, possess certain common characteristics which allow them to be considered together. These characteristics are as follows.

A. None accumulates material which replaces all or any of the nutritional requirements of any strain of *Neurospora* or of *E. coli* thus far tested.

B. Culture filtrates of these mutants exhibit ultra-

violet absorption spectra which are indistinguishable from that of wild-type *Neurospora*.

C. All strains in this class exhibit a multiple aromatic requirement. The strains do not grow on shikimic acid or quinic acid.

D. Of a number of crosses between mutants of this class, all were sterile.

Strain 13-II-1A, from which the group is named, requires tryptophan, phenylalanine, tyrosine, and *p*-aminobenzoic acid. Indole, anthranilic acid, and *N*-(*o*-carboxyphenyl)glycine were found to be highly active as substitutes for tryptophan. *N*-methyl anthranilic acid shows slight activity. Kynurenine, 5-hydroxyanthranilic acid, shikimic acid, and quinic acid show no activity as substitutes for tryptophan. Phenylpyruvic acid acts as a substitute for phenylalanine in the nutrition of this mutant.

Strain 13-II-2a was frequently used as an assay organism during the course of this work. This mutant shows no obvious metabolic differences from 13-II-1A, but is morphologically more convenient than the latter because of its superior conidiation. Under the assay conditions described on p. 59, DL-tryptophan at a concentration of about 12 $\mu\text{g./ml.}$ allows half-maximal growth when the other nutrients are supplied in excess. The corresponding concentration of DL-phenylalanine was found to be about 15 $\mu\text{g./ml.}$ For L-tyro-

sine, 8 µg./ml. allows half-maximal growth, and for *p*-aminobenzoic acid, 0.003 µg./ml. suffices. Anthranilic acid was found to be approximately twice as effective on a molar basis as DL-tryptophan. Phenylpyruvic acid appeared to be somewhat less effective than DL-phenylalanine. Folic acid was found to substitute for *p*-aminobenzoic acid, but since commercial preparations of the former compound are often contaminated with considerable amounts of the latter, this finding is subject to question. The following compounds were found to be inactive at concentrations up to 5 µg./ml. in replacing *p*-aminobenzoic acid: anthranilic acid, *p*-hydroxybenzoic acid, quinic acid, aminogallic acid, 2-hydroxy-4-aminobenzoic acid, 3-hydroxy-4-aminobenzoic acid, 3-amino-4-hydroxybenzoic acid, 2-amino-5-hydroxybenzoic acid, 2-hydroxy-5-aminobenzoic acid, and 2-hydroxy-3-aminobenzoic acid.

Strain 76A, as previously discussed, was found to require the four aromatic compounds under investigation, and in addition, riboflavin. Quinic acid was found to be inactive as a substitute for any one of the compounds under study. Shikimic acid was found to replace tyrosine, but only after a lag of four or five days, and even then it allowed only about 10% of maximal growth. It was observed to be completely ineffective in replacing tryptophan, phenylalanine, or *p*-aminobenzoic acid.

Strains 76R3a, 76R4A, and 76R9A were obtained from crosses of 76A to wild type. These three strains exhibit a

quadruple requirement, showing that the quintuple requirement of the parent strain is due to the presence of more than one altered gene. These derived strains appear to be metabolically indistinguishable from 13-II-1A.

Strain 15-IV-3a requires only tryptophan, phenylalanine, and tyrosine for growth. It seems probable that the lack of a requirement for p-aminobenzoic acid is due to preferential conversion of metabolites, as described by Davis (see p. 7). In other respects, this mutant conforms to the criteria of the 13-II-1 class.

The remaining strains which were assigned to this class have not been further examined.

Ten mutant strains, designated as the 6D6 class, have been grouped together on the basis of the following properties.

A. Culture filtrates of these mutants contain protocatechuic acid. The latter may be detected by its characteristic ultraviolet absorption spectrum.

B. These strains require tryptophan, phenylalanine, tyrosine, and p-aminobenzoic acid. None was found to grow on shikimic acid or quinic acid.

Strain 6D6a requires approximately 2 $\mu\text{g.}/\text{ml.}$ of DL-tryptophan, 3 $\mu\text{g.}/\text{ml.}$ of DL-phenylalanine, and 2 $\mu\text{g.}/\text{ml.}$ of L-tyrosine for half-maximal growth. In the absence of p-aminobenzoic acid, there is usually slight growth observable, the amount depending on the size of the inoculum. Indole, anthranilic acid, N-methyl anthranilic acid, N-(o-

carboxyphenyl)glycine, kynurenine, and quinic acid will replace tryptophan in the nutrition of this mutant. Shikimic acid was found to be inactive for this purpose. Phenylpyruvic acid shows phenylalanine-replacing activity for this strain. The following compounds were shown to be inactive in replacing phenylalanine: trans-cinnamic acid, p-hydroxybenzoic acid, p-hydroxyphenylacetic acid, gallic acid, α -phenylglycine, chloramphenicol, DL-threo-1-phenyl-2-amino-1,3-propanediol, * D-threo-1-(p-nitrophenyl)-2-amino-1,3-propanediol, * DL-1-hydroxy-2-amino-3-phenylpropane, + 3,4-dihydroxyphenylalanine, 2,3-dihydroxyphenylalanine, and 2,5-dihydroxyphenylalanine. p-Hydroxyphenylpyruvic acid was found to replace tyrosine in the nutrition of 6D6a.

This strain and others in the class accumulate no detectable materials for growth of strains of the 13-II-1 class, for the two strains of the C-86 class (see p. 98), or for one another. Strain 6D6a appears to accumulate material at the very borderline of detection by E. coli strain 83-1, which responds to shikimic acid and 5-dehydroshikimic acid. The uncertainty of the actual presence of growth-promoting

*The author wishes to acknowledge the generosity of Dr. George W. Moersch of Parke, Davis & Company in furnishing these compounds.

+The author is much indebted to Dr. D. W. Woolley of the Rockefeller Institute for Medical Research for supplying this compound.

material has thus far discouraged any attempt to elucidate its nature.

The isolation of protocatechuic acid from culture filtrates of 6D6a has been described on p. 66. Small amounts of vanillic acid have also been detected on paper chromatograms. The latter is present in concentrations too low to be detected in raw culture filtrates, but may easily be concentrated by adsorption on Norite A and subsequent elution with alcoholic ammonia.

