The Biosynthesis of Aromatic Compounds by *Neurospora crassa*

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The findings of Davis (1955) have shed much light on the mode of synthesis of aromatic compounds in *Escherichia coli* and other bacteria. These studies have established dehydroquinic acid, dehydroshikimic acid, shikimic acid and prephenic acid as successive precursors in the formation of the benzene ring by *E. coli*. The purpose of the present paper is to outline some of the similarities and contrasts in the synthesis of the benzene ring by *Neurospora*. Tatum (1949) and Tatum, Gross, Ehrensvaerd & Garnjobst (1954) have given evidence for the involvement of dehydroshikimic acid and shikimic acid in the metabolism of *Neurospora*, and the isolation of prephenic acid from this organism has been described (Metzenberg & Mitchell, 1956).

EXPERIMENTAL

Bacterial strains used in assays. E. coli strains 156–53M2 and 83–1, and *Aerobacter aerogenes* strain A-170-143-S1 were kindly furnished for this study by Dr B. D. Davis. The first of these strains responds only to shikimic acid as a substitute for aromatic compounds; the second can respond alternatively to shikimic acid or dehydroshikimic acid, whereas the *Aerobacter* mutant responds not only to both these compounds, but also to quinic acid and dehydroquinic acid. These strains permitted detection of any of these compounds which might be present in culture filtrates of *Neurospora* mutants, and also allowed the accumulated materials to be classified as shikimic acid, dehydroshikimic acid, or either quinic acid or dehydroquinic acid on the basis of nutritional properties for the bacterial mutants.

The medium used in these tests was that described by Davis & Mingioli (1950). Minimal medium (5 ml.) in 6 in. test tubes was supplemented with 0·5 ml. of *Neurospora* culture filtrate to be tested. The tubes were pasteurized at 70° for 15 min. and inoculated with a bacterial test strain. After incubation for 24 hr. at 35°, growth was noted by inspection. Examination of the growth of the bacterial strains with various concentrations of authentic shikimic acid revealed that the procedure used would detect 10 μg. of this compound.

Mutant strains of Neurospora. Strains 75001 and 5212, which have nutritional requirements for tryptophan and phenylalanine respectively, were available at the inception of this study and have been previously described (Bonner, 1948; Haskins, 1951). Additional mutants of *Neurospora* which require tryptophan, phenylalanine, tyrosine, *p*-aminobenzoic acid or all four of these compounds were selected by the technique of Lein, Mitchell & Houlahan (1948), and by a modification of the technique of Woodward, DeZeeuw & Srb (1954). Twenty-six strains which required the multiple aromatic supplement for growth were isolated. In addition, some 29 tryptophan-requiring strains, two phenylalanine-requiring strains and four tyrosine-requiring strains were found.

Of the 26 mutants which had multiple aromatic requirements, none showed any growth stimulation by shikimic acid, even when additionally supplemented with tyrosine and phenylalanine. Culture filtrates of 16 of these strains showed ultraviolet-absorption spectra which were indistinguishable from that of wild-type *Neurospora*. Crosses between members of this group were invariably sterile and, as a result, it has not been possible to obtain evidence for or against allelism of these strains. A typical member of this group was saved and designated as C-161.

Filtrates of the remaining 10 mutants which required a multiple aromatic supplement exhibited strong ultraviolet-absorption spectra characteristic of protocatechuic acid (3:4-dihydroxybenzoic acid). Crosses were made between several members of this group. Over 400 spores were examined and all proved to be mutant. Therefore it seems probable that these strains are allelic with respect to the altered gene. An example of this group was designated as C-163.

One of the phenylalanine-requiring strains isolated was subjected to further examination. This strain, designated as C-165, grew vigorously when supplemented with phenylpyruvic acid, and showed slow growth in the presence of tyrosine. Unlike mutant 5212 it did not accumulate prephenic acid or phenylpyruvic acid.

A tyrosine-requiring strain, C-167, has already been described in part (Metzenberg & Mitchell, 1956). Crosses were made between this strain and each of the other tyrosine-requiring strains. Between 300 and 700 spores from each cross were examined and in no case were any wild types found. These four strains have therefore been assumed to be allelic.

