Degradation of Phenylalanine and Tyrosine by Sporobolomyces roseus

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(Received 25 July 1967)

Ammonia-lyase activity for L-phenylalanine, m-hydroxyphenylalanine and L-tyrosine was demonstrated in cell-free extracts of Sporobolomyces roseus. Cultures of this organism converted DL-[ring-14C]phenylalanine and L-[U-14C]tyrosine into the corresponding cyanide acids. Tracer studies showed that these compounds were further metabolized to [14C]protocatechuic acid. Benzoic acid and p-hydroxybenzoic acid were intermediates in this pathway. Washed cells of the organism readily utilized cinnamic acid, p-coumaric acid, caffeic acid, benzoic acid and p-hydroxybenzoic acid. Protocatechuic acid was the terminal aromatic compound formed during the metabolism of these compounds. The cells of S. roseus were able to convert m-coumaric acid into m-hydroxybenzoic acid, but the latter compound, which accumulated in the medium, was not further metabolized. 4-Hydroxycoumarin was identified as the product of o-coumaric acid metabolism by this organism.

Among the wide variety of micro-organisms that can metabolize phenylalanine and tyrosine, the majority appear to degrade these amino acids via homogentisic acid with the initial formation of the corresponding phenylpyruvic acid, a route characteristic of mammals (Kluver & Van Zijp, 1951; Evans, 1963; Rogoff, 1961; Treccani, 1963). The recent report on the occurrence of ammonia-lyases in fungi that catalyse the non-oxidative deamination of these aromatic amino acids to the corresponding cyanide acids (Power, Towers & Neish, 1965) has stimulated us to investigate the metabolic fate of phenylalanine and tyrosine in fungi. Evidence for the conversion of phenylalanine into cinnamic acid and its subsequent transformation into protocatechuic acid was obtained in this Laboratory by a study of the metabolism of wood-degrading fungi, e.g. Schizophyllum commune (Moore, Subba Rao & Towers, 1967) and Sporobolomyces roseus. The present paper provides evidence for the degradation by S. roseus of phenylalanine and tyrosine via cinnamic acid, benzoic acid, p-hydroxybenzoic acid and protocatechuic acid. The metabolism of m-hydroxyphenylalanine, m-coumaric acid and o-coumaric acid was also examined.

MATERIALS AND METHODS

Chemicals. DL-[ring-14C]Phenylalanine, L-[U-14C]Tyrosine, [1-14C]Benzoic acid and 1,4-bis-(5-phenyloxazol-2-yl)benzene were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. [3-14C]Cinnamic acid and [2-14C]Cinnamic acid were available commercially from Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A. Bacto-soytone, malt extract and yeast extract were purchased from Difco Laboratories, Detroit, Mich., U.S.A. 4-Hydroxycoumarin was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. DL-m-Hydroxyphenylalanine and DL-m-hydroxybenzoic acid were from Fluka A.-G., Buchs SG, Switzerland. 2,5-Diphenyloxazole was supplied by Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A. All the other chemicals were the best grades available from commercial sources.

Chromatography. Solvent A (benzene-acetic acid-water; 10:7:3, by vol.) and solvent B (100% formic acid-water; 1:49, v/v) were used for the chromatographic separation of various cinnamic acids and benzoic acids on Whatman no. 1 filter paper. The spots were located on chromatograms by spraying with p-nitroaniline reagent (Ibrahim & Towers, 1960). The separation of amino acids was achieved by developing the paper chromatograms two-directionally in solvents C (phenol-water; 5:1, v/v) and D (butanol-acetic acid-water; 20:5:11, by vol.). For chromatograms on silicic acid (20 cm. × 20 cm. plates) solvent E (benzene-acetic acid; 9:1, v/v) was used.

Ultraviolet spectra. A Unicam SP.800 recording spectrophotometer was used for obtaining ultraviolet spectra.

Infrared spectra. Infrared spectra were taken in a Unicam SP.400 spectrophotometer, KBr disks being used.