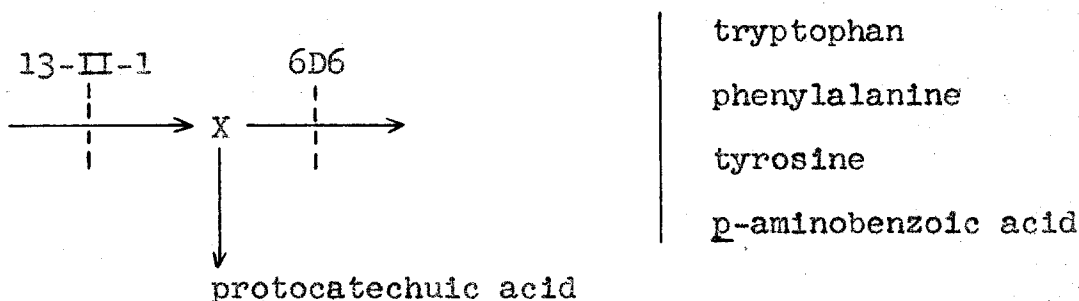
Strain C-120A, kindly made available by Dr. George R. Dubes, requires somewhat higher concentrations of nutrients in order to grow than does 6D6a. The "borderline" accumulation of growth factors for E. coli appears to be absent in C-120A. No other metabolic differences have been noted. Evidence has been presented that these strains are probably allelic.

Strain 6-IV-3a accumulates large amounts of protocatechuic acid. No material active for growth of any of the bacterial strains could be detected in culture filtrates of this mutant.

Other strains that were assigned to the 6D6 class on the basis of nutritional requirements and of ultraviolet absorption spectra, have not been further studied.

The double mutants 13-II-1/6D6 and 15-IV-3/C-120 were prepared as described on p. 53. Neither of these double mutants accumulates protocatechuic acid. It appears, there-

fore, that the presence of a gene of the 13-II-1 class suppresses the accumulation which is characteristic of the 6D6 class. For this reason, the metabolic lesion associated with the 13-II-1 class has been assigned a position earlier in the biosynthetic sequence than that associated with the 6D6 class. The diagram below illustrates this situation.



Two strains will be referred to as comprising the C-86 class. Of these, both will grow on a variety of aromatic compounds, and neither exhibits a multiple requirement. C-86 has been described by Gordon *et al.* (5), and on p. 4 of this thesis. The other strain in this class, 15-IV-1a, was examined for nutritional requirements. Fifty-nine compounds, including the commonly-occurring amino acids, vitamins, purines, pyrimidines, nucleosides, and a variety of aromatic and hydroaromatic compounds were tested for ability to support the growth of these two mutants. Of these com-

pounds, tryptophan, anthranilic acid, N^{α} -acetylkynurenine, quinic acid, phenylalanine, phenylpyruvic acid, tyrosine, and nicotinamide were found to support the growth of both mutants. C-86 grows well on indole, whereas 15-IV-1a was found to grow only a trace under the same conditions. Conversely, benzoic acid was observed to support the growth of 15-IV-1a, but not of C-86. The optimum concentration of benzoic acid for the former strain is about 50 $\mu\text{g./ml.}$ At higher concentrations, growth appears to be inhibited. It is to be noted that even at optimum concentration, growth on benzoic acid is only about 20% of that obtainable with tryptophan.

Culture filtrates of C-86 and of 15-IV-1a were found to possess no nutritional activity for any of the requirements of 13-II-1A, 13-II-2a, 76A, or 6D6a; the culture filtrates were observed to be similarly inactive for the bacterial assay strains.

Isolation of the double mutant 6D6/C-86 has been discussed on p.55. The double mutant shows the nutritional requirements characteristic of the 6D6 class, and shows no growth on quinic acid. The double mutant accumulates protocatechuic acid. On the basis of these observations, the lesion associated with the 6D6 class was tentatively placed before that of the C-86 class in the biosynthetic sequence. More recently, it has been found that culture filtrates of this mutant will relieve the tyrosine requirement of strain

76A in the same manner as does shikimic acid (see p. 94). When these culture filtrates were tested with the bacterial assay strains, an abundant accumulation of material with the nutritional properties of shikimic acid was noted. Chromatographic evidence has been presented on p. 69 that this material is indeed shikimic acid. As was mentioned, small amounts of a second material could also be detected. In view of the fact that 6D6a accumulates, at most, traces of activity for the bacterial strains, and C-86 and 15-IV-1a accumulate none, the behavior of the double mutant is difficult to interpret.

A *Neurospora* mutant, 75001, which requires only tryptophan, was available at the beginning of these studies. This strain will grow on anthranilic acid or indole, and is therefore considered to have its metabolic lesion rather early in the biosynthetic pathway of tryptophan, but after the branch point at which synthesis of the aromatic metabolites diverges. Culture filtrates of this mutant contain small amounts of material which will replace phenylalanine in the nutrition of 13-II-2a. When strain 75001 was grown in neutral medium as described on p.91 and the culture filtrates assayed for prephenic acid as described on p. 62, the latter compound appeared to be present in amounts of about 80 µg./ml. Phenylpyruvic acid was also detected as such in slightly lesser amounts. It is extremely likely that the former compound would be completely converted

to the latter under the conditions of biological assay. The accumulation of phenylalanine precursors by a tryptophan mutant is difficult to interpret at present, but is probably due to some secondary upset in metabolic balance, rather than to the usual relationship between a metabolic lesion and an accumulation of biologically active material (see p. 3).

Strain 44A grows maximally on DL-phenylalanine at a concentration of 50 $\mu\text{g./ml.}$ and half maximally at roughly half this concentration. This mutant will also grow about half-maximally on L-tyrosine at a concentration of 25 $\mu\text{g./ml.}$, but growth is not increased by increasing the concentration of L-tyrosine. When both DL-phenylalanine and L tyrosine are furnished, half-maximal growth occurs at much lower concentrations, although the exact levels have not been determined. There is some doubt, therefore, as to whether the mutant should be classified as a phenylalanine-requiring strain, or a phenylalanine + tyrosine-requiring strain. For purposes of nomenclature, it has been arbitrarily been classified as the former. Phenylpyruvic acid was found to satisfy the nutritional requirements of this strain.

Culture filtrates of this strain were observed to be inactive in relieving any of the nutritional requirements of 13-II-2a or 76A. These filtrates also failed to promote the growth of any of the bacterial assay strains. No fluorescence or ultraviolet-absorbing materials were detected. After six days' growth, culture filtrates were treated with Nite A,

and the adsorbed materials eluted with alcoholic ammonia. Chromatographic study of this eluate revealed that traces of vanillic acid were present. No other accumulated materials were detected in these filtrates.

A mutant designated as 5212 was available to these studies from their inception. This strain, which grows half-maximally on DL-phenylalanine at a concentration of about 2 $\mu\text{g./ml.}$, has been described previously (32). Strain 5212 was found to accumulate materials which satisfy the tryptophan requirement and the phenylalanine requirement of 13-II-2a, 76A, and 6D6a. Culture filtrates of 5212 were not observed to contain materials which could replace all the nutritional requirements of the bacterial assay strains.

