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Metabolism of DL-(±)-Phenylalanine by *Aspergillus niger*

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A fungus capable of degrading DL-phenylalanine was isolated from the soil and identified as *Aspergillus niger*. It was found to metabolize DL-phenylalanine by a new pathway involving 4-hydroxymandelic acid. D-Amino acid oxidase and L-phenylalanine: 2-oxoglutaric acid aminotransferase initiated the degradation of D- and L-phenylalanine, respectively. Both phenylpyruvate oxidase and phenylpyruvate decarboxylase activities could be demonstrated in the cell-free system. Phenylacetate hydroxylase, which required reduced nicotinamide adenine dinucleotide phosphate, converted phenylacetic acid to 2- and 4-hydroxyphenylacetic acid. Although 4-hydroxyphenylacetate was converted to 4-hydroxymandelate, 2-hydroxyphenylacetate was not utilized until the onset of sporulation. During sporulation, it was converted rapidly into homogentisate and oxidized to ring-cleaved products. 4-Hydroxymandelate was degraded to protocatechuate via 4-hydroxybenzoylformate, 4-hydroxybenzaldehyde, and 4-hydroxybenzoate.

Microbial metabolism of aromatic compounds has been the subject of intense research for several years, as evidenced by the review articles that have appeared in literature (4, 7, 22, 23). However, studies on the fungal degradation of phenylalanine have received only scant attention. The major fate of phenylalanine, in most of the fungi studied, is its degradation to cinnamic acid and to phenylpyruvic acid (10, 14-16, 22, 23). Cinnamate thus formed is converted to protocatechuate through benzoate, whereas phenylpyruvate is converted to homogentisate before ring cleavage (22, 23). Among the other pathways suggested for the phenylalanine metabolism is the one involving 4-hydroxymandelate (3, 18). Perrin and Towers (18) have detected radioactive 4-hydroxymandelate in the culture filtrates of *Polyporous hispidus* grown on phenylalanine or tyrosine. On the basis of 2,5-dihydroxybenzoylformate formation from both 4-hydroxymandelate and 4-hydroxyphenylacetate, Crowden (3) has suggested that 4-hydroxymandelate is formed from 4-hydroxyphenylacetate. In a preliminary communication (12), we have reported the isolation and identification of 4-hydroxymandelate as a key intermediate in the catabolism of phenylalanine by *Aspergillus niger*. In this paper we wish to report our detailed investigations on the pathway.

MATERIALS AND METHODS

Chemicals. DL-Phenylalanine, sodium phenylpyruvate, 2-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 4-hydroxymandelic acid (DL), 3,4-dihydroxybenzoic acid, homogentisic acid, thiamine pyrophosphate (TPP), nicotinamide adeninine dinucleotide phosphate (NADP), flavine adenine dinucleotide (FAD), riboflavine 5'-phosphate (FMN), pyridoxal phosphate (PALPO), phenazine methosulfate (PMS), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were from Sigma Chemical Co., St. Louis, Mo. 4-Hydroxybenzoic acid was supplied by K & K Laboratories, Inc., California.

All the other chemicals used were of analytical grade available commercially and purified when necessary.

Organism. *A. niger* isolated from the soil and identified by N. G. K. Karnath, of the Microbiology and Pharmacology Laboratory of the Indian Institute of Science, was used throughout the course of these investigations.

Cultivation. The organism was grown on a modified synthetic medium (1) containing the following nutrients: glucose, 50 g; KNO_3, 5 g; KH_2PO_4, 2.5 g; $MgSO_4 \cdot 7H_2O$, 1 g; Na_2SO_4, 1 g; DL-phenylalanine, 1 g (as the inducer); $FeCl_3 \cdot 6H_2O$, 20 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; $MnSO_4 \cdot 4H_2O$, 3 mg; $Na_2MoO_4 \cdot 2H_2O$, 1.5 mg; and $CuSO_4 \cdot 5H_2O$, 1 mg, in 1 liter of water. The pH of the medium was adjusted to 5.5 with 3 N NaOH. The medium was distributed in 500-ml conical flasks (60 ml per flask) and sterilized by autoclaving at 120°C) for 15 mins. Stock cultures were maintained on slants of the same medium solidified with 2% agar. The flasks were inoculated with a heavy suspension of spores in sterile water, obtained from agar slants 96 or more h old and incubated at 30°C for various intervals of time, ranging from 26 to 60 h, depending upon the experiment to be done. The mycelia were harvested before sporulation after decanting out the unspent medium and washing it with distilled water. The mycelia were then squeezed and dried between the foldings of a blotting paper. The dried mycelia could be used directly for preparation of enzymes and in vivo experiments or stored at −20°C until required.

In vivo experiments. Mycelial felts of *A. niger*, during various phases of growth, were taken, washed as described above, and suspended in solutions containing 1 mg of the metabolite of interest per ml in 0.025 M sodium phosphate buffer, pH 7.0 (hereafter referred to as the "replacement medium"). The culture suspensions were placed on a rotary shaker at 30°C for 4 to 6 h, and the products formed were analyzed.

Isolation of phenolic compounds. For the isolation of phenolic compounds from the aqueous solution, the mycelial felts were removed and the medium was filtered through glass wool. The filtrate was acidified to pH 2 with 2 N HCl and extracted repeatedly with peroxide-free distilled diethylether. The ethereal layers were pooled and resolved into the acidic and neutral fractions by extraction with 5% $NaHCO_3$. The acidic fraction was reextracted into ether after acidification to neutralize the bicarbonate. Both the fractions were dried over anhydrous sodium sulfate, concentrated, and subjected to chromatography.

Chromatography. The following solvent systems were used for the chromatographic separation (ascending) and identification of the phenolic compounds: solvent A; benzene-acetic acid-water (10:7:3 [vol/vol], organic phase); solvent B, formic acid-water (2:98 [vol/vol]; and solvent C, isopropanol-ammonia-water (20:1:2 [vol/vol]). The chromatograms were run at 30°C on Whatman no. 3 filter paper.