Radioactivity measurements. Suitable portions of radioactive samples in methanol were transferred to scintillation vials containing 15 ml. of scintillation fluid (1,4-bis-(5-phenyloxazol-2-yl)benzene; 4 g.; 2,5-diphenyloxazole, 50 mg.; toluene, 11.) and the radioactivity was measured in a Nuclear-Chicago 720 series liquid-scintillation spectrometer.

Organism. The culture of S. roseus (U.B.C. 901) was grown with shaking at 25° in a nutrient medium containing: malt extract, 3%; Bacto-soytone, 0-5%; yeast, 0-1%. The
fungus was maintained on slante of the same medium supplemented with agar (1·5%). Subcultures were grown in 50 ml. of the medium for 48 hr. and used for inoculating large batches of medium distributed in 11. flasks. After 72 hr. the cells were collected by centrifuging at 10000 g for 15 min. and washed thrice with distilled water. The wet packed cells were used for studying the metabolism of various aromatic compounds.

Preparation of cell-free extracts. Wet cells (10 g) were homogenized in a mortar with an equal weight of aluminium oxide and extracted with 0·05 M-tris-HCl buffer, pH 8 (20 ml). The resulting slurry was centrifuged at 20000 g for 20 min. and the supernatant was used as the crude enzyme preparation.

Assay for the non-oxidative deamination of aromatic amino acids. The reaction mixture containing 0·5 ml. of 0·05 M-tris-HCl buffer, pH 8·8, 0·5 ml. of crude enzyme and 5 μmol of substrate (L-phenylalanine, L-tyrosine, DL-m-hydroxyphenylalanine, DL-o-hydroxyphenylalanine or DL-3,4-dihydroxyphenylalanine) in a final volume of 2 ml. was incubated at 30°C. After 1 hr. the reaction mixture was acidified with 1 ml. of N-HCl and extracted with 5 ml. of peroxide-free ether. Suitable samples from the ethereal layer were evaporated to dryness under a stream of air and the amount of cinnamic acid or p-coumaric acid was determined spectrophotometrically (Koukol & Conn, 1961; Neish, 1961) after dissolving the residue in 3 ml. of 0·05 N-NaOH. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard.

Assay for catechol oxidase and protocatechuic oxidase. To study the disappearance of protocatechuc acid or catechol, 1 ml. of crude enzyme was incubated at 30°C with 2 μmol of substrate and 0·8 ml. of 0·1 M-sodium phosphate buffer, pH 7, in a final volume of 2 ml. After 30 min. incubation at 30°C, the reaction was stopped by the addition of 0·5 ml. of 30% (w/v) trichloroacetic acid and the enzyme activity was assayed colorimetrically (Nair & Vaidyanathan, 1964).

Enzymic synthesis of [14C]p-coumaric acid. The crude enzyme (15 ml.) was treated with 2%, protamine sulphate (2 ml.) and the precipitate was removed by centrifugation and discarded. To the supernatant (20 ml.) was added 10 ml. of 0·05 M-tris-HCl buffer and 20 μC of L-[14C]-tyrosine (sp. activity 3·6 mCl/m-mole) and incubated for 3 hr. at 30°C. The reaction mixture was adjusted to pH 12 with N-HCl and the [14C]p-coumaric acid was extracted twice with equal volumes of peroxide-free ether. After shaking with anhydrous Na2SO4, the solvent was removed in vacuo. The residue was dissolved in 0·5 ml. of 95% ethanol and subjected to thin-layer chromatography on silicic acid plates with solvent E. The radioactive band corresponding to p-coumaric acid was eluted with 95% ethanol, the eluent evaporated to dryness in vacuo and the residue redissolved in 5 ml. of water. A sample was used for radioactivity determinations in the concentration of the preparations at a 4-3 μC of radioactive p-coumaric acid was obtained.