The filtrates had been found by Haskins (32) to contain fluorescent materials, and evidence was given that anthranilic acid is present, explaining the tryptophan-replacing activity of the filtrates. In addition, the mutant was observed by Haskins to exhibit self-feeding, i.e., to accumulate materials which are used by the mutant itself for growth. This peculiar situation becomes more easily understood by growing the mutant in medium of pH 7. Under these conditions, it is found that prephenic acid is present in the culture filtrates in amounts up to 200 $\mu\text{g./ml.}$ Under normal conditions of pH (mildly acid), this compound is rapidly converted to phenylpyruvic acid in the medium. Phenylpyruvic acid has been found to satisfy the phenylalanine requirement of this strain.

Therefore it may be hypothesized that the metabolic lesion is due to the absence or inactivity of the enzyme which normally converts prephenic acid into phenylpyruvic acid. It seems probable, then, that this strain does not actually accumulate materials which can be used as such in its nutrition, but rather excretes accumulated material into the medium, where it is rapidly converted non-enzymatically into a compound that can be utilized for growth of the strain.

The double mutant 44/5212 was isolated as described on p. 54. It was found not to accumulate any fluorescent material, nor any phenylpyruvic acid or prephenic acid. It was also found that this double mutant resembles strain 44A in its inability to accumulate any materials active in replacing the tryptophan or phenylalanine requirements of strains of the 13-II-1 class. On these grounds, it was felt that the metabolic lesion associated with the 44A gene is earlier in the biosynthetic pathway of phenylalanine than is the lesion associated with the 5212 gene. More recently, it has been found that the double mutant accumulates considerable amounts of material which will satisfy all of the nutritional requirements of the two bacterial strains that will respond to 5-dehydroshikimic acid. Under these conditions, the strain which is specific for shikimic acid shows no growth, and it therefore seems clear that the former compound, or something that is nutritionally similar, is accumulated by the double mutant. The significance of the accu-

mulation of this compound is difficult to assess, but it appears that there is some conflict with Davis' interpretation of the data obtained from E. coli strains. As has been noted, 5-dehydroshikimic acid has been pictured as occurring rather early in the biosynthetic pathway of the aromatic amino acids, and several steps before the branch point.

One strain, designated as 13-II-3a, has been classified as a phenylalanine-requiring mutant. This strain requires a concentration of DL-phenylalanine of about 25 µg./ml. for half-maximal growth. The mutant shows slight growth on tyrosine at lower concentrations, but this effect is not increased by increasing the concentration. Of a variety of other compounds tested, none was found to satisfy the nutritional requirements of this mutant. Phenylpyruvic acid was observed to be totally inactive in promoting growth. This is the only strain among twelve tested that fails to utilize phenylpyruvic acid as a substitute for phenylalanine. This characteristic has been considered sufficient to set 13-II-3a in a separate class. It appears that this mutant may be lacking the transaminase which normally converts phenylpyruvic acid into phenylalanine. For this reason, 13-II-3a has tentatively been assumed to possess a lesion later in the pathway of phenylalanine synthesis than does 5212.

It must be noted that 13-II-3a accumulates no materials active for the nutrition of 13-II-2a, 76A, 6D6a, C-86, or the bacterial assay strains. The ultraviolet absorption

spectrum of culture filtrates of 13-II-3a is identical with that of wild-type *Neurospora*. It is perhaps surprising that this strain, presumed to be blocked in the conversion of phenylpyruvic acid into phenylalanine, does not accumulate the former compound. The relative infertility of this mutant in crosses has hampered attempts to examine the possibility of allelism of this strain with other phenylalanine-requiring mutants. Its designation as a separate class must therefore remain provisional, pending further information on its metabolic capacities and genetic behavior.

A group of four strains, designated as the 3-IV-3 class, were found to require tyrosine. Evidence has been presented on p. 52 that these strains (3-IV-3a, 16A, 73A, and 74A) are probably alleles. For this reason, only 3-IV-3a has been studied.

This mutant was found to grow half-maximally at a tyrosine concentration of 2 $\mu\text{g./ml.}$ *p*-Hydroxyphenylpyruvic acid was found to be an effective substitute for tyrosine. It was noted that the growth of this strain was somewhat inhibited by the other aromatic amino acids at concentrations of 50 $\mu\text{g./ml.}$ Therefore, the mutant was tested for growth and for inhibition of growth by a variety of amino acids, vitamins, and miscellaneous compounds. The "basal" medium for these tests contained 0.5 $\mu\text{g./ml.}$ of tyrosine, which allows about 25% of maximal growth. Therefore, either stimulation or inhibition of growth could be detected. No

compounds except tyrosine and *p*-hydroxyphenylpyruvic acid were found to stimulate growth, but tryptophan, phenylalanine, serine, glutamic acid, cystine, arginine, alanine, methionine, isoleucine, leucine, glycine, asparagine, ornithine, citrulline, phenylserine, 3,4-dihydroxyphenylalanine, α -phenylglycine, quinic acid, and protocatechuic acid were found to inhibit growth at concentrations of 50 $\mu\text{g./ml.}$ The imino acids, proline and hydroxyproline, were found not to inhibit growth of 3-IV-3a. The vitamins, at concentrations of 5 $\mu\text{g./ml.}$, were observed to be non-inhibitory. This behavior closely resembles that of a tyrosine-requiring strain described by DeBusk and Wagner (77).

Strain 3-IV-3 was found to accumulate materials which would replace tryptophan and phenylalanine in the nutrition of 13-II-1 class mutants and 6D6a. The medium was found to be highly blue-green fluorescent. Evidence has been presented on p. 78 that some of the fluorescence can be attributed to the presence of anthranilic acid. The latter also accounts for the tryptophan-replacing material observed to be present in the medium. The phenylalanine-replacing activity appears to be due almost entirely to the presence of phenylpyruvic acid in the medium. Isolation of the latter from culture filtrates of this mutant has been described on p. 75. It was desired to know whether the phenylpyruvic acid in the culture filtrates is formed from excreted prephenate by the acidity of the medium, or whether phenylpyruvic acid is excreted as such. The strain was grown at

neutrality as previously described and the culture filtrates examined for the presence of these two compounds. It appears that, in the case of 3-IV-3a, phenylpyruvic acid is excreted as such, and that there is little, if any, prephenic acid accumulated by this mutant.

The double mutant 75001/44 was isolated as described on p. 57. This strain accumulates no materials active for the nutrition of 76A or of the bacterial assay strains. The ultraviolet absorption spectra of culture filtrates show no differences from those of wild type. Traces of vanillic acid could be detected by the methods previously described.