Detection. Aromatic compounds were located on the chromatograms by ultraviolet (UV) light. Phenolic compounds could be identified by spraying either with (i) freshly prepared, diazotized 4-nitroaniline reagent (3 ml of 0.3% solution of 4-nitroaniline in 0.8 N HCl plus 0.5 ml of 5% sodium nitrite, mixed before use) followed by alkali or (ii) a 1% ferric chloride-ferricyanide mixture (1:1). Carbonyl compounds could be recognized by their reaction with 2,4-dinitrophenyl hydrazine (0.1% 2,4-dinitrophenylhydrazine in 2 N HCl) followed by alkali.

The various phenolic compounds that were resolved by paper chromatography were eluted from the filter paper with ether. The ether extract was concentrated and subjected to repeated rechromatography so as to isolate a pure material.

UV spectra. A Cary-14 recording spectrophotometer was used for obtaining spectra of the various compounds in both the UV and visible regions of the spectrum.

Infrared spectra. Infrared (IR) spectra were determined in a spectrophotometer (Infracord, model 700, The Perkin-Elmer Corp.), using Nujol mull.

Estimation of carbonyl compounds. Carbonyl compounds were estimated using a modification of the colorimetric method of Friedemann and Haugen (6) for the estimation of 2-keto acids.

Estimation of 3,4-dihydroxybenzoic acid. 3,4-Dihydroxybenzoic acid was estimated by the method of Nair and Vaidyanathan (17).

Estimation of protein. The method of Lowry et al. (13) was used for the determination of protein. Bovine serum albumin was used as the standard.

Tracer studies. In these studies, mycelial felts of *A. niger* were placed in a medium containing 1 μCi of [U-^{14}C]phenylalanine, and the phenolics formed after 4 h were isolated in a manner similar to that described under in vivo experiments. The isolated phenolics were subjected to chromatography on Whatman no. 3 filter paper, using the solvent system A/B, and the chromatogram was kept over an X-ray film for 2 weeks. The film was then developed, and the radioactive zones were localized.

Estimation of concentrations of phenolic compounds during various phases of growth. The concentrations of phenolic metabolites formed during various phases of growth were determined as follows. The phenolic compounds extracted from the culture medium were subjected to quantitative two-dimensional chromatography using Whatman no. 3 paper and solvent system A/B. The individual compounds were eluted from the chromatogram using hot ether. After evaporating the ether, they were used for estimation. Determinations of 2- and 4-hydroxyphenylacetic acids were done by the 4-nitroaniline method described under Assay of phenylacetate hydroxylase. 3,4-Dihydroxybenzoic acid was estimated by the method of Nair and Vaidyanathan (17), and 2,5-dihydroxyphenylacetic acid was estimated, by the method of Fellman et al. (5).

Preparation of cell-free extract of *A. niger*. The cell-free extract of *A. niger* was obtained as follows. Mycelial felts, 10 g, were mixed with an equal weight of glass powder and ground to a fine slurry in a mortar. The slurry was uniformly suspended in 30 ml of 0.025 M sodium phosphate buffer, pH 7.0, and the cell debris as well as insoluble particles were removed by centrifugation at 27,000 × *g* for 30 min. The supernatant obtained was designated as the "crude extract" and used as an enzyme source. For preparation of the particulate fraction, the crude extract was subjected to ultracentrifugation at 100,000 × *g* for 60 min. The supernatant obtained was designated as the "soluble fraction," and the pellet, after suspension in 0.025 M sodium phosphate buffer, was referred to as the "particulate fraction."

Unit of enzyme activity. One unit of enzyme activity is defined as the amount that catalyzes the transformation of 1 μmol of the substrate or the formation of 1 μmol of the product per min under the conditions of the assay. Specific activity is expressed as milliunits of enzyme activity per milligram of protein.

Enzyme assays. All the assays reported in this section measure the activities of the corresponding enzymes under standard conditions (initial velocities, saturating conditions, etc.). D-Amino acid oxidase activity was measured by incubating, at 30°C for 15 min, 0.5 ml of a reaction mixture consisting of D-phenylalanine (0.5 μmol), sodium phosphate buffer, pH 7.6 (60 μmol), and enzyme (1 mg of protein). The reaction was terminated by the addition of 0.1 ml of 8 N HCl, and the precipitated protein was removed by centrifugation at 5,000 × *g* for 5 min. From the supernatant, 0.25 ml was treated with 0.1 ml of 2,4-dinitrophenylhydrazine reagent, and the solution was placed in a boiling water bath for 2 min. The solution was cooled to room temperature, and the hydrazone formed was estimated after addition of 1 ml of 10% NaOH, by measuring its absorption at 460 nm as described earlier. Reaction mix-

tures to which the substrate was added at the end of the incubation period served as a control.

(i) **L-Phenylalanine: 2-oxoglutarate aminotransferase.** The activity of L-phenylalanine aminotransferase was determined by measuring the increase in absorption at 660 nm of 1 ml of a reaction mixture consisting of L-phenylalanine (1 μmol), 2-oxoglutarate (1 μmol), PALPO (0.1 μmol), PMS (1 μmol), sodium phosphate buffer, pH 7.0 (60 μmol), and enzyme (1 mg of protein) against the blank, in which phenylalanine was omitted.

(ii) **Phenylpyruvate decarboxylase.** A reaction mixture (0.5 ml) consisting of sodium phenylpyruvate (0.2 μmol), TPP (0.05 μmol), sodium phosphate buffer, pH 7.0 (30 μmol), and enzyme (1 mg of protein) was incubated at 30°C for 30 min. The reaction was terminated by the addition of 0.1 ml of 8 N HCl, and the hydrazones of phenylpyruvate and phenylacetaldehyde were obtained. The solutions were extracted with ether, and the ether extract was subjected to a second extraction with 5% $NaHCO_3$. The absorbance of the bicarbonate layer at 460 nm was measured. Similarly, the ether layer was dried over anhydrous sodium sulphate, and the ether was evaporated in vacuo. One milliliter of 10% NaOH was added to the dry material, and its absorbance at 460 nm was measured.

(iii) **Phenylpyruvate oxidase.** A 3.1-ml reaction mixture consisting of sodium phenylpyruvate (3.0 μmol), FAD (0.2 μmol), sodium phosphate buffer, pH 7.0 (140 μmol), and enzyme, (8 mg of protein) was placed in the Clark oxygen electrode cell, and the oxygen tension was recorded as a function of time.

The disappearance of phenylpyruvate was measured by its reaction with 2,4-dinitrophenylhydrazine as described earlier.

(iv) **Phenylacetate hydroxylase;** The activity was determined by incubating a mixture consisting of sodium phenylacetate (1 μmol), sodium phosphate buffer, pH 7.0 (40 μmol), NADPH (0.2 μmol), glucose-6-phosphate (0.25 μmol), 0.2 U of glucose-6-phosphate dehydrogenase, and enzyme (3 mg of protein) in a final volume of 1 ml for 30 min at 30°C. The reaction was arrested by the addition of 0.1 ml of 8 N HCl. The precipitated proteins were removed by centrifugation at 5,000 × *g* for 5 min, and 0.75 ml of the supernatant was subjected to three extractions with 2-ml portions of ether. The ether extracts were pooled, and the ether was evaporated off. The residue was dissolved in 0.5 ml of 95% ethanol, treated with 0.2 ml of diazotized 4-nitroaniline and, after 2 min, with 1 ml of 5% sodium carbonate solution. The orange-red color developed was measured at 540 nm in a spectrophotometer (Pye Unicam) against a blank from a reaction mixture in which the reaction was arrested immediately after mixing and taken through the same treatments.

(v) **4-Hydroxymandelate dehydrogenase.** A reaction mixture consisting of DL-4-hydroxymandelate (1 μmol). NAD^+ or $NADP^+$ (1 μmol), sodium phosphate buffer, pH 7.0 (40 μmol), and enzyme (1 mg of protein) was placed in a 1-ml cuvette, and the increase in absorbance at 340 nm was monitored as a function of time against a blank devoid of the substrate. An alternative assay, based on the reaction of 4-hydroxybenzoylformic acid with 2,4-dinitrophenylhydrazine, was also employed.

(vi) **4-Hydroxybenzoylformic acid decarboxylase.** The enzyme was assayed by incubating in a total volume of 1.2 ml, 50 μmol of sodium phosphate buffer (pH 6), 0.1 μmol of TPP, 1 μmol of 4-hydroxybenzoylformic acid, and enzyme (1 mg of protein) at 30°C for 30 min. Substrate was added to the blank after stopping the reaction with 0.2 ml of 2 N HCl. A portion of the reaction mixture, after removal of the precipitated protein, was extracted with 2 ml of carbon tetrachloride, which solubilizes benzaldehyde, leaving 4-hydroxybenzoylformic acid in the aqueous layer. The two layers were separated, and a 0.2-ml portion of the aqueous layer was diluted to 1 ml with water and estimated as its 2,4-dinitrophenylhydrazone (9).

(vii) **4-Hydroxybenzaldehyde dehydrogenase.** The dehydrogenase activity could be assayed by incubating, in a total volume of 1 ml, 0.2 μmol of 4-hydroxybenzaldehyde, 0.2 μmol of NAD^+, 40 μmol of sodium phosphate buffer (pH 7.0), and enzyme (1 mg of protein) for 30 min at 30°C. The reaction was terminated by the addition of 0.1 ml of 8 N HCl, and the activity was assayed by hydrazone formation.

(viii) **4-Hydroxybenzoate-3-hydroxylase.** One-milliliter reaction mixtures containing the following components were incubated at 30°C for 30 min: 4-hydroxybenzoate (0.2 μmol), tris(hydroxymethyl)-aminomethane-maleate buffer (pH 6.2; 28 μmol), FAD (0.02 μmol), NADPH (0.2 μmol), glucose-6-phosphate (0.2 μmol), glucose-6-phosphate dehydrogenase (2 mU), and enzyme (1 mg of protein). The reaction was terminated by the addition of 0.2 ml of 2 N HCl, and the precipitated proteins were removed by centrifugation at 5,000 × *g* for 5 min. Protocatechuate formed was estimated by the method of Nair and Vaidyanathan (17).

(ix) **3,4-Dihydroxybenzoate oxygenase.** The oxygenase activity was estimated by incubating 1 ml of a reaction mixture consisting of 3,4-dihydroxybenzoate (0.5 μmol), tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.2; 40 μmol), and enzyme (1 mg of protein) at 30°C for 30 min. The reaction was arrested by the addition of 0.2 ml of 2 N HCl, and the amount of 3,4-dihydroxybenzoic acid that had disappeared was estimated by the method of Nair and Vaidyanathan (17).

(x) **2,5-Dihydroxyphenylacetate oxygenase.** Homogentisate oxygenase activity was conveniently measured in a Clark oxygen electrode using a 3.1-ml reaction mixture containing the following components: sodium phosphate buffer (pH 7.2; 140 μmol), ascorbic acid (5 μmol), enzyme (6 mg of protein), and homogentisic acid (3 μmol) at 30°C. The oxygen uptake was measured during the progress of the reaction. In the same reaction mixture, increase in absorbance at 330 nm could be monitored as a function of enzyme activity, but the activities were not significant in view of the contaminating isomerase.

The enzyme protein used in this assay was isolated from 60-h-old cultures that had partially sporulated.

Isolation of the product of degradation of protocatechuic acid by cell-free extracts. One hundred milliliters of a reaction mixture described for the

assay of 3,4-dihydroxybenzoate oxygenase was incubated with 1 mmol of 3,4-dihydroxybenzoate for 3 h, and the reaction was stopped by the addition of 20 ml of 6 N H_2SO_4. The protein was removed by centrifugation, and the supernatant was saturated with $(NH_4)_2SO_4$ and repeatedly extracted with diethylether. The ether layers were combined, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was recrystallized twice from ethylacetate-benzene to yield about 20 mg of final product.

RESULTS

Analysis of culture filtrates. The neutral fraction of the phenolic compounds isolated from 30-h-old cultures contained a single phenolic compound, which could be identified as 4-hydroxybenzaldehyde by its UV and IR spectra, color reaction with 2,4-dinitrophenylhydrazine, and diazotized 4-nitroaniline, as well as its chromatographic mobility in several solvent systems (Table 1). The concentration of 4-hydroxybenzaldehyde in 36-h-old cultures was maximum and there was a rapid disappearance after 37 h.

The acidic fraction of the 30-h-old cultures, by two-dimensional chromatography in solvent systems A/B and C/A, could be resolved into five components that were identified as 2- and 4-hydroxyphenylacetic acids, 4-hydroxymandelic acid, 4-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid. The UV and IR spectra of each of these five components corresponded well with those of the authentic samples.

Maximum concentrations of 4-hydroxymandelic acid were detected in 32-h-old cultures. Beyond 40 h, there were no detectable amounts of 4-hydroxymandelic acid left in the culture medium. The variation in 4-hydroxymandelic acid concentration as a function of time is depicted in Fig. 1. The IR and UV spectra of 4-hydroxymandelic acid isolated from the culture filtrate and the spectra of an authentic sample are given in Fig. 2 and 3. The concentration of 3,4-dihydroxybenzoic acid in the culture medium attained the highest concentrations at around 36 h (Fig. 1).

2-Hydroxyphenylacetic acid accumulated in the culture medium, reaching a constant high concentration beyond 36 h (Fig. 4). It was not utilized until after 42 h of growth. Beyond 44 h, i.e., with the advent of sporulation, this pheno-

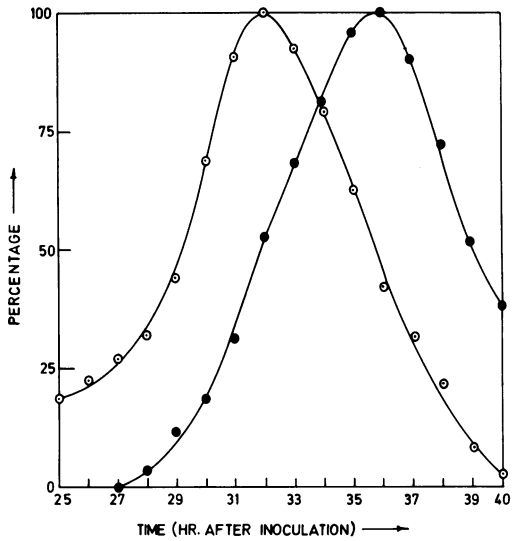

FIG. 1. *Time course for the accumulation of 4-hydroxymandelic acid* (○) *and protocatechuic acid* (●) *in the medium. Estimations were done as described under Materials and Methods. The concentration of each phenolic compound is expressed as the percentage of its highest concentration. One hundred percent of 4-hydroxymandelic acid = 150 mg/liter; 100% of 3,4-dihydroxybenzoic acid = 54 mg/liter.*

TABLE 1. *R_f values and color reactions of phenolic compounds*

Name of compound	R_f values in solvent systems						Color reaction with diazotized 4-nitroaniline/NaOH
	A[a]		B		C		
	a	i	a	i	a	i	
2-Hydroxyphenylacetic acid	0.46	0.46	0.88	0.88	0.52	0.51	Crimson red
4-Hydroxyphenylacetic acid	0.30	0.31	0.82	0.83	0.42	0.43	Bluish-violet
4-Hydroxymandelic acid	0.01	0.01	0.89	0.88	0.23	0.23	Brick red
4-Hydroxybenzaldehyde	0.40	0.40	0.58	0.58	0.38	0.37	Light brown (fading)
4-Hydroxybenzoic acid	0.36	0.35	0.60	0.60	0.35	0.36	Light brown (fading)
3,4-Dihydroxybenzoic acid	0.02	0.03	0.58	0.59	0.03	0.02	Light blue fading to brown
2,5-Dihydroxyphenylacetic acid	0.02	0.03	0.88	0.88	—[b]	—[b]	Light blue fading to brownish-yellow

[a] a, Authentic; i, isolated.
[b] Trailing with oxidation.

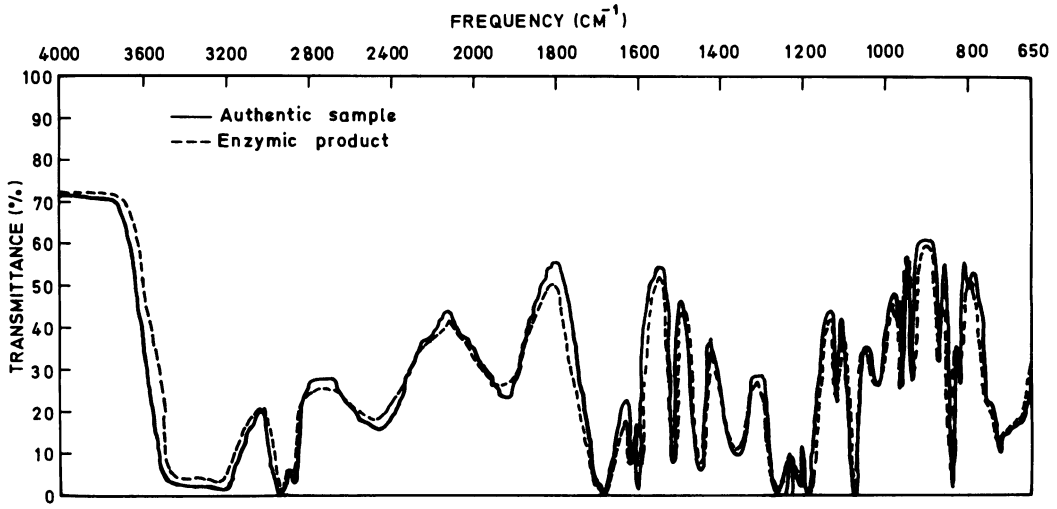

FIG. 2. *IR spectrum of 4-hydroxymandelic acid in Nujol mull:* (——)*authentic sample;* (----) *enzymic product.*

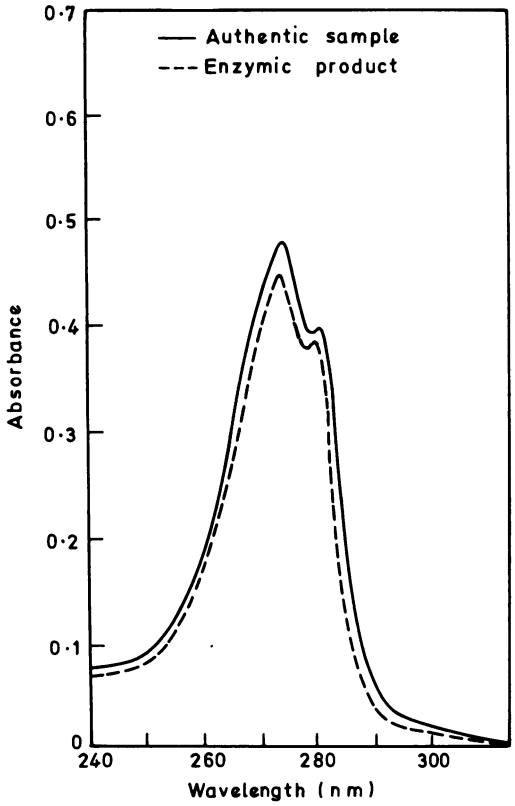

FIG. 3. *UV absorption spectrum of 4-hydroxymandelic acid in ethanol:* (——) *authentic sample;* (----) *enzymic product.*

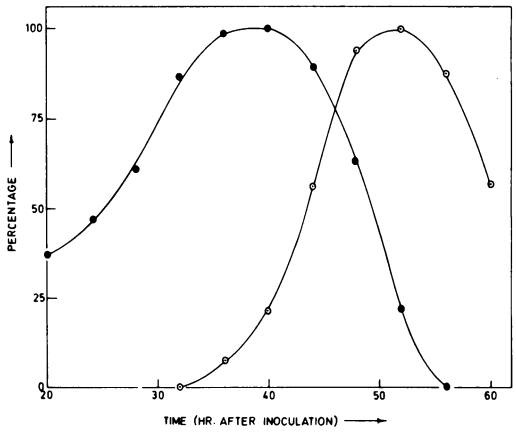

FIG. 4. *Time course for the accumulation of 2-hydroxyphenylacetic acid* (○) *and 2,5-dihydroxyphenylacetic acid* (●) *in the medium. Estimations were done as described under Materials and Methods. The concentration of each phenolic compound is expressed as percentage of its highest concentration. One hundred percent of 2-hydroxyphenylacetic acid = 250 mg/liter; 100% of 2,5-dihydroxyphenylacetic acid = 65 mg/liter.*

lic compound was rapidly degraded, and only trace amounts could be detected after 52 h.

From the 50-h-old cultures, homogentisic acid was isolated and its identity was established by spectral, chromatographic, and chemical criteria. After 65 h of growth, very low amounts of phenolics were present in the culture medium. This corresponded to the mid-sporulation phase of this organism.

All the data that have been reported here have been compared with those obtained from glucose-grown cells.

In vivo experiments. The capacity of *A. niger* to utilize DL-phenylalanine as a carbon source was demonstrated in experiments using whole mycelia. Cells grown on glucose medium

alone could not metabolize DL-phenylalanine immediately but could do so after a lag, as judged by the phenolic concentration of the "replacement medium." Cells grown on glucose supplemented with DL-phenylalanine could, however, rapidly metabolize the aromatic amino acids. Such cells could also rapidly metabolize any of the intermediates described above. Table 2 summarizes the results of the in vivo experiments.

Identification of the ring cleaved product of 3,4-dihydroxybenzoate. The isolated product melted at 120°C (uncorrected), and the melting point was not depressed by admixture with authentic 3-oxoadipic acid, which also melted at the same temperature, with decomposition.

Tracer studies. The radioautogram of the phenolic compounds isolated from the replacement medium containing [U-^{14}C]phenylalanine is shown in Fig. 5. This establishes that the degradation of phenylalanine by *A. niger* proceeds through the various intermediates mentioned above. Approximately 70% of the total incorporated radioactive material is accounted for by these phenolics and 20% is accounted for by the trichloroacetic acid-precipitable materials.

Enzymes of the pathway. Cell-free extracts of *A. niger* possessed D-amino acid oxidase activity. By the action of this enzyme, D-phenylalanine could be metabolized to phenylpyruvic acid. That the pathway for the metabolism of the D as well as of the L isomer is the same was confirmed by exposing *A. niger* cells to the two isomers separately.

The metabolism of L-phenylalanine to phenylpyruvic acid was initiated by an aminotransferase that employed 2-oxoglutarate as the amino group acceptor. Phenylpyruvic acid acts as the converging point for the degradation of D- and L-phenylalanine by *A. niger*.

Cell-free extracts of *A. niger* did not possess any amino acid racemase activity. Neither phenylalanine-ammonia lyase nor phenylalanine-4-hydroxylase activity could be detected in the cell-free preparations. There was no significant incorporation of label in the reisolated cinnamic acid and tyrosine when the organism was incubated with labeled phenylalanine and cold cinnamic acid and tyrosine were added after the incubation time.

Both phenylpyruvate decarboxylase and phenylpyruvate oxidase activities could be demonstrated in the crude extracts of *A. niger*.

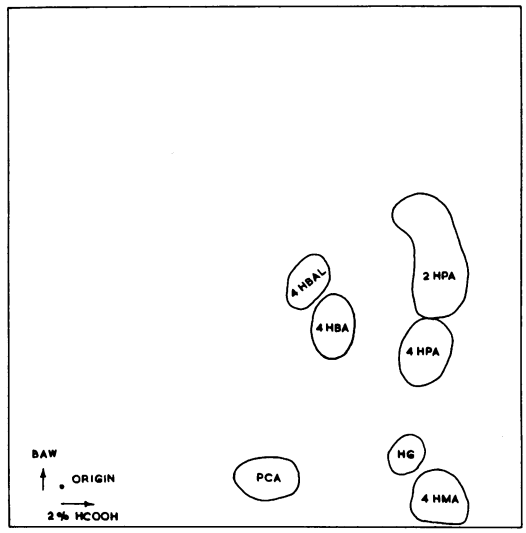

FIG. 5. *Autoradiogram of the radioactive phenolics synthesized from [U-^{14}C]phenylalanine by A. niger. A. niger cells grown for 30, 40, and 50 h were mixed and incubated with labeled phenylalanine for 4 h. At the end, the phenolics were isolated and chromatographed using solvent system A/B. The paper chromatogram was kept on an X-ray film for 2 weeks and developed. 4 HBAL, 4-Hydroxybenzaldehyde; 4HBA, 4-hydroxybenzoic acid; 4 HPA, 4-hydroxyphenylacetic acid; 2HPA, 2-hydroxyphenylacetic acid; PCA, protocatechuic acid; HG, homogentisic acid; 4 HMA, 4-hydroxymandelic acid.*

TABLE 2. *Results of in vivo experiments with 32-h-old-cultures*

Compounds added to replacement medium	Phenolic compounds detected					
	2-Hydroxyphenyl acetic acid	4-Hydroxyphenyl acetic acid	4-Hydroxymandelic acid	4-Hydroxybenzaldehyde	4-Hydroxybenzoic acid	Protocatechuic acid
D-Phenylalanine	3+	3+	+	+	3+	2+
L-Phenylalanine	3+	3+	+	+	3+	2+
Phenylpyruvic acid	3+	3+	2+	+	3+	2+
Phenylacetic acid	3+	3+	2+	+	3+	2+
2-Hydroxyphenylacetic acid	3+	−	−	−	−	−
4-Hydroxyphenylacetic acid	−	3+	3+	+	2+	3+
4-Hydroxymandelic acid	−	−	3+	+	3+	3+
4-Hydroxybenzaldehyde	−	−	−	3+	3+	3+
4-Hydroxybenzoic acid	−	−	−	−	3+	3+
Protocatechuic acid	−	−	−	−	−	3+

The former enzyme employed TPP as a cofactor, whereas the latter showed an absolute requirement for FAD. Phenylpyruvate decarboxylase decarboxylated phenylpyruvate to phenylacetaldehyde in a nonoxidative decarboxylation reaction. Phenylpyruvate oxidase could bring about the conversion of phenylpyruvate to phenylacetate. The enzyme was not activated by NAD^+, $NADP^+$, or TPP. FAD could be replaced by other flavins, significantly by FMN, which could function as efficiently as FAD itself. The enzyme exhibited a high degree of specificity toward phenylpyruvic acid and could not utilize other 2-keto acids, notably benzoylformic acid, as its substrate.

Hydroxylations of phenylacetic acid at the 2- and 4-positions, yielding 2- and 4-hydroxyphenylacetic acids, could be demonstrated in the cell-free extracts of *A. niger*. It has not been possible to resolve these two activities so far. The enzyme required NADPH as the donor of reducing equivalents for hydroxylation reaction. NADH could not replace NADPH for hydroxylation.

In spite of the rapid conversion of 4-hydroxyphenylacetic acid to 4-hydroxymandelic acid in vivo, attempts at constituting this enzyme activity in cell-free preparations were unsuccessful. In spite of this, positive evidence for the formation of 4-hydroxymandelic acid from 4-hydroxyphenylacetic acid has been obtained by in vivo tracer studies and spectral characteristics as well as chemical reactions of this metabolite.

The ability to oxidize 4-hydroxymandelic acid to 4-hydroxybenzoylformic acid was associated with both the particulate as well as the soluble fractions of *A. niger*. Both fractions were found to utilize both pyridine nucleotides as cofactors for the reaction. $NADP^+$ elicited a higher reaction rate than NAD^+. The stereospecificities of the reactions could not be checked since the D- and L-enantiomers of 4-hydroxymandelic acid were not available.

Crude extracts of *A. niger* could decarboxylate 4-hydroxybenzoylformic acid to 4-hydroxybenzaldehyde. The enzyme required TPP as a cofactor. The requirements for divalent metal ions in crude preparations could not be demonstrated. The enzyme could also decarboxylate benzoylformic acid.

4-Hydroxybenzaldehyde dehydrogenase, an enzyme that required NAD^+ as a cofactor, was capable of bringing about the oxidation of the aldehyde to the acid.

Hydroxylation of 4-hydroxybenzoic acid by the enzyme 4-hydroxybenzoate-3-hydroxylase was NADPH dependent and required FAD as a cofactor. The product of this reaction, viz., 3,4-dihydroxybenzoic acid, has all the necessary prerequisites for undergoing the ring cleavage reaction. This is accomplished by a dioxygenase, 3,4-dihydroxybenzoate 3,4-dioxygenase, which effected an intradiol cleavage of the substrate.

Inducible nature of the enzymes of the pathway for phenylalanine metabolism in *A. niger*. All the enzymes of the pathway hitherto described were strictly inducible, and no activity could be detected in the extract of mycelium grown on glucose alone, by the methods employed, except the D-amino acid oxidase and L-phenylalanine amino transferase that were present even in the absence of the inducer. Nevertheless, the presence of either D-phenylalanine, L-phenylalanine, phenylpyruvic acid, or 4-hydroxyphenylacetic acid either in the growth medium or in the replacement medium led to a distinct increase in the activity of these enzymes. The specific activities of the enzymes of the phenylalanine pathway are given in Table 3.

DISCUSSION

The results presented in Table 1 suggest the formation of certain phenolic compounds from DL-phenylalanine due to the metabolic activity of the fungus *A. niger*. These compounds were identified as 2- and 4-hydroxyphenylacetic acids, 4-hydroxymandelic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid. In addition to these phenolics, the 50-h-old cultures contain homo-

TABLE 3. *Specific activities of enzymes of the phenylalanine pathway*

Enzyme	Sp act (mU/mg protein)	
	Glucose grown	Phenylalanine grown
D-Amino acid oxidase	50.0	120.0
L-Phenylalanine: 2 oxoglutarate aminotransferase	70.0	132.0
Phenylpyruvate oxidase	0.0	78.0
Phenylpyruvate decarboxylase	0.6	1.1
Phenylacetate hydroxylase (total)	2.0	40.0
4-Hydroxymandelate dehydrogenase	0.0	37.0
4-Hydroxybenzoylformate decarboxylase	0.0	23.0
4-Hydroxybenzaldehyde dehydrogenase	0.0	31.0
4-Hydroxybenzoate-3-hydroxylase	0.0	7.0
3,4-Dihydroxybenzoate dioxygenase	0.0	28.2
Homogentisate oxidase	0.0	12.0

gentisic acid as well. The identity of these phenolics has been further confirmed by IR data.

The results of in vivo experiments using cold, as well as labeled, phenylalanine provide overwhelming evidence for the formation of these intermediates from phenylalanine by *A. niger*. A tentative scheme based on these data may therefore be drawn up for the metabolism of DL-phenylalanine by *A. niger*, and this is presented in Fig. 6.

Studies on the enzymes of the pathway have shown that D-phenylalanine is oxidized by means of a specific oxidase that is a soluble enzyme. The L-isomer, on the other hand, is metabolized by an aminotransferase, that can employ 2-oxoglutarate as the keto acid substrate. The absence of racemase rules out the possibility of interconversions of the isomers and indicates that the enzymes mentioned above initiate the metabolism of their respective substrates. The converging point in the metabolism of DL-phenylalanine by *A. niger* is, therefore, at the level of phenylpyruvate. Such a view is further substantiated by the fact that the metabolites formed from both D- and L-phenylalanine are the same (Table 2).

Lack of phenylalanine hydroxylase in *A. niger* precludes the possibility of the conversion of the aromatic amino acid to tyrosine before undergoing further metabolism. This appears to be the case not only for this organism, but also for most other fungi. Several fungi are known to degrade phenylalanine via the cinnamate pathway (10, 14–16, 22, 23). The operation of this pathway has, however, been shown to be restricted to *Basidiomycetes* and a few fungi imperfecti. None of the *Ascomycetes* tested elaborate this pathway.

The significance of the production of nonoxidative decarboxylase and an oxidative decarboxylase for the degradation of phenylpyruvic

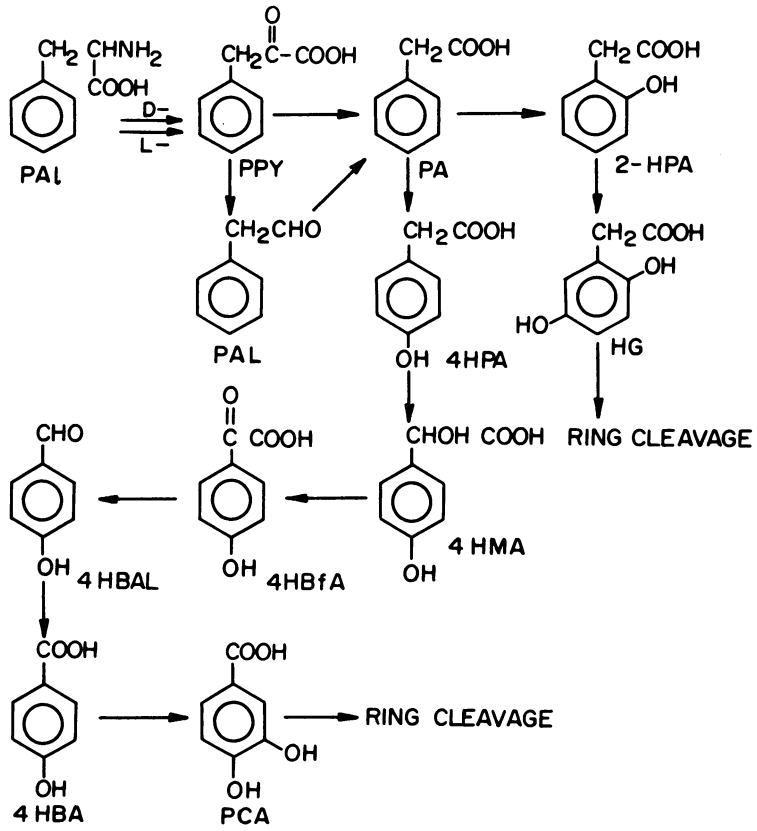

FIG. 6. *Proposed pathway for the metabolism of DL-(±)-phenylalanine in A. niger. PAL, Phenylalanine; PPY, phenylpyruvic acid; PA, phenylacetic acid; PAL, phenylacetaldehyde; 4 HBfA, 4-hydroxybenzoylformic acid; 4 HBAL, 4-hydroxybenzaldehyde; 4HBA, 4-hydroxybenzoic acid; 4 HPA, 4-hydroxyphenylacetic acid; 2HPA, 2-hydroxyphenylacetic acid; PCA, protocatechuic acid; HG, homogentisic acid; 4HMA, 4-hydroxymandelic acid. A double arrow indicates that the mechanisms of conversion of the D- and L-isomers are different.*

acid is not clear. The available evidence strongly suggests the existence of two separate proteins for the indicated activities. Thus, the nonoxidative decarboxylase is activated by TPP, whereas the oxidative decarboxylase is stimulated by FAD or FMN. The nonoxidative decarboxylase is a labile enzyme, whereas the oxidase is extremely stable. TPP has no effect on the rates of the reaction catalyzed by the partially purified oxidase. Phenylacetate hydroxylation requires NADPH and results in the formation of both 2- and 4-hydroxyphenylacetic acids. However, 2-hydroxyphenylacetic acid is formed in larger amounts compared with 4-hydroxyphenylacetic acid.

The conversion of 4-hydroxyphenylacetate to 4-hydroxymandelate has been firmly established. Although the enzyme involved in this conversion could not be demonstrated in the cell-free extract, results of other experiments provide strong support for such a contention. Presumably the enzymatic machinery involved in this conversion should be analogous to dopamine-β-hydroxylase (11) or ω-hydroxylase of fatty acids (19).

Although the metabolic origin of mandelic acids had been restricted to plants, several fungi also seem to synthesize them. Thus, their formation had been suggested by Crowden in *Polyporous tumuloses* (3) and by Perrin and Towers in *Polyporous hispidus* (18). However, the results presented here provide convincing proof for the formation of 4-hydroxymandelic acid from phenylalanine by *A. niger*.

The metabolic fate of 4-hydroxymandelate biosynthesized from phenylalanine is similar to that reported earlier for mandelic acid by Jamaluddin et al. (9). Two dehydrogenases, differing in their subcellular distribution, bring about the dehydrogenation of 4-hydroxymandelate to 4-hydroxybenzoylformic acid. Both dehydrogenases showed a requirement for pyridine nucleotides. This is in contrast to the earlier reports on the mandelate oxidases of *A. niger* (20; M. Ramanarayanan, Ph.D. thesis, Indian Institute of Science, Bangalore, India, 1972). These investigators had shown that *A. niger* grown on mandelate possesses two distinct oxidases, a particulate D-mandelate oxidase, whose cofactor requirement could not be identified, and a soluble L-mandelate oxidase, which showed an absolute requirement for flavins. Both enzymes utilized oxygen as the most efficient electron acceptor. The 4-hydroxymandelate dehydrogenases of the *A. niger* strain under investigation, however, showed a requirement specifically for pyridine nucleotides and could not transfer electrons to oxygen. $NADP^+$ was the preferred electron acceptor in both the particulate and soluble enzymes. The differences may be attributable to the strain variations.

4-Hydroxybenzoylformate decarboxylase, like benzoylformate decarboxylase of *A. niger* in earlier reports (9), showed a requirement for TPP. The same enzyme could decarboxylate benzoylformate. The product of the reaction, viz., 4-hydroxybenzaldehyde, was rapidly metabolized by crude extracts in the presence of pyridine nucleotides to 4-hydroxybenzoic acid.

It may be interesting to point out that *Pseudomonas convexa* (S. G. Bhat, Ph.D. thesis, Indian Institute of Science, Bangalore, India, 1975) is capable of bringing about a direct conversion of 4-hydroxymandelate to 4-hydroxybenzaldehyde. Such a direct conversion could not be demonstrated in *A. niger*.

Although the presence of 4-hydroxybenzaldehyde dehydrogenase was established, no attempts were made to characterize the specific cofactor requirements of this enzyme. According to Jamaluddin et al. (9), *A. niger* contains two distinct benzaldehyde dehydrogenases, one showing a specific requirement for NAD^+ and the other for $NADP^+$. Similar observations have been made by Gunsalus et al. (8) and Stachow et al. (21) with *Pseudomonas putida*. With the formation of 4-hydroxybenzoic acid, the required structure has been achieved for a subsequent series of oxygenation reactions. Thus, 4-hydroxybenzoate-3-hydroxylase brings about hydroxylation of the aromatic nucleus of 4-hydroxybenzoic acid, yielding 3,4-dihydroxybenzoic acid. The enzyme, as Cain (2) and his co-workers have already reported, utilizes both NADPH and NADH as electron donors for the hydroxylation reaction. FAD was an essential cofactor. 3,4-Dihydroxybenzoate 3,4-dioxygenase of *A. niger* brought about a conversion of 3,4-dihydroxybenzoate to 3-carboxy *cis, cis*-muconic acid, and this was further metabolized to 3-oxoadipic acid.

A. niger grown in the presence of DL-phenylalanine could oxidize 3,4-dihydroxybenzoate but not catechol. This organism, therefore, belongs to a group of fungi wherein the protocatechuate and catechol branches of aromatic metabolism merge at the level of 3-oxoadipate. In certain other fungi, the merger is accomplished at the level of catechol by the action of 3,4-dihydroxybenzoate decarboxylase, which converts 3,4-dihydroxybenzoate to catechol (2). In the case of the former group, the product of the oxygenase reaction is 3-carboxy-*cis, cis*-muconate.

As mentioned earlier, the second product of hydroxylation of phenylacetate is 2-hydroxyphenylacetate. One of the pathways that has

been described for the metabolism of 2-hydroxyphenylacetate involves its conversion to homogentisate. Although the presence of such an enzyme system could not be demonstrated in cell-free extracts, in vivo experiments showed a conversion of 2-hydroxyphenylacetate to homogentisate by cells in the sporulating phase. Such a conversion, however, did not occur in the presporulation phase of the organism. Since the metabolism of 2-hydroxyphenylacetate does not occur before the onset of sporulation, even if it is exogenously supplied in the medium, it can be concluded that the genes are derepressed only in the sporulation phase either because of the high rates of anabolism, necessitating the catabolism of all available secondary metabolites, or because of the incorporation of the aromatic nucleus into some structural materials like the melanins of the spore. The latter possibility appears to be remote, in view of the detection of homogentisate oxygenase in the cell-free extracts.

The evidences presented in the foregoing discussion justify the metabolic scheme drawn up in Fig. 6. The microbial metabolism of phenylalanine can be broadly classified into metabolism via (i) phenylpyruvate and (ii) cinnamate pathways. Most bacteria studied and a few fungi employ the former pathway, whereas most fungi, as mentioned earlier, follow the cinnamate pathway. *A. niger* belongs to a group of fungi that employ the phenylpyruvate pathway. Microorganisms, within the former framework, may follow either the homoprotocatechuate pathway or the homogentisate pathway (23). Phenylacetate can be hydroxylated at the 2-, 3-, or 4-positions and subsequently to the dihydroxyphenylacetates mentioned above. A third variant of the phenylpyruvate pathway is provided by the demonstration of protocatechuate branch operating in *A. niger*. Similar pathways had been postulated for the metabolisms of [^{14}C]tyrosine in *Telletiopsis washingtonsensis* (23), 2,5-dihydroxyphenylacetate in *Polyporous tumuloses* (3), and L-phenylalanine by *Polyporous hispidus* (18). The present studies, however, constitute the first report on the clear-cut demonstration of the existence of such a pathway in microbes.

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