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Involvement of 4-Hydroxymandelic Acid in the Degradation of Mandelic Acid by *Pseudomonas convexa*

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A microorganism capable of degrading DL-mandelic acid was isolated from sewage sediment by enrichment culture and was identified as *Pseudomonas convexa*. It was found to metabolize mandelic acid by a new pathway involving 4-hydroxymandelic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid as aromatic intermediates. All the enzymes of the pathway were demonstrated in cell-free extracts. L-Mandelate-4-hydroxylase, a soluble enzyme, requires tetrahydropteridine, nicotinamide adenine dinucleotide phosphate, reduced form, and Fe^{2+} for its activity. The next enzyme, L-4-hydroxymandelate oxidase (decarboxylating), a particulate enzyme, requires flavine adenine dinucleotide and Mn^{2+} for its activity. A nicotinamide adenine dinucleotide-dependent, as well as a nicotinamide adenine dinucleotide phosphate-dependent, benzaldehyde dehydrogenase has been resolved and partially purified.

Mandelic acid (phenylglycolic acid) is an aromatic α-hydroxy acid of plant origin (4, 5). Both D(−)- and L(+)-isomers occur in nature. A number of microorganisms can degrade mandelic acid (9, 10, 16, 18-20). In all the cases so far studied, mandelic acid is converted to benzoic acid by a similar set of enzyme-catalyzed reactions. The chemical events of the mandelate pathway in each organism so far studied are as follows: mandelic acid → benzoylformic acid → benzaldehyde → benzoic acid.

In bacteria, benzoic acid is further metabolized via the catechol branch of 3-oxoadipate pathway, whereas fungi convert the benzoic acid to 4-hydroxybenzoic acid, which is metabolized via the protocatechuate branch of the 3-oxoadipate pathway. By analogous chemical events, 4-hydroxymandelic acid is also shown to be degraded in microorganisms (11, 18). The pathway followed for the degradation of 4-hydroxymandelic acid is as follows: 4-hydroxymandelic acid → 4-hydroxybenzoylformic acid → 4-hydroxybenzaldehyde → 4-hydroxybenzoic acid. It has been shown that the same set of enzymes as those responsible for the oxidation of mandelic acid to benzoic acid are involved in the oxidation of 4-hydroxymandelic acid to 4-hydroxybenzoic acid (18, 30).

We have isolated an organism from sewage sediment by enrichment on mandelic acid and identified it as *Pseudomonas convexa*. From the preliminary studies it was found that 4-hydroxymandelic acid, 4-hydroxybenzoic acid, and protocatechuic acid were the metabolites that accumulated in culture medium of *P. convexa*, grown in the presence of mandelic acid. This observation led us to a more detailed investigation of the mandelate pathway in *P. convexa*, the results of which are reported in the present paper. Part of the work presented here has been published earlier (1).

MATERIALS AND METHODS

Chemicals. DL-Mandelic acid and benzoylformic acid were purchased from Aldrich Chemical Co., Milwaukee, Wis. D(−)-Mandelic acid, L(+)-mandelic acid, and DL-4-hydroxymandelic acid were obtained from Sigma Chemical Co., St. Louis, Mo. 2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine and tetrahydrofolic acid were purchased from Calbiochem., Los Angeles, Calif.

Isolation and identification of microorganism. Enrichments were set up using basal salt medium (23), containing 0.3% DL-mandelic acid as carbon source, and inoculated with 1 g of sewage sediment. After several transfers at weekly intervals, the cultures were streaked onto a similar medium solidified with 2% agar. The developing colonies were picked and purified by the conventional techniques. The isolates were again checked for growth on mandelic acid-containing medium. Selected strains were tested for the mode of metabolism of mandelic acid by analysis of the culture medium for the products of mandelic acid degradation, as described later. One strain that was found to convert mandelic acid to 4-hydroxymandelic acid was chosen for further studies. The morphological, cultural, physiological, and biochemical characters of this bacterial strain were studied by adopting the recommended methods (6). *Bergey's Manual* (2) and Skerman's key (26) were referred to for the identification of the organism.

Maintenance of the organism. *P. convexa* was maintained on nutrient agar slants. Stock cultures were also maintained in mineral salts medium (described below) on a rotary shaker.

Conditions of growth of the organism. *P. convexa* was grown in the mineral salts medium described by Seubert (25), which contains: K_2HPO_4, 6.3 g; KH_2PO_4, 1.82 g; NH_4NO_3, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.06 g; $MnSO_4$, 0.06 g; and DL-mandelic acid, 3 g (when mandelic acid is used as sole carbon source); or DL-mandelic acid, 1.0 g; and glucose, 5.0 g (when mandelic acid is used as inducer and glucose as the main carbon source) per liter of distilled water. The medium was boiled and filtered, and the pH was adjusted to 7.0. The medium was then dispensed in 200-ml amounts in 500-ml Erlenmeyer flasks and sterilized by autoclaving for 15 min at 115.5°C (10 lb/in²). The flasks were inoculated with 10 ml of a 24-h-old culture prepared in the same way. All flasks were incubated on a rotary shaker at 30°C.

Manometry. Oxygen uptake by the cells was measured by using conventional manometric techniques (31). The experiments were conducted at 30°C in Warburg vessels with 0.2 ml of 20% KOH in the central well and air as the gas phase. Each flask contained 1.2 ml of 0.1 M sodium phosphate buffer, pH 7.0, and 1.5 ml of aqueous cell suspension (10 mg [wet weight]/ml). Oxygen uptake was recorded at regular intervals after 0.3 ml of 50 mM substrate was tipped in from the side arm.

Analysis of culture medium and replacement medium. The organism was grown in the mineral salts medium described above. After 18 h of growth, the cells were harvested by centrifugation and washed twice with 0.1 M sodium phosphate buffer, pH 7.0, and retained for further metabolic studies. The supernatant fluid, which is referred to as culture medium, was then adjusted to pH 2.0 with 2 N HCl and extracted thrice with an equal volume of peroxide-free ether. The ether layers were pooled, dried over anhydrous sodium sulfate, and evaporated to dryness under suction. The residue was dissolved in a small volume of ethylacetate and used for chromatography.

The washed cells (4.5 g) were resuspended in 150 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 150 mg of DL-mandelic acid (henceforth referred to as replacement medium; the pH of the medium was adjusted to 7.0 with 1 N NaOH). The replacement medium was incubated at 30°C for 12 h with gentle agitation. Samples (20 ml) from replacement medium were removed aseptically at different time intervals. The samples were treated as above to isolate the product(s) of mandelic acid metabolism.

Preparation of cell-free extract. To study the individual enzymes of the mandelic acid pathway, *P. convexa* was always grown on Seubert's medium containing 0.5% glucose and 0.1% mandelic acid. Cultures in the mid-log phase of growth were harvested and washed twice with 0.1 M sodium phosphate buffer, pH 7.0. The harvested cells were suspended in 4 volumes of 0.025 M sodium phosphate buffer, pH 7.0, and then subjected to sonication for 4 min at 0 to 5°C in a Branson sonifier by keeping the current strength at 5 A. The sonic extract was centrifuged at 10,000 × *g* for 10 min. The supernatant was designated as crude cell-free extract.

Preparation of particulate and supernatant fractions. The crude cell-free extract was centrifuged at 100,000 × *g* for 1 h in a Beckman model L-3-50 preparative ultracentrifuge. The cream-colored pellet so obtained was resuspended in 0.025 M sodium phosphate buffer, pH 7.0, and again centrifuged at 100,000 × *g* for 1 h. The pellet obtained was uniformly dispersed in 0.025 M sodium phosphate buffer, pH 7.0, with the help of a fine hypodermic syringe. This preparation is referred to as the particulate fraction, and the earlier 100,000 × *g* supernatant fluid is referred to as the supernatant fraction.

Enzyme assays. (i) Mandelate racemase. Mandelate racemase catalyzes the interconversion of two stereoisomers of mandelic acid. A washed preparation of a particulate fraction prepared from *Aspergillus niger* grown in the presence of mandelic acid contains an oxidase that is specific for the D(−)-isomer of mandelic acid (18). The rate of enzyme-catalyzed racemization of L(+)-mandelic acid may therefore be measured by use of an excess of D-mandelate oxidase in a coupled reaction. The mandelate racemase activity was assayed in the supernatant fraction as follows. The reaction mixture contained 70 μmol of sodium phosphate buffer, pH 7.0, 1 μmol of L(+)-mandelic acid (aqueous neutralized solution) and washed particulate fraction (a quantity sufficient to oxidize a saturating quantity of D-mandelic acid at 20 times the racemase rate measured; 3 mg of protein particulate fraction of *A. niger*), and enzyme (supernatant fraction, 6 mg of protein) in a final volume of 1.5 ml. The reaction mixture was incubated at 30°C for 30 min. The amount of benzoylformic acid formed was estimated as its 2,4-dinitrophenylhydrazone in alkali (18).

(ii) Mandelate-4-hydroxylase. Mandelate-4-hydroxylase activity was assayed by measuring the amount of 4-hydroxymandelic acid formed using a modification of the colorimetric method of Bray et al. (2). The reaction mixture contained 70 μmol of citrate-phosphate buffer, pH 5.4, 0.1 μmol of ferrous sulfate, 0.25 μmol of DL-mandelic acid, 0.02 μmol of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine (in 0.1 ml of 10^{-3} M β-mercaptoethanol), 0.1 ml of a reduced-form nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (NADPH-generating system contains 50 μmol of sodium phosphate buffer, pH 7.0, 2.0 μmol of $NADP^+$, 2.5 μmol of glucose-6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase in a final volume of 1 ml; incubated at 30°C for 15 min), and enzyme (supernatant fraction, 4 mg of protein) in a final volume of 1.5 ml. Incubation was carried out in a Dubnoff metabolic shaker with air as the gas phase for 30 min at 30°C. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 6% and centrifuged to remove denatured protein. A sample (0.5 ml) was pipetted out and

diluted to 1 ml with water. To this, 1 ml of 95% ethanol, 0.2 ml of diazotized *p*-nitroaniline (freshly prepared by mixing 25 ml of 0.3% *p*-nitroaniline solution in 0.8 N HCl and 1.5 ml of 5% $NaNO_2$), and 2 ml of water were added. After 2 min, the mixture was made alkaline by adding 1 ml of 5% Na_2CO_3 solution. The red color that developed was read immediately or within 5 min against a reagent blank in a Klett-Summerson photoelectric colorimeter using a no. 54 filter (500 to 570 nm).

(iii) L-4-Hydroxymandelate oxidase (decarboxylating). The enzyme was assayed in the particulate fraction by estimating the 4-hydroxybenzaldehyde, the product of the reaction, as its 2,4-dinitrophenylhydrazone in alkali. The assay system contained 80 μmol of sodium phosphate buffer, pH 6.6, 1 μmol of DL-4-hydroxymandelic acid (neutralized solution), 0.1 μmol of flavine adenine dinucleotide (FAD), and 0.2 μmol of $MnSO_4$ and enzyme (particulate fraction, 0.225 μg of protein) in a total volume of 1.5 ml. The reaction mixture was incubated at 30°C for 1 h. The reaction was stopped by the addition of 0.3 ml of 2 N HCl. The mixture was then centrifuged to remove the precipitated protein, and 0.4 ml of the supernatant layer was pipetted out into a test tube, which was made up to 1 ml with water. A 0.25-ml amount of 2,4-dinitrophenylhydrazine reagent (0.1% 2,4-dinitrophenylhydrazine [wt/vol] in 2 N HCl) was added and kept in a boiling-water bath for 10 min. After cooling to room temperature, 1 ml of 10% aqueous NaOH was added. The wine-red color that developed was measured at 450 nm in a Coleman Junior II spectrophotometer against a reagent blank.

(iv) NAD^+-4-hydroxybenzaldehyde dehydrogenase and $NADP^+$-4-hydroxybenzaldehyde dehydrogenase. Since 4-hydroxybenzaldehyde has a high extinction at 340 nm, the 4-hydroxybenzaldehyde dehydrogenase activity could not be assayed by measuring the reduction of nicotinamide adenine dinucleotide (NAD^+) or $NADP^+$ spectrophotometrically at 340 nm. Therefore, the enzymes were assayed by following the disappearance of substrate, 4-hydroxybenzaldehyde. The assay system contained 50 μmol of tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.8, 0.2 μmol of 4-hydroxybenzaldehyde, 0.2 μmol of NAD^+ or $NADP^+$, and enzyme (approximately 6 milliunits) in a total volume of 1 ml. The reaction mixture was incubated at 30°C for 20 min. The reaction was stopped by adding 0.2 ml of 2 N HCl. Substrate was added to the blank after stopping the reaction. The blank and experimental samples were centrifuged at low speed to remove the precipitated protein, and the amount of 4-hydroxybenzaldehyde present in both samples was determined colorimetrically as its 2,4-dinitrophenylhydrazone in alkali, as described above.

(v) 4-Hydroxybenzoate-3-hydroxylase. 4-Hydroxybenzoate-3-hydroxylase activity was measured by following the formation of the product, namely, 3,4-dihydroxybenzoic acid. A reaction mixture (1 ml) containing 50 μmol of sodium phosphate buffer, pH 7.0, 0.2 μmol of 4-hydroxybenzoic acid, 0.1 ml of an NADPH-generating system (the preparation of NADPH generating system is described above), 0.02 μmol of FAD, and enzyme (supernatant fraction, 3 mg of protein) was incubated for 15 min at 30°C. The reaction was stopped by the addition of 0.2 ml of 30% trichloroacetic acid, and the mixture was centrifuged to remove the precipitated protein. A sample of the supernatant (0.4 ml) was pipetted out into a test tube and made up to 1 ml with water. The amount of 3,4-dihydroxybenzoic acid formed was estimated colorimetrically by the method of Nair and Vaidyanathan (22).

(vi) Protocatechuate-3,4-oxygenase. Protocatechuate-3,4-oxygenase activity was measured in the supernatant fraction by incubating for 15 min at 30°C, a reaction mixture (1 ml) containing 50 μmol of tris-(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, 0.4 μmol of protocatechuic acid, and enzyme (supernatant fraction, 3 mg of protein). The reaction was stopped by adding 0.2 ml of 30% trichloroacetic acid. Substrate was added to the blank after stopping the reaction. The precipitated protein was removed by centrifugation. A sample (0.2 ml) was taken and made up to 1 ml with water, and the amount of 3,4-dihydroxybenzoic acid that disappeared was determined colorimetrically by the method of Nair and Vaidyanathan (22).

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 enzymes per milligram of protein.

Protein determination. The protein content of various preparations was estimated by the method of Lowry et al. (21).

RESULTS

Identification of isolated bacterium. The important taxonomic characters of the isolated bacterial strain are presented in Table 1. Based on this, the bacterium was identified as *P. convexa*.

Induction of oxidizing activities of *P. convexa* with different substrates. The ability of *P. convexa* grown in the presence or absence of mandelic acid to utilize L-mandelic acid, D-mandelic acid, DL-4-hydroxymandelic acid, catechol, and protocatechuic acid has been evaluated by measuring the oxygen uptake. From Fig. 1 and 2 it can be seen that *P. convexa* grown in the absence of mandelic acid metabolized both isomers of mandelic acid, DL-4-hydroxymandelic acid, catechol, and protocatechuic acid with a lag period, whereas the same organism grown in the presence of mandelic acid used both isomers of mandelic acid, DL-4-hydroxymandelic acid, and protocatechuic acid without any lag period; on the contrary, catechol was used with a lag period. This suggests that glucose-grown cells are devoid of enzymes responsible for the degradation of the above-

TABLE 1. *Taxonomic characters of P. convexa*

Criteria	Description
Cell morphology	Short thick rods with rounded ends occurring singly or occasionally in short chains. Motile, possessing a polar flagellum.
Staining	Gram negative.
Colonial morphology	Gelatin colonies, circular, convex, greenish, glistening. The medium becomes blue, fluorescent. Agar slant colonies are moist, glistening, greenish yellow. On broth: turbid, becoming yellowish green.
Physiological and cultural	Gelatin not liquefied. Bromocresol purple milk alkaline. No coagulation. Nitrite not produced from nitrate. Indole not produced. No acid and no gas from glycerol, lactose, maltose, fructose, sorbitol, inositol, mannitol, and sucrose. Citrate is not utilized as source of carbon. Methyl red negative. Acetylmethylcarbinol not produced. Arginine is utilized. Starch is hydrolyzed. Grows best at 25 to 30°C and poorly at 