Isolation of the double mutant 75001/5212 has been discussed on p. 58. It was found that this strain accumulates material which will replace only phenylalanine in the nutrition of 13-II-2a and 76A. Strain 75001/5212 was grown in neutral medium as previously described. Under these conditions, prephenic acid was found in the culture filtrates in concentrations of approximately 100 $\mu\text{g./ml}$. Little if any phenylpyruvic acid is accumulated as such by this strain. Culture filtrates of 75001/5212 were examined for accumulation of materials active for the multiple requirement of the bacterial assay strains. No such material was found.

The preparation of the tryptophan-tyrosine-requiring double mutant 75001/3-IV-3 has been described on p. 55. This strain accumulates material which will replace phenyl-

alanine in the nutrition of 13-II-2a and 76A. It accumulates no fluorescent materials, nor does it accumulate material which will satisfy the multiple requirement of bacterial assay strains. Growth of 75001/3-IV-3 in medium of pH 7 and analysis of culture filtrates as previously described has shown that little or no prephenic acid is accumulated, but that phenylpyruvic acid is accumulated as such in concentrations of approximately 250 µg./ml.

Preparation of the double mutant 44/3-IV-3 has been described on p. 56. This strain does not accumulate fluorescent material, nor does it accumulate any material active for the nutrition of 13-II-2a or 76A. Culture filtrates of 44/3-IV-3 contain material which relieves the multiple requirement of two of the three bacterial assay strains, and this material is therefore believed to be 5-dehydroshikimic acid. Culture filtrates of this double mutant after four days' growth exhibit ultraviolet absorption spectra which differ only slightly from those of wild-type *Neurospora*. However, after six days' growth, filtrates of this strain can be seen to contain considerable quantities of protocatechuic acid. Vanillic acid has also been detected in these filtrates by methods previously described.

The double mutant 5212/3-IV-3 was isolated as described on p. 56. It was noted that this strain accumulates intensely fluorescent materials, as do both the parent strains. Culture filtrates contain materials which replace tryptophan

and phenylalanine in the nutrition of all the Neurospora assay strains. Isolation of anthranilic acid from these filtrates has been discussed on p. 79. Chromatography of culture filtrates has shown the presence of phenylpyruvic acid, a compound known to be accumulated by both parent strains. It was desired to know whether this compound is accumulated as such by the double mutant, or whether it is the result of degradation of prephenic acid by the acidity of the medium. Unfortunately, this strain grows so poorly in neutral medium that it has not been possible to resolve this question. However, it may be surmised that the presence of the 5212 gene in this strain would favor the accumulation of prephenic acid rather than phenylpyruvic acid.

Preparation of the triple mutant 75001/44/3-IV-3 has been discussed on p. 57. This strain does not accumulate materials which satisfy any of the nutritional requirements of Neurospora strains 13-II-2a or 76A. However, it accumulates a considerable amount of material which satisfies all the nutritional requirements of two of the three bacterial assay strains. Chromatographic evidence has been presented on p. 70 that this material is 5-dehydroshikimic acid. After five days' growth in either flasks or in carboys under forced aeration, protocatechuic acid could be observed in large amounts in the culture filtrates. Isolation and identification of the latter compound and of vanillic acid from culture filtrates of this strain have been described on p. 71 and on

p. 73 respectively. Evidence has been given on p. 75 that p-hydroxyphenylacetic acid is also present in these filtrates. As this compound is known to be a catabolite of tyrosine in certain organisms (78), it was desired to know whether its presence in these filtrates is due to accumulation behind a metabolic lesion or to catabolism of added tyrosine. Five 125 ml. Erlenmeyer flasks were charged with 20 ml. of Fries medium containing DL-tryptophan (25 μ g./ml.), and DL-phenylalanine (25 μ g./ml.). This basal medium was supplemented with L-tyrosine at five different concentrations. The flasks were inoculated with strain 75001/44/3-IV-3 and allowed to grow for 96 hours at 25^o. At the end of this time, the mycelium was removed, pressed, dried, and weighed. The culture filtrates from the four flasks in which growth occurred were poured into small columns containing 400 mg. of Norite A and 1600 mg. of H1-flo Supercel. The columns were washed with water to remove non-aromatic materials. They were then eluted with alcoholic ammonia (10 ml.). The eluates were evaporated, dissolved in water (0.2 ml.), and subjected to chromatography. The chromatograms were sprayed with diazotized sulfanilic acid. It was observed that all the protocatechuic acid had been oxidized during the elution with alcoholic ammonia, but vanillic acid and p-hydroxyphenylacetic acid could readily be observed. The relative amounts present in the different eluates were noted by inspection of the chromatograms. The results are given below.

Flask #	Concentration of tyrosine	Weight of mycelium	Vanillic acid	p-Hydroxyphenylacetic acid
1	10 µg./ml.	25 mg.	++++	±
2	25 µg./ml.	35 mg.	++++	+
3	50 µg./ml.	41 mg.	+++	++
4	100 µg./ml.	41 mg.	++	+++
5	250 µg./ml.	trace	--	--

It will be noted that the strain did not grow at the highest concentration of tyrosine. Vanillic acid shows behavior typical of an accumulation, in that there is less of it present when the organism is replete with respect to one or more of its nutrients. p-Hydroxyphenylacetic acid, on the other hand, is present in amounts which vary directly as the concentration of tyrosine. For this reason, it is believed that it is a catabolite of tyrosine. This phenomenon has not yet been studied with wild-type *Neurospora*. Final proof that this is a catabolite of tyrosine awaits studies with radioactive tyrosine.

It was desired to find if there were any abnormal accumulations in the mycelia of this strain. For this purpose, mycelium (92 g. wet weight) from the carboy from which protocatechuic acid was isolated, was frozen in dry ice and homogenized in a dry ice-cooled Osterizer. The frozen homogenate was carefully added to boiling 95% ethanol (380 ml.). The precipitated material was discarded and the filtrate saved

for future study. Mycelial extracts of wild-type 5297a were prepared under identical conditions for purposes of comparison. Chromatography of these extracts and detection of compounds with the Hanes-Isherwood spray for phosphates, the periodate spray, and ninhydrin showed no significant differences between extracts of the mutant and of wild type. However, it was noted that acidification of extracts of mutant mycelium resulted in a copious precipitate which did not appear on acidification of wild-type extracts. This material was found to be insoluble in water, moderately soluble in alcohol, and easily soluble in ether and in ligroin. It was found to dissolve slowly in 1N NaOH. The nature of this material has not been further investigated, and its significance is difficult to assess at this time.