RESULTS

Evidence that the observed metabolic requirements of the Neurospora *strains is due to a single gene alteration in each case.* Each of the mutants was crossed to wild-type *Neurospora* of the opposite mating type on slants of medium (Westergaard & Mitchell, 1947) supplemented with DL-tryptophan, DL-phenylalanine, L-tyrosine (each at 100 μg./ml.) and *p*-aminobenzoic acid (0·5 μg./ml.). The crosses were incubated until ripe, the asci then dissected

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and the spores induced to germinate by heat treatment on plates of minimal agar. The germinated spores could easily be classified as mutant or wild type by the length of the hyphae after growth for 12 hr. The results are given in Table 1. It will be seen that in each case, the data are consistent with the interpretation that the observed nutritional requirements are due to a single gene alteration.

Preparation of Neurospora *strains containing more than one mutant gene.* Strains C-161 and C-163 of opposite mating types were crossed, asci dissected and the separated spores induced to germinate. One ascus was found to contain two mutant spore pairs and two wild-type spore pairs. Each of the mutant spores may therefore be assumed to contain both the C-161 and C-163 genes. This assumption was confirmed by crossing one of the presumed double-mutant strains to wild type and observing several asci containing more than two mutant spore pairs. This double mutant will be referred to as C-161/C-163.

The phenylalanine-requiring strains C-165 and 5212 were crossed and a double mutant was obtained. The genetic constitution of the double mutant C-165/5212 was established in the same manner as with C-161/C-163.

The multiple mutants C-165/C-167, 75001/C-165, 75001/C-167, 75001/C-165/C-167 and 75001/5212/C-167 were obtained from crosses in a similar manner. In each case the nutritional requirements were those to be expected from the component genes, and therefore it was not felt necessary to confirm the genotypes of these multiple strains by genetic analysis. The triple mutant 75001/5212/C-167 has previously been described with respect to accumulation of prephenic acid (Metzenberg & Mitchell, 1956).

Order of the series of Neurospora *mutants in the biosynthetic pathway.* No cross-feeding behaviour could be detected between C-161 and C-163, the two mutants which exhibit a multiple aromatic requirement for growth. This situation is in contrast with that described by Davis in *E. coli*. It has been mentioned that C-163 accumulates a compound with the absorption spectrum of protocatechuic acid, and isolation and identification of this material is presented in a later section of this paper. The catabolism of this compound has been studied in detail by Gross, Gafford & Tatum (1956). It was found that the double mutant C-161/C-163 accumulates no protocatechuic acid and thus resembles C-161. Therefore it appears that the metabolic lesion associated with C-161 occurs earlier in the biosynthetic sequence than that associated with C-163. This technique of establishing the order of a series of mutant genes in a pathway has been used previously (Mitchell & Houlahan, 1946; Haas, Mitchell, Ames & Mitchell, 1952).

The phenylalanine-requiring strains C-165 and 5212 may be placed in order by the observation that both strains readily utilize phenylpyruvic acid as a substitute for phenylalanine. It has been shown (Metzenberg & Mitchell, 1956) that 5212 excretes prephenic acid, which is converted into phenylpyruvic acid by the acidity of the media usually used for culture of *Neurospora*. Since 5212 apparently lacks prephenic decarboxylase, and C-165 apparently does *not* lack the transaminase required to convert phenylpyruvic acid into phenylalanine, C-165 must represent an earlier lesion in the pathway to phenylalanine than does 5212. This conclusion is corroborated by the behaviour of the double mutant C-165/5212, which accumulates neither prephenic acid nor phenylpyruvic acid and thus resembles C-165. The order of metabolic lesions concluded from these data is presented in Fig. 1.

Table 1. *Evidence that nutritional requirements of* Neurospora *mutants are due to single gene alterations*

Neurospora strain	No. of asci observed	Asci containing more than two mutant spore pairs	Asci showing second-division segregation
C-161	29	0	5
C-163	14	0	1
C-165	9	0	3
C-167	26	0	20

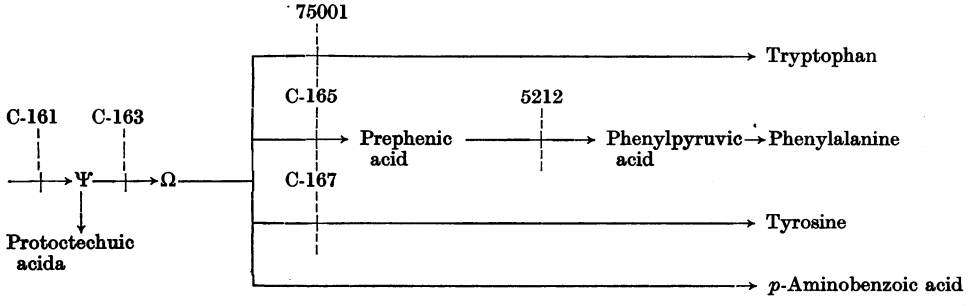

Fig. 1. Order of metabolic lesions in the biosynthetic sequence.