Experiments with radioactive compounds. Each radioactive compound was added under sterile conditions to a 25 ml. flask containing 1 g. (wet wt.) of freshly harvested cells of S. roseus suspended in 5 ml. 0·01 M-phosphate buffer, pH 7. The flasks were incubated at 25°C for 1–6 hr. with constant agitation. The reaction was stopped by the addition of 1 ml. of N-HCl and the cells were separated by centrifugation.

The supernatant was collected and the residue was washed with 10 ml. of distilled water and again centrifuged. The combined washings were extracted twice with 25 ml. of peroxide-free ether. The ethereal layer was evaporated to dryness at room temperature after removal of traces of water with anhydrous Na2SO4. The residue was dissolved in 1 ml. of methanol. A portion (0·1 ml.) of the methanolic solution was transferred to a scintillation vial containing 15 ml. of scintillation fluid and the radioactivity measured. The remaining methanolic solution was applied to Whatman no. 1 filter paper and developed two-directionally with solvent A in the first direction and solvent B in the second direction. Radioautographs were prepared with Kodak Medical X-ray film (Estar base), and the corresponding radioactive spots were cut out from paper chromatograms and placed in scintillation vials together with 15 ml. of scintillation fluid for radioactivity determinations.

Experiments with non-radioactive compounds. Washed cells (3 g, wet wt.) were added aseptically to 500 ml. Erlenmeyer flasks containing 100 ml. of substrate in 0·01 M-sodium phosphate buffer, pH 7 (100 ml.), and incubated at 25°C for 24 hr. Samples (10 ml.) were withdrawn at various intervals and acidified with N-HCl (1 ml.), and the cells were separated by centrifugation. The disappearance of each substrate and formation of protocatechuic acid (m-hydroxybenzoic acid in the case of m-coumaric acid) were assayed as described below.

Cinnamic acid was determined spectrophotometrically (Koukol & Conn, 1961). Benzoic acid was determined by measuring the extinction at 229 mμ after diluting suitable portions with 95% ethanol (Webley, Duff & Farmer, 1955). For the determination of m-coumaric acid, p-coumaric acid, caffeic acid, m-hydroxybenzoic acid and p-hydroxybenzoic acid, samples (10 ml.) removed from the reaction mixture at various periods were extracted twice with 25 ml. of peroxide-free ether. The solvent was removed in vacuo and the residue taken up in 1 ml. of ethanol. Suitable samples were applied to Whatman no. 1 filter paper and developed in solvent B. The band corresponding to each compound was cut out, eluted with 95% ethanol and the concentration determined spectrophotometrically by measuring the extinction at 273, 333, 325, 300 and 258 mμ respectively for m-coumaric acid, p-coumaric acid, caffeic acid, m-hydroxybenzoic acid and p-hydroxybenzoic acid. Standard graphs were prepared by subjecting different concentrations of authentic samples to similar manipulations. The colorimetric method of Nair & Vaidyanathan (1964) was used for the direct assay of protocatechuic acid. However, when cells were incubated with caffeic acid, the concentrations of protocatechuic acid at various time-intervals was followed spectrophotometrically at 260 mμ after chromatographic separation with solvent B and elution with 95% ethanol.

Isolation of the metabolite from o-coumaric acid. Freshly harvested cells (60 g) were dispensed in 21. of 0·01 M-sodium phosphate buffer, pH 7, containing 2 g. of o-coumaric acid and incubated for 24 hr. on a metabolic shaker at 25°C. Samples (10 ml.) were removed at 3 hr. intervals for paper-chromatographic analysis of the ether-extractable metabolites with solvents A and B. The remaining medium was acidified after 24 hr. and the cells were separated by centrifugation. The clear supernatant solution was concentrated in vacuo at room temperature to 100 ml. and extracted twice with 200 ml. portions of ether. The organic layer was separated out and evaporated to dryness. The residue, after
being taken up in hot water and treatment with charcoal, gave a white crystalline material that was further purified by recrystallization.