The triple mutant 75001/5212/3-IV-3 was obtained as described on p. 58. This mutant does not accumulate materials which can act as precursors of tryptophan for the Neurospora assay strains, but accumulates very large amounts of a material which satisfies the phenylalanine requirement of 13-II-2a, 76A, and 6D6a. When this strain is grown in Fries medium, the accumulated material can be identified as phenylpyruvic acid by ultraviolet absorption spectra in acid and base, by a green color reaction with ferric chloride, and by chromatography. Growth of the strain in neutral medium as previously described results in accumulation of prephenic acid in the culture filtrates in concentrations of up to

350 $\mu\text{g./ml.}$ This is by far the largest accumulation of this compound observed in any of the available strains. The isolation of prephenic acid from culture filtrates of this mutant has been described on p. 81. Under conditions of growth in carboys, as described for isolation of this compound, concentrations as high as 600 $\mu\text{g./ml.}$ may be attained in the culture filtrates.

This strain was found to accumulate material which satisfies all the nutritional requirements of two of the three bacterial assay strains; this material is therefore nutritionally identical with 5-dehydroshikimic acid. The concentration of this material in culture filtrates of 75001/5212/3-IV-3 seems to be smaller than in culture filtrates of 75001/44/3-IV-3.

Strains 25A and 65A require guanine or guanosine. Guanylic acid, kindly purified for this work by Dr. Paul R. Whitfeld, does not support growth of these mutants. Evidence has been presented that these strains are probably allelic (see p. 52) and therefore only strain 25A was studied further. The exact concentrations of nutrients required for growth has not been determined, but it was noted that strain 25A will grow luxuriantly in medium supplemented with guanine at a concentration of only 1 $\mu\text{g./ml.}$ This strain shows perceptible growth on minimal after several days. The amount of this growth shows some proportionality to the size of the inoculum. In view of the low requirement for guanine, it was found advisable to carry the stock on slants containing guanine at a concentra-

tion of 1 $\mu\text{g.}/\text{ml.}$ This strain fails to grow on adenine, adenosine, uridine, cytidine, thymidine, or a wide variety of amino acids and vitamins. Slow growth was observed in the presence of phenylalanine, tryptophan, riboflavin, and thiamin hydrochloride, but these effects were sporadic and not always reproducible.

Examination of the ultraviolet absorption spectra of culture filtrates of 25A in acid and in base revealed no differences from wild type.

To the knowledge of this author, guanine mutants in *Neurospora* have not been previously described, and for this reason, the above discussion has been included in this thesis. However, it is recognized that these observations are probably not germane to the problem of the biosynthesis of aromatic compounds.

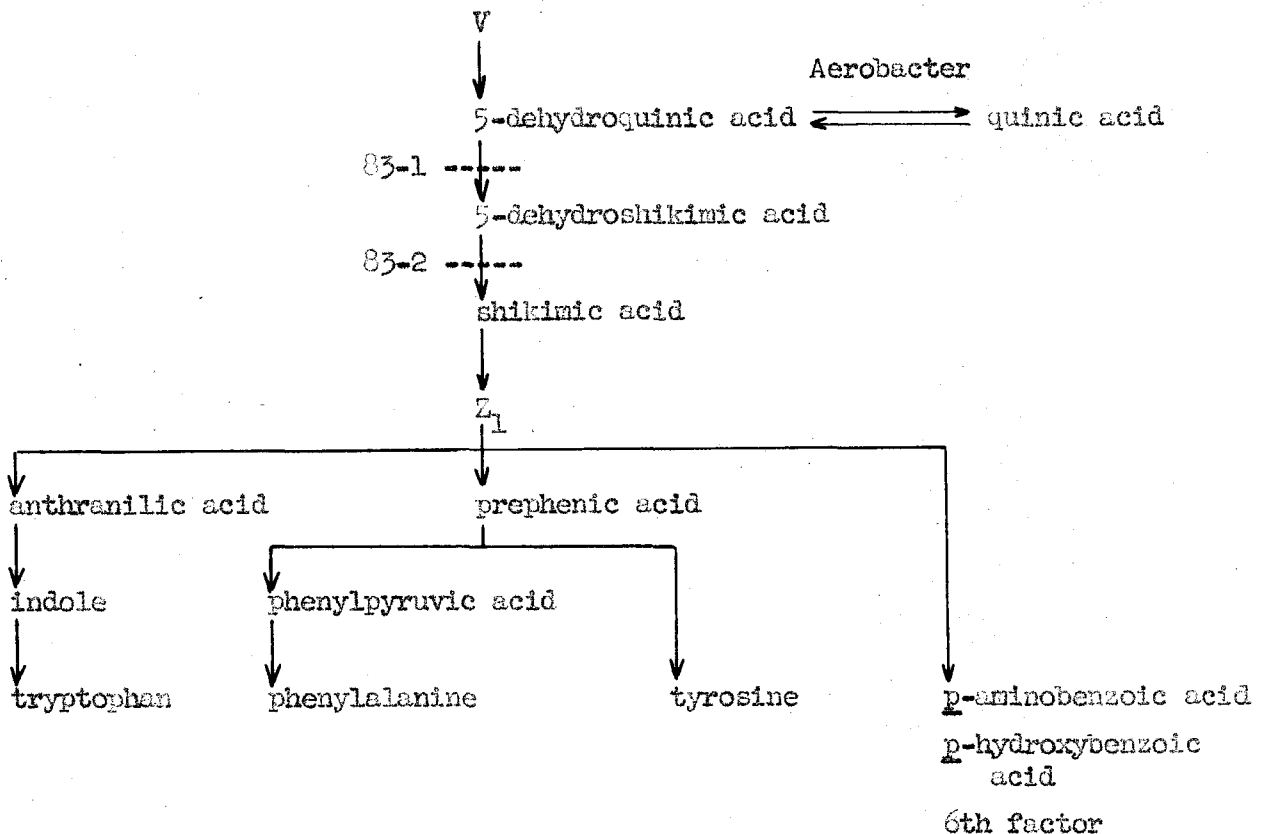
In conclusion of this section, it should be mentioned that the involvement of phosphorylated intermediates that do not escape from the mycelium might explain some of the observed anomalies in crossfeeding behavior. In order to test this hypothesis, strains 5297a (wild type), 13-II-1A, 13-II-2a, 15-IV-3a, 6D6a, 6-IV-3a, C-86a, 15-IV-1a, 75001A, 5212A, 13-II-3A, 3-IV-3a, 75001/5212, 75001/3-IV-3, 5212/3-IV-3, and 75001/5212/3-IV-3 were grown on Fries medium supplemented with DL-tryptophan (25 $\mu\text{g.}/\text{ml.}$), DL-phenylalanine (25 $\mu\text{g.}/\text{ml.}$), L-tyrosine (25 $\mu\text{g.}/\text{ml.}$), and p-aminobenzoic acid (0.1 $\mu\text{g.}/\text{ml.}$) under the usual conditions. After four days' growth the

mycelia were squeezed dry,, ground with sand under 70% ethanol, and the homogenates heated in a boiling water bath for one minute. The coagulated material was removed by centrifugation, and the supernatant solutions evaporated and redissolved in a small volume of water. These extracts were subjected to chromatography in a solvent system consisting of two parts 95% ethanol, one part n-amyl alcohol, and one part 1N acetic acid. The dried chromatograms were sprayed with the Hanes-Isherwood reagent for detection of phosphates. In no case was any mutant found to accumulate phosphorus-containing compounds not present in wild-type *Neurospora*. However, it should be noted that the sensitivity of the method leaves much to be desired, and that heavier loading of the chromatograms results in smearing. These results indicate that there are no gross abnormal accumulations of phosphorylated intermediates in the mycelia of these mutants. It is probable that small accumulations of such compounds would have escaped detection.