It is apparent that the triple-blocked strain 75001/C-165/C-167 might well accumulate the last precursor (i.e., the 'branch-point' compound Ω), which is common to the three aromatic amino acids and *p*-aminobenzoic acid. Such a compound would perhaps be difficult to demonstrate by studies of an organism in which genetic manipulation is not feasible. It was found that this triple-blocked strain accumulated no material which would satisfy all or any of the nutritional requirements of C-161; however, this strain did accumulate protocatechuic acid, vanillic acid and dehydroshikimic acid. Evidence for the presence of these compounds in culture filtrates of *Neurospora* mutants is presented below.

Identification of materials accumulated by mutant strains

Protocatechuic acid. Erlenmeyer flasks (125 ml.) were charged with 20 ml. each of Fries medium (Fries, 1938) supplemented with DL-tryptophan, DL-phenylalanine and L-tyrosine, each at a concentration of 5 μg./ml., and *p*-aminobenzoic acid (0·1 μg./ml.). The flasks were inoculated with conidia of C-163. After 5 days' growth at 25°, the culture filtrates were pooled and agitated with sufficient Norit to remove the characteristic ultraviolet absorption (approx. 3 g./l.). The Norit was treated with boiling ethanol in an atmosphere of nitrogen for 10 min. The ethanolic eluate was evaporated *in vacuo*. The residue was taken up in a little water and extracted with ligroin. Evaporation of the latter gave white crystals, which were further purified by sublimation onto a solid carbon dioxide 'cold finger' *in vacuo*.

The material was recrystallized from water. The isolated protocatechuic acid had m.p. 201–202°, unchanged by admixture with an authentic sample (Found: C, 54·9; H, 4·3. $C_7H_6O_4$ requires C, 54·6; H, 3·9%). Melting points were uncorrected.

The ultraviolet-absorption spectrum of the isolated protocatechuic acid was found to be identical with that of an authentic sample. Chromatographic comparison of these in three solvent systems [propanol–aq. M-NH_3 soln. (3:1, v/v); propanol–aq. M-acetic acid (3:1, v/v); butanol–ethanol–water (4:1:1, by vol.)] and detection by spraying with aq. ferric chloride (1%, w/v) revealed no differences.

Culture filtrates of the triple-blocked strain 75001/C-165/C-167 were prepared in the following manner. The strain was grown in a carboy containing 15 l. of the medium of Westergaard & Mitchell (1947) supplemented with 25 μg./ml. each of DL-tryptophan, DL-phenylalanine and L-tyrosine. Protocatechuic acid was isolated essentially as described above. The material was characterized as before by absorption spectrum, melting point and chromatographic behaviour.

Vanillic acid. During the course of isolation of protocatechuic acid from 75001/C-165/C-167, the mother liquors from crystallization of the above compound were examined chromatographically for the presence of other compounds. In a solvent system consisting of 2-methylpropan-2-ol–aq. M-NH_3 soln. (30:10, v/v) two compounds which migrated more rapidly than protocatechuic acid were revealed by spraying the chromatogram with diazotized sulphanilic acid (Ames & Mitchell, 1952). The material of intermediate mobility, which was present in considerable amounts, gave an orange colour with this reagent. The liquors were applied to strips of Schleicher and Schüll no. 470 paper (Carl Schleicher und Schüll, Dassel, Germany), and the chromatogram was developed by the ascending method with the solvent system mentioned above. Guide strips were cut and the position of the material of intermediate mobility was determined by spraying with diazotized sulphanilic acid. The remainder of the material was then eluted with water 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 material was recrystallized six times from dilute 2-methylpropan-2-ol and once from ligroin–*n*-propanol. The neutralization equivalent was found to be 172, in reasonably good agreement with the theoretical value of 168. The ultraviolet-absorption spectra in acid and in alkali were identical with those of vanillic acid synthesized by the method of Pearl (1946). The chromatographic behaviour of the two preparations was identical in five solvent systems [butanol–ethanol–water (4:1:1, by vol.); propanol–pyridine–benzene–water (3:1:1:1, by vol.); 2-methylpropan-2-ol–aq. M-NH_3 soln. (3:1, v/v); 2-methylpropan-2-ol–aq. M-NH_3 soln.–pyridine–benzene–water (6:1:1:1:1, by vol.); 2-methylpropan-2-ol–pyridine–water (6:1:1, by vol.)]. The sample isolated had m.p. 205–206°. The mixed m.p. with synthetic vanillic acid (m.p. 207–208°) was 205–207°.

Dehydroshikimic acid. Culture filtrates of 75001/C-165/C-167 were examined for the presence of materials which would stimulate growth of the bacterial strains. It was found that these filtrates promoted rapid growth of strains 83-1 and A-170-143-S 1, but no growth of 156–53M2. On these grounds, it was suspected that dehydroshikimic acid was present in filtrates of this triple-blocked mutant. In order to obtain further evidence for the nature of the material, filtrate (50 ml.) from the carboy from which protocatechuic acid and vanillic acid were isolated was evaporated to dryness and

redissolved in water (5 ml.). Methanol (50 ml.) was added and the precipitate of inorganic salts removed by centrifuging. The supernatant liquid was evaporated and the residue was dissolved in water (1 ml.).

For purposes of comparison, a concentrate containing dehydroshikimic acid was prepared from culture filtrates of *E. coli* strain 83–2, which is known to accumulate the latter compound. Erlenmeyer flasks (125 ml.) were charged with 40 ml. of the medium recommended by Salamon & Davis (1953). The flasks were inoculated and incubated at 35° for 2 days with gentle agitation. The cells were removed by centrifuging and the supernatant medium was prepared for chromatography as described above.

The desalted preparation from the triple-blocked *Neurospora* mutant and the dehydroshikimic acid preparation from the *E. coli* mutant were subjected to paper chromatography in three solvent systems. These solvents were: *A*, ethanol–pentan-1-ol–M-acetic acid (2:1:1, by vol.); *B*, 2-methylpropan-2-ol–M-acetic acid (3:1, v/v); *C*, 2-methylpropan-2-ol–88% formic acid–water (2:1:1, by vol.).

Shikimic acid was also applied to chromatograms for purposes of comparison. After development, the chromatograms were cut horizontally into ten equal sections, and the sections eluted with 2 ml. of the medium of Davis & Mingioli (1950), and supplemented with DL-phenylalanine and L-tyrosine (each 25 μg./ml.) to increase the sensitivity of the assay. The tubes were pasteurized as before and inoculated with *Aerobacter aerogenes* strain A-170-143-S 1. On examination of the assay tubes from the chromatograms, it was apparent that the biologically active material in the *Neurospora* culture filtrate was chromatographically identical with dehydroshikimic acid. The results are presented in Table 2. It will be apparent that the second decimal place given for each R_F value is of only relative significance.

The *Neurospora* strains described in this paper were examined for the presence of biologically active materials by use of the bacterial-assay strains. The results are given in Table 3.

Table 2. *Chromatographic properties of shikimic acid and dehydroshikimic acid from* Escherichia coli, *and biologically active material from* Neurospora *strain* 75001/*C*-165/*C*-167

	R_F		
	Solvent *A*	Solvent *B*	Solvent *C*
Shikimic acid	0·65	0·45	0·75
Dehydroshikimic acid	0·40	0·05–0·1	0·05–0·1
Neurospora material	0·40	0·05–0·1	0·05–0·1

Table 3. *Biologically active materials accumulated by* Neurospora *mutants*

Degree of growth is indicated by the symbols + +, approx. 10^8 bacteria/ml.; +, approx. 10^7 bacteria/ml.; ±approx. 10^6 bacteria/ml.; −, fewer than 10^6 bacteria/ml.

Culture filtrate from *Neurospora* strain	Growth of bacterial strains		
	156–53 M-2	83-1	A-170-143-S 1
C-161	−	−	−
C-163	−	±	±
75001	−	−	−
C-165	−	−	−
5212	−	−	−
C-167	−	−	−
C-165/5212	−	+ +	+ +
C-165/C-167	−	+ +	+ +
75001/C-165	−	−	−
75001/C-167	−	−	−
75001/C-165/C-167	−	+ +	+ +
75001/5212/C-167	−	+	+

DISCUSSION

It will be seen from Table 3 that, except for C-163, which possibly accumulates a biologically active material at the very borderline of detection, none of the *Neurospora* strains examined containing a single metabolic lesion has been found to excrete shikimic acid, dehydroshikimic acid, dehydroquinic acid or quinic acid. On the other hand, a number of the multiple mutants accumulate material with the nutritional characteristics of dehydroshikimic acid. It is also to be noted that shikimic acid is not accumulated by these strains. However, the data presented here should not be taken as evidence against the participation of shikimic acid in the biosynthesis of the aromatic compounds, as the strains examined may be impermeable to shikimic acid. The provisional pathway outlined in Fig. 1 suggests the hypothesis that the 'branch-point' compound is not accumulated by a singly blocked mutant, e.g. C-165, for the reason that it is diverted into the production of tryptophan or tyrosine; in the strains with multiple blocks this diversion does not occur, and the compound is accumulated. The presence of biologically active material in filtrates of C-165/5212 is somewhat anomalous and remains to be fully explained. However, it is tempting to speculate from the behaviour of the *Neurospora* mutants that dehydroshikimic acid or a closely related compound may be the 'branch-point' compound Ω in *Neurospora*, rather than being a relatively early precursor of aromatic compounds, as with *E. coli*.

The origin of protocatechuic acid and vanillic acid deserves some comment. These compounds are at the oxidation level of dehydroshikimic acid, and are probably detoxification products of the latter. Neither compound promotes the growth of any of

the *Neurospora* or bacterial mutants. In addition, it seems improbable on structural grounds that these benzenoid compounds would be intermediates in the synthesis of such hydroaromatic compounds as prephenic acid. It is not known at present whether vanillic acid is formed by methylation of the 3-hydroxyl group of dehydroshikimic acid followed by dehydration, or by direct methylation of protocatechuic acid. Gross *et al.* (1956) have shown that vanillic acid is a potent inducer of protocatechuic acid oxidase in *Neurospora*, and therefore it is of added interest that this compound occurs naturally in *Neurospora*.

SUMMARY

1. Some strains of *Neurospora* showing a nutritional requirement for aromatic compounds have been obtained. Preparation of multiple mutants is described.

2. Evidence for the presence of protocatechuic acid, vanillic acid and dehydroshikimic acid in culture filtrates of some of these mutants is presented.

3. The possibility of a central role for dehydroshikimic acid in the synthesis of aromatic compounds by *Neurospora* is discussed.

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Enzymic Determination of Acetaldehyde in Blood

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The determination of acetaldehyde in blood is of interest in the study of ethanol metabolism, where this substance may be expected to occur as an intermediate, especially during treatment with substances such as tetraethyl thiuram disulphide, [bis(diethylthiocarbamoyl) disulphide], which presumably interfere with aldehyde breakdown. The methods available are, however, not entirely satisfactory. Among the purely chemical methods the most reliable is undoubtedly that of Stotz (1943) based on the colour reaction with *p*-hydroxydiphenyl in strong sulphuric acid. The procedure requires, however, distillation involving special apparatus, and is very sensitive to trace contamination. Moreover, interference by various normal metabolic products cannot be excluded. The elegant method of Burridge, Hine & Schick (1950) involves the use of Conway micro-diffusion units. Acetaldehyde is caught in semicarbazide in a buffer solution and the light absorption of the semicarbazone at 224 mμ is used for the estimation. Unfortunately this method, though very sensitive, is not sufficiently specific. Acetoacetate in the blood will give rise to acetone under the analytical conditions, and this substance interferes in the measurement (see below).

Enzymic methods for determination of acetaldehyde have also been suggested (Holzer, Holzer & Schultz, 1955; Racker, 1957). The oxidation of reduced diphosphopyridine nucleotide (DPN) by aldehydes, catalysed by yeast alcohol dehydrogenase, may be used quantitatively, although the