Studies on the fate of the side chain of [2-14C]cinnamic acid. A suspension of wet cells (3 g.) of S. roseus in 5 ml. of 0.01 M sodium phosphate buffer, pH 7, was incubated for 24 hr. with 2 μC of [2-14C]cinnamic acid. The cells were collected by centrifugation, macerated in a mortar with 3 g. of aluminium oxide and extracted with 0.1 M sodium phosphate buffer, pH 7 (10 ml.). The resulting slurry was centrifuged at 4100 g for 15 min. and the supernatant was placed on a column of Dowex 50 (H+ form), washed with 11. of distilled water and eluted with 4% (w/v) NH3. The eluate was concentrated, applied to Whatman no. 1 chromatography paper and developed two-directionally in solvents C and D. A radioautograph was prepared from the chromatograms.

Determination of evolution of 14CO2. Fresh cells (2 g. wet wt.) were dispensed into a flask containing 1 μC of DL-[ring-14C]phenylalanine in 5 ml. of 0.01 M sodium phosphate buffer, pH 7. The 14CO2 produced during a total incubation period of 6 hr. at 25° was collected in a solution of Hyamine hydroxide (10 ml.) and the radioactivity measured in a liquid-scintillation spectrometer.

RESULTS

Enzymic formation of cinnamic acids. Cell-free preparations from S. roseus deaminated L-phenylalanine, DL-m-hydroxyphenylalanine and L-tyrosine to the corresponding cinnamic acids (Table 1). There was no ammonia-lyase activity when the crude preparations were incubated with DL-o-hydroxyphenylalanine or DL-3,4-dihydroxyphenylalanine.

Degradation of DL-[14C]phenylalanine to 14CO2. Fig. 1 shows the time-course of 14CO2 evolution when growing cultures of the organism were given DL-[ring-14C]phenylalanine. It is apparent that the aromatic ring of phenylalanine can be degraded to carbon dioxide by S. roseus.

Feeding experiments with radioactive compounds. When DL-[ring-14C]phenylalanine was administered to washed-cell suspensions of S. roseus, the radioactivity was incorporated into cinnamic acid, benzoic acid, p-hydroxybenzoic acid and protocatechuic acid (Table 2). Radioautography revealed the presence of four radioactive spots on chromatograms corresponding to these acids. In view, however, of the poor resolution of cinnamic acid and benzoic acid on paper chromatograms the radioactivities of these two compounds are added together in Table 2. Under the experimental conditions, neither [14C]phenylpyruvic acid nor [14C]p-hydroxyphenylpyruvic acid could be detected. Further, these compounds added to the washed cells along with the [14C]phenylalanine failed to trap any radioactivity.

The conversion of cinnamic acid into benzoic acid, p-hydroxybenzoic acid and protocatechuic acid was established by using [3-14C]cinnamic acid.

Table 1. Enzymic formation of cinnamic acids in the presence of cell-free preparations from S. roseus

| Substrate                     | Product         | Specific activity
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Phenylalanine</td>
<td>Cinnamic acid</td>
<td>0.87</td>
</tr>
<tr>
<td>m-Hydroxyphenylalanine</td>
<td>m-Coumaric acid</td>
<td>0.30</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>p-Coumaric acid</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Fig. 1. Release of 14CO2 from DL-[ring-14C]phenylalanine in the presence of washed cells of S. roseus. Experimental details are given in the text.

No other radioactive spot could be detected on radioautographs. Table 2 shows that, when [1-14C]benzoic acid (ring-labelled) was given to washed whole cells of S. roseus, it was completely utilized in 1 hr.: 18% of the radioactivity was detected in 14CO2, whereas 9.4% was recovered in p-hydroxybenzoic acid and protocatechuic acid. The remaining radioactivity was present in the aqueous fraction probably incorporated into various organic and amino acids.

p-Coumaric acid, p-hydroxybenzoic acid and protocatechuic acid were intermediates in the degradation of tyrosine as evidenced by the changes in radioactivity of L-[U-14C]tyrosine during a 6 hr. period. The distribution of radioactivity in
Table 2. Metabolism of radioactive phenylalanine, cinnamic acid and benzoic acid by the cells of S. roseus

Experimental details are given in the text.