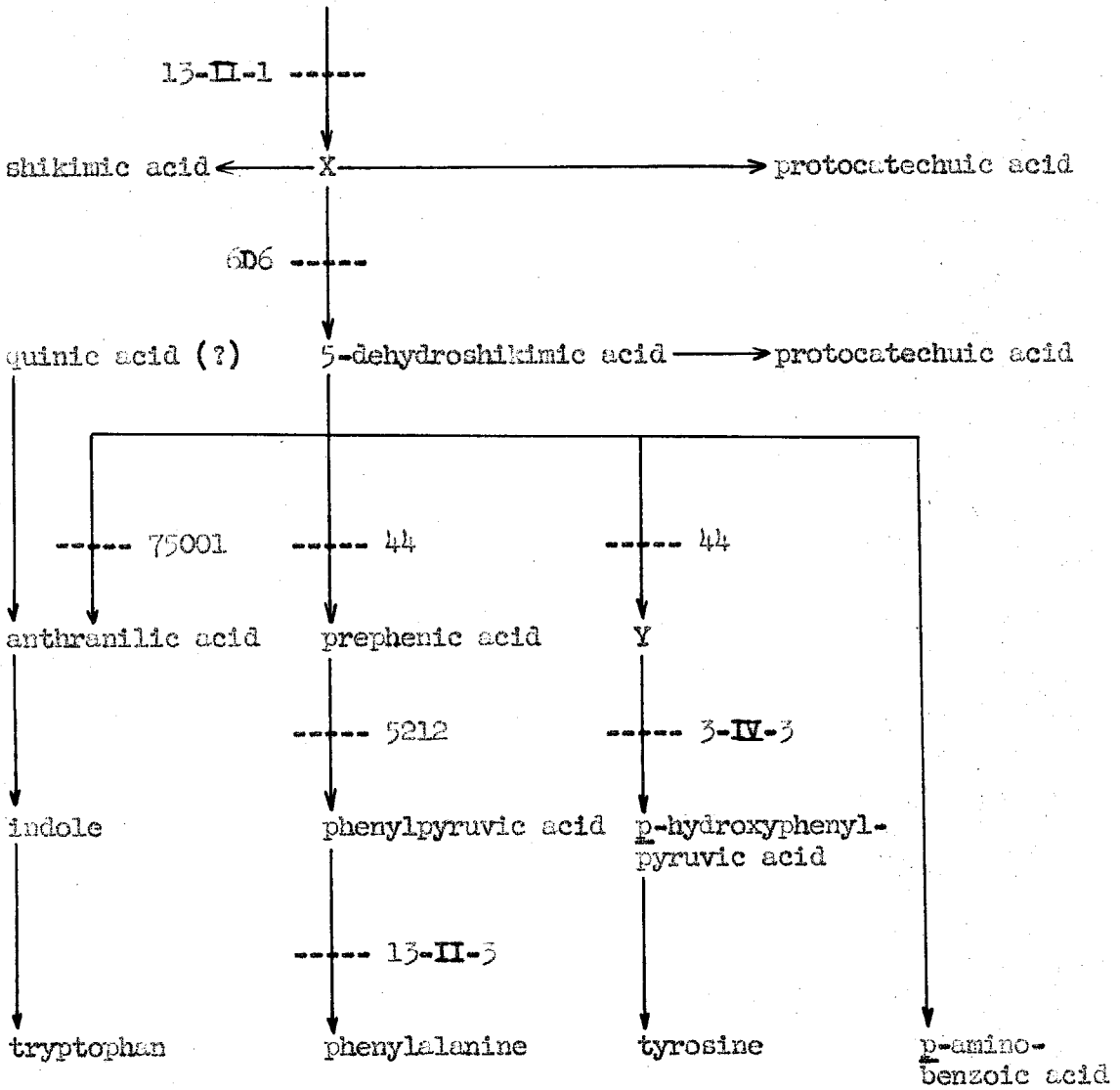
VII DISCUSSION

Evidence presented in previous sections of this thesis makes it appear that the biosynthesis of aromatic compounds in *Neurospora* shows both similarities and contrasts with biosynthesis of the same compounds in bacteria. Comparison of the biosynthetic pathways as visualized by Davis (13) in *E. coli* and as visualized by the present author in *Neurospora* are shown diagrammatically below.

E. coli:



Neurospora crassa:



The characteristics of the mutant strains of *Neurospora* which resemble those of *E. coli* may be summarized as follows.

(a) Mutants may be obtained which exhibit nutritional requirements for tryptophan, phenylalanine, tyrosine, and p-aminobenzoic acid.

(B) Under certain conditions, materials identified as shikimic acid, 5-dehydroshikimic acid, and prephenic acid are found to be accumulated by some of the mutant strains.

Those features of aromatic biosynthesis in *Neurospora* which differ most sharply from the pathway in *E. coli* suggested by Davis are enumerated below.

(A) Strains of the 13-II-1 class and of the 6D6 class have been shown to be non-allelic with respect to the altered gene, yet do not exhibit crossfeeding behavior. A number of bacterial mutants showing comparable nutritional requirements have been found to crossfeed abundantly.

(B) Of 26 strains found to require a multiple aromatic supplement, none was found to utilize shikimic acid as a substitute for this multiple aromatic supplement. It is evident that no *Neurospora* mutant of the type described by Tatum (6) has been obtained in the present investigation.

(C) Strains of the C-86 class will utilize quinic acid as the sole supplement. No strain of bacteria showing the nutritional properties of C-86 has been isolated.

(D) The compound designated as 5-dehydroshikimic acid appears to be located at (or near) the point in the pathway

at which synthesis of the various aromatic metabolites diverges. In the strains of bacteria investigated by Davis and co-workers, 5-dehydroshikimic acid has been identified as a precursor of shikimic acid, and therefore as a relatively "early" precursor of the aromatic metabolites.

(E) Prephenic acid has been found in culture filtrates of a tryptophan-requiring strain, a phenylalanine-requiring strain, and certain double and triple mutants. In E. coli, prephenic acid has been found in culture filtrates of phenylalanine-requiring strains, and tyrosine-requiring strains.

It seems fitting to discuss the above differences in somewhat greater detail.

The reason for the lack of crossfeeding between strains of the 13-II-1 class and those of the 6D6 class is not known. It seems possible that one or more of the complications mentioned on p. 4 is in operation. It is also to be noted that if these mutants do indeed accumulate compounds which, due to considerations of permeability, do not cause crossfeeding of the *Neurospora* strains, these compounds are clearly not shikimic acid, 5-dehydroshikimic acid, 5-dehydroquinic acid, or quinic acid. The latter four compounds would have been detected by assay of the culture filtrates with the bacterial strains provided by Davis.

The data resulting from the present investigation suggest that shikimic acid is not an obligatory intermediate in the biosynthesis of aromatic compounds in *Neurospora*. It has been

observed (see p. 94) that under certain conditions, shikimic acid may be slowly utilized as a substitute for tyrosine. In the same strain, however, it does not satisfy any of the other nutritional requirements. The fact that it may be used as a substitute for tyrosine, a major metabolite, and yet does not relieve the relatively minute requirement for *p*-aminobenzoic acid makes it appear unlikely that the failure of shikimic acid to satisfy all the nutritional requirements of this strain is due to its failure to enter the cell. The substitution of shikimic acid for single metabolites in other strains has not been investigated. The accumulation of shikimic acid by strain 6D6/C-86 has already been discussed briefly. This observation implicates shikimic acid in the biosynthesis of aromatic compounds by *Neurospora*, but does not seem sufficient evidence to warrant its classification as an obligatory intermediate. One may speculate that 6D6 accumulates an unknown intermediate which is normally detoxified by dehydrogenation to protocatechuic acid. Evidence obtained by Haskins (32) indicates that C-86 probably has a partial block in the conversion of tryptophan into nicotinic acid. Therefore it seems possible that 6D6/C-86 has a relatively low supply of nicotinic acid and hence a low supply of the pyridine nucleotides which would be needed for dehydrogenation of the unknown intermediate to protocatechuic acid. For this reason, an appreciable amount of the unknown material might be detoxified by conversion into shikimic acid. The author wishes

to emphasize that he fully appreciates the speculative nature of this interpretation, and suggests that the hypothesis might be experimentally tested by growing strain 6D6/C-86 on medium supplemented with tryptophan, phenylalanine, tyrosine, *p*-aminobenzoic acid, and in addition, nicotinic acid or nicotinamide. Culture filtrates of the mutant grown in the above medium would then be assayed with the bacterial strains for the presence of shikimic acid. If the hypothesis is correct, addition of nicotinic acid or nicotinamide to the medium should favor dehydrogenation of the unknown material to protocatechuic acid, and therefore the accumulation of shikimic acid should be suppressed.

The nutritional requirements of strains of the C-86 class are difficult to reconcile with those of strains which require four aromatic compounds. The following hypothesis, based largely on the work of Haskins (32) and Gordon, Haskins, and Mitchell (5) is offered, pending further investigation of this problem. It is presumed that C-86 and 15-IV-1 are partially blocked in the catabolism of tryptophan, and that the true metabolic lesion is the result of a dearth of nicotinamide and of the coenzymes which contain this vitamin. Because the block is incomplete, any factor which tends to increase the amount of precursor accumulating behind the blocked reaction will, by mass action, increase the "leakage" through the block and thus allow increased synthesis of nicotinic acid. This explains the fact that mutants of this

class will grow on nicotinamide at low concentration, but require a much larger concentration of tryptophan. These strains have also been noted to grow on phenylalanine and on tyrosine, though less rapidly than on tryptophan. It seems reasonable that addition of phenylalanine or tyrosine to the growth medium would tend to divert common aromatic precursors into the synthesis of tryptophan, and thus promote the synthesis of nicotinic acid in the same manner as does tryptophan, although less directly. The growth of the C-86 class mutants on quinic acid may be interpreted in a number of ways. The observation that quinic acid will relieve the tryptophan requirement of 6D6a suggests that quinic acid may, at least under certain conditions, be converted into tryptophan, or into a compound with tryptophan-sparing ability. The growth of the C-86 class mutants on quinic acid might therefore be due to the occurrence of this conversion. It has been noted that 15-IV-1 shows appreciable growth on benzoic acid. As the latter compound was of primary standard purity, it seems highly unlikely that this surprising effect is due to the presence of biologically active impurities. As yet, not even the most speculative hypothesis has been devised to explain the participation of benzoic acid in the biosynthesis of aromatic compounds.

The accumulation of 5-dehydroshikimic acid presents some problems of interpretation. It will be noted that this compound does not appear in the culture filtrates of any strain

containing only one metabolic lesion, but appears in filtrates of both triple mutants investigated, and in filtrates of several of the double mutants. For this reason, it is felt that 5-dehydroshikimic acid may be the compound at the branch point, as shown on p. 117.

It must be admitted that culture filtrates containing this compound do not satisfy any of the amino acid requirements of strains 13-II-2a or 76A. However, it is possible that this material fails to enter the cell. In fresh Fries medium (pH ca. 5.5), 5-dehydroshikimic acid would presumably be almost totally dissociated. If the anion does not enter the Neurospora cell, it is not contradictory to find that this material is nutritionally inert for Neurospora. It would perhaps be of interest to investigate the activity of this possible "branch point compound" for the Neurospora assay strains at the lower limit of pH tolerance of Neurospora. It is possible that under these conditions, culture filtrates of a mutant such as 75001/44/3-IV-3 would support the growth of such assay strains as 13-II-1.

The accumulation of protocatechuic acid by 75001/44/3-IV-3 has been mentioned. The latter compound can be seen to differ from 5-dehydroshikimic acid by the elements of water. It is probable that the protocatechuic acid accumulated by this strain is formed by dehydration of 5-dehydroshikimic acid, perhaps as a detoxification mechanism.

It has been noted that the tryptophan-requiring strain 75001 accumulates detectable amounts of prephenic acid and

phenylpyruvic acid. This does not appear sufficient grounds for classifying these compounds as tryptophan precursors. It seems more likely that the lack of production of tryptophan causes increased amounts of the common precursors to be diverted toward the production of phenylalanine, and that the conversion of prephenic acid into phenylpyruvic acid and of the latter into phenylalanine are rate-limiting reactions under these conditions. Weiss et al. (23) have reported that prephenic acid is accumulated by certain tyrosine mutants, and have concluded that prephenic acid is probably a precursor of tyrosine as well as of phenylalanine. In the opinion of this author, such a conclusion is somewhat premature. In order to interpret these data as establishing that prephenic acid is a precursor of tyrosine, one must also concede that the former is a precursor of tryptophan. On structural grounds, this seems unlikely.

Similarly, the accumulation of anthranilic acid by the phenylalanine-requiring strain 5212 and by the tyrosine-requiring strain 3-IV-3a should not be taken as evidence that anthranilic acid is a precursor of phenylalanine and tyrosine. It seems more likely that anthranilic acid is accumulated by these mutants as the result of increased amounts of common precursors being channeled into the production of tryptophan, and that removal of anthranilic acid becomes the rate-limiting reaction in this "overloaded" pathway. The same explanation is favored for accumulation of phenylpyruvic acid by 3-IV-3a

and 75001/3-IV-3.

It should be mentioned that the presence of the 75001 gene in any strain completely suppresses accumulation of tryptophan-replacing metabolites. It seems possible that the metabolic lesion in this strain occurs immediately after the branch point, and that the equilibria and dynamics of the system favors conversion of the "branch point compound" into such materials as prephenic acid and phenylpyruvic acid. Introduction of the 5212 gene suppresses conversion of the former compound to the latter, and causes prephenic acid to be accumulated. The presence of the 44 gene suppresses all accumulation of tryptophan-replacing material, and of phenylalanine-replacing material. The reason for the suppression of the accumulation of anthranilic acid by the presence of this gene is not clear. One may suppose that possibly the introduction of this gene alters the complex dynamic equilibria which, in its absence, result in the accumulation of anthranilic acid. It is noteworthy that 3-IV-3a accumulates no detectable tyrosine-replacing material, nor do other strains. The accumulation of shikimic acid by 6D6/C-86 and its very slow utilization by 76A as a substitute for tyrosine may be taken as an exception to the above statement, but it is probable that even this crossfeeding would escape detection in an ordinary four day growth test. It seems likely that 3-IV-3a is blocked in the synthesis of tyrosine fairly close to the branch point. The strong sparing effect exerted

by tyrosine in the nutrition of 44A has led to the belief that the latter mutant has a partial block in the synthesis of tyrosine as well as of phenylalanine. Various considerations already discussed have led to the hypothesis that this block occurs very soon after the branch point.

It seems fitting to place the 3-IV-3a block immediately after the partial block in tyrosine synthesis caused by the 44 gene.

The accumulation of vanillic acid by some of the mutants is of interest. It is probably formed by methylation of proto-catechuic acid, perhaps as a detoxification of the latter. Methylation of phenolic compounds probably is a reaction of relatively minor importance in *Neurospora*, but is of considerable interest in the biosynthesis of anthocyanins and alkaloids in higher plants. It is possible that further study of the formation of vanillic acid in *Neurospora* would shed some light on analogous reactions in the biosynthesis of other compounds containing the methoxy group.

In concluding the discussion of the data presented in this thesis, it is perhaps appropriate to consider some possible approaches in future investigation of the problem of biosynthesis of aromatic compounds in *Neurospora*. First, it is clear that there are many more reactions in this biosynthetic pathway than are represented by the number of genetically distinct mutants obtained in this study. It is by no means certain that the twenty-seven available mutants

which require a multiple aromatic supplement fall into only two genetic classes. Limitations of time have thus far prohibited a thorough investigation of these, but it seems possible that examination of some of these strains for heterocaryosis might, with a more modest investment of labor, reveal the presence of more classes of mutants than have as yet been demonstrated. It would also be of considerable interest to obtain new examples of strains requiring phenylalanine or tyrosine. It has been noted that only one class of mutant requiring only tyrosine has been found in these studies. The author believes that new mutants requiring these amino acids will allow new insights into their biosynthesis. Techniques for selection of mutants of *Neurospora* have been discussed in an earlier section of this thesis, and it will be appreciated that mutants can now be obtained in large numbers with relatively little labor. It is felt that many new mutants would be obtained from a small selection program, and that the information to be gained from study of these mutants would be well worth the trouble of isolating them.

Another approach that might be considered is the preparation of additional double and triple mutants. Only two triple mutants, 75001/44/3-IV-3 and 75001/5212/3-IV-3, have been prepared. It is evident that only one tryptophan-requiring strain, one tyrosine-requiring strain, and two phenylalanine-requiring strains are represented in these triple mutants.

The nutritional properties and the materials accumulated by such multiple mutants as C-86/75001, C-86/44, C-86/5212, C-86/3-IV-3, C-86/44/3-IV-3 and C-86/5212/3-IV-3 might be of considerable interest. Other strains requiring tryptophan, phenylalanine, or tyrosine might likewise be incorporated into multiple mutants and result in the discovery of new intermediates in the biosynthesis of aromatic amino acids. In addition, strains which require the vitamin p-aminobenzoic acid are available. These might be crossed to triple mutants to give quadruple mutants of interest.

Two nutritionally inactive compounds, designated by Davis as Z_1 and 5-phosphoshikimic acid (see pp. 13, 14) have been found in culture filtrates of E. coli. As yet, no attempt has been made to detect these materials in filtrates of the Neurospora mutants. It is felt that this question is worthy of investigation.

Enzymatic studies of the Neurospora mutants have not been attempted. Extracts of various mutants might effect some very interesting transformations of such compounds as 5-dehydroshikimic acid and 5-dehydroquinic acid, which, due to permeability barriers, have thus far not been noted. It seems possible that extracts of the triple mutant 75001/5212/3-IV-3 might be capable of condensing 5-dehydroshikimic acid with pyruvic acid or a similar three-carbon compound to form prephenic acid. Thus enzymatic analysis might be a very valuable complement to the in vivo studies which have been initiated.

Last of all, it must be emphasized that although knowledge has been gained through study of mutants of bacteria and Neurospora, the largest and most important group of organisms which carry out these syntheses--the higher plants--remain virtually unexamined. The great variety of aromatic compounds found in the higher plants assures that an untold number of interesting reactions will be available for study.

VIII PERMANENT NOMENCLATURE OF THE NEW MUTANT STRAINS
RESULTING FROM THIS INVESTIGATION

A number of the newly isolated strains are being saved for possible future work by other investigators. These strains have been assigned official numbers, which are given below.

Nomenclature used in this thesis	Permanent nomenclature	Nutritional requirements
13-II-1A	C-161A	tryptophan, phenylalanine, tyrosine, p-aminobenzoic acid
15-IV-3a	C-162a	tryptophan, phenylalanine, tyrosine
6D6a	C-163a	tryptophan, phenylalanine, tyrosine, p-aminobenzoic acid
15-IV-1a	C-164a	"C-86 type requirements"
44A	C-165A	phenylalanine
13-II-3a	C-166a	phenylalanine
3-IV-3a	C-167a	tyrosine
76R7*	C-168*	riboflavin
25A	C-169A	guanine

*The mating type of this culture has not been determined.

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