<table>
<thead>
<tr>
<th>Compound administered</th>
<th>Incubation time (hr.)</th>
<th>Total radioactivity of ether extract (mCi)</th>
<th>[14C]Cinnamic acid + [14C]benzoic acid (mCi)</th>
<th>[14C]p-Hydroxy[14C]-benzoic acid (mCi)</th>
<th>[14C]Protocatechuic acid (mCi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL[ring-14C]Phenylalanine (1 μC)</td>
<td>2</td>
<td>7.1</td>
<td>2-1</td>
<td>3-6</td>
<td>1-4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.8</td>
<td>2-2</td>
<td>3-5</td>
<td>2-1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9.4</td>
<td>3-0</td>
<td>4-2</td>
<td>2-2</td>
</tr>
<tr>
<td>[3-14C]Cinnamic acid (1 μC)</td>
<td>1</td>
<td>140</td>
<td>27</td>
<td>99</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>120</td>
<td>10</td>
<td>84</td>
<td>26</td>
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<tr>
<td></td>
<td>4</td>
<td>74</td>
<td>3</td>
<td>57</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>53</td>
<td>2</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>[1-14C]Benzoic acid (1 μC)</td>
<td>1</td>
<td>94</td>
<td>0</td>
<td>69</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>84</td>
<td>0</td>
<td>60</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>69</td>
<td>0</td>
<td>42</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>49</td>
<td>0</td>
<td>24</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 3. Metabolism of radioactive tyrosine and p-coumaric acid by the cells of S. roseus

Experimental details are given in the text.

<table>
<thead>
<tr>
<th>Compound administered</th>
<th>Incubation time (hr.)</th>
<th>Total radioactivity of ether extract (mCi)</th>
<th>[14C]p-Coumaric acid (mCi)</th>
<th>[14C]p-Hydroxy[14C]-benzoic acid (mCi)</th>
<th>[14C]Protocatechuic acid (mCi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-[U-14C]Tyrosine (1 μC)</td>
<td>1</td>
<td>7</td>
<td>5-4</td>
<td>1-6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9-8</td>
<td>5-6</td>
<td>1-6</td>
<td>2-6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9-7</td>
<td>4-0</td>
<td>2-4</td>
<td>3-3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>7-9</td>
<td>1-6</td>
<td>2-9</td>
<td>3-4</td>
</tr>
<tr>
<td>[U-14C]p-Coumaric acid (0-86 μC)</td>
<td>1</td>
<td>160</td>
<td>123</td>
<td>21</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>150</td>
<td>106</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>140</td>
<td>93</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>130</td>
<td>68</td>
<td>37</td>
<td>25</td>
</tr>
</tbody>
</table>

The metabolism of the various acids is summarized in Table 3. Incubation of washed-cell suspensions of the organism with [U-14C]p-coumaric acid also resulted in its conversion into p-hydroxybenzoic acid and protocatechuic acid.

Metabolism of non-radioactive cinnamic acids and phenolic acids by S. roseus. The pathway leading to the oxidation of phenylalanine and tyrosine to protocatechuic acid was further established by giving non-radioactive compounds. Incubation of washed cells of S. roseus under sterile conditions with cinnamic acid, p-coumaric acid, benzoic acid or p-hydroxybenzoic acid resulted in a steady utilization of these compounds in a 24 hr. period. In each case, the formation and disappearance of protocatechuic acid was unequivocally established (Fig. 2). The concentration of protocatechuic acid invariably reached an optimum after 9 hr. and approached zero at the end of the experimental period. Cell-free preparations of the fungus also rapidly destroyed protocatechuic acid. However, under the same experimental conditions, catechol was not metabolized.

m-Coumaric acid was oxidized to m-hydroxybenzoic acid. The latter compound, which accumulated in the medium, however, was not further metabolized (Fig. 3).

Although DL-3,4-dihydroxyphenylalanine was not deaminated by S. roseus the corresponding cinnamic acid, caffeic acid, was readily converted into protocatechuic acid by washed cells (Fig. 4).

Metabolic fate of o-coumaric acid. o-Coumaric acid was not degraded to salicylic acid. On the contrary, it served as a precursor for the biosynthesis of 4-hydroxycoumarin. The latter compound was isolated from incubation media in pure crystalline...
Fig. 2. Time-course of the disappearance of substrate (○) and formation of protocatechuic acid (○) in the presence of washed cells of S. ro8eu8. The substrates used were: (a) cinnamic acid; (b) benzoic acid; (c) p-hydroxybenzoic acid; (d) p-coumaric acid. Experimental details are given in the text.

Fig. 3. Time-course of the conversion of m-coumaric acid (○) into m-hydroxybenzoic acid (○) catalysed by the cells of S. ro8eu8. Experimental details are given in the text.

Fig. 4. Time-course of the disappearance of caffeic acid (○) and formation of protocatechuic acid (○) in the presence of washed cells of S. ro8eu8. Experimental details are given in the text.
Scheme 1. Pathways for the degradation of phenylalanine and tyrosine by S. roseus. (I) Phenylalanine; (II) cinnamic acid; (III) benzoic acid; (IV) p-hydroxybenzoic acid; (V) protocatechuic acid; (VI) tyrosine; (VII) p-coumaric acid; (VIII) m-hydroxyphenylalanine; (IX) m-coumaric acid; (X) m-hydroxybenzoic acid; (XI) o-coumaric acid; (XII) 4-hydroxycoumarin.

form and its identity established by analysis and by comparing its ultraviolet, infrared and nuclear-magnetic-resonance spectra and melting point with those of an authentic sample.

Metabolism of the side chain of cinnamic acid. To follow the metabolic changes in the side chain of cinnamic acid, the organism was incubated with [2-14C]cinnamic acid. Analysis of the aqueous layer, after separation of the ether-soluble fraction, gave ninhydrin-positive spots when subjected to
paper chromatography. Radioautography revealed that most of the radioactivity was confined to glutamic acid.

**DISCUSSION**

Pathways for the degradation of phenylalanine and tyrosine by *S. roseus* are depicted in Scheme 1. The initial step involves the formation of the corresponding cinnamic acid (Tables 1, 2 and 3) by enzymes similar to those present in vascular plants (Koukol & Conn, 1961; Neish, 1961). The pathways reported hitherto differ from those reported for mammals (Henderson, Gholson & Dalgliesh, 1962) and micro-organisms (Evans, 1963; Rogoff, 1961; Treccani, 1963) where the initial step in phenylalanine and tyrosine degradation is the formation of phenylpyruvic acid and p-hydroxyphenylpyruvic acid respectively.

Whiting & Carr (1959) observed that *Lactobacillus pastorianus* was able to reduce the side chain of cinnamic acid and hydroxycinnamic acid with subsequent decarboxylation. A non-oxidative decarboxylase specific for cinnamic acids bearing a 4-hydroxyl group was reported by Finkle, Lewis, Corse & Lundin (1962).

The principal constituents, before the rupture of the benzene ring of *trans*-cinnamic acid by a soil pseudomonad, were o-hydroxyphenylpropionic acid and 2,3-dihydroxyphenylpropionic acid (Coulson & Evans, 1959). Blakley & Simpson (1964), in a study of the metabolism of cinnamic acid by a pseudomonad, suggested that the first step is the reduction of the double bond in the side chain, giving rise to phenylpropionic acid, which undergoes hydroxylation in either the *ortho* - or *meta*-position. *Achromobacter* degrades *β*-phenylpropionic acid by a pathway involving o-hydroxyphenylpropionic acid and 2,3-dihydroxyphenylpropionic acid as intermediates (Dagley, Chapman & Gibson, 1965). Neither phenylpropionic acid nor its hydroxylation products were identified as possible intermediates in the degradation of cinnamic acid by *S. roseus*. Under the experimental conditions described in the text, this organism converted cinnamic acid into benzoic acid (Table 2).

Webley, Duff & Farmer (1955, 1956, 1957, 1958), Henderson & Farmer (1955) and Henderson (1961) reported that cinnamic acid and related compounds could be converted by micro-organisms into the corresponding benzoic acids by a process involving *β*-oxidation. Zenk (1965, 1967) and Zenk & Müller (1964) have provided evidence for the conversion of cinnamic acids into the corresponding benzoic acids by the leaves of *Salix* and *Catalpa* in a process involving the removal of a C2 fragment from the side chain as acetate. These workers suggested that benzoic acid, salicylic acid and p-hydroxybenzoic acid are formed from the parent cinnamic acid by *β*-oxidation in a manner analogous to fatty acid degradation (Stumpf, 1965). Similar assumptions were made by Vollmer, Reisené & Grisebach (1965), who demonstrated the incorporation of [1-14C]-acetic acid into glutamic acid in wheat when [1-14C]p-coumaric acid was administered. The formation of radioactive glutamic acid from [2-14C]cinnamic acid by *S. roseus* also suggests that acetate may be a product of the reaction involving the conversion of cinnamate into benzoate.

Although *p*-coumaric acid and *o*-coumaric acid may be produced by *Aspergillus niger* supplied with cinnamic acid on prolonged incubation (Bocks, 1967a), there was no evidence for the hydroxylation of cinnamic acid by *S. roseus* in the present study. Benzoic acid, however, was readily hydroxylated in the *para*-position. There was never any detectable formation of either *o* - or *m*-hydroxybenzoic acid. Protocatechuic acid may be converted into catechol by *Aerobacter aerogenes* before ring-cleavage (Pittard, Gibson & Doy, 1962). Although protocatechuic acid was a conspicuous metabolite of phenylalanine, benzoic acid or *p*-hydroxybenzoic acid in *S. roseus*, catechol was never detected among the products. The disappearance of protocatechuic acid, but not catechol, with cell-free preparations of the organism is further evidence that protocatechuic acid is the terminal aromatic compound in the degradation of phenylalanine or tyrosine.

Evidence presented in this study showed that the degradation of *m*-tyrosine proceeded via the formation of *m*-coumaric acid and ended with the accumulation of *m*-hydroxybenzoic acid (Fig. 3). Bocks (1967b) reported that *m*-hydroxybenzoic acid can be converted into protocatechuic acid and ultimately into catechol by *Aspergillus*, but attempts to demonstrate the further metabolism of this compound by *S. roseus* were unsuccessful.

*S. roseus* was also able to convert *o*-coumaric acid into 4-hydroxyconmarin. This is similar to the results of Bocks (1967a) and Mead, Smith & Williams (1958) with other organisms.

The system capable of oxidizing the side chain of cinnamic acid seems to be rather unspecific, since *m*-coumaric acid, *p*-coumaric acid and caffeic acid were readily converted into the corresponding benzoic acids. The hydroxylation system involved in the conversion of *p*-hydroxybenzoic acid into protocatechuic acid appears to be quite specific, since *m*-hydroxybenzoic acid did not serve as substrate. A hydroxylation system for *p*-coumaric acid is not evident in *S. roseus*, in contrast with *Lentinus lepideus*, in which caffeic acid is a metabolite of *p*-coumaric acid (Power et al. 1965).

The authors thank Dr R. J. Bandoni, of this Department, for providing the culture, and Mrs Mary Coleman for her...
technical assistance. Financial assistance from the National Research Council, Canada, is gratefully acknowledged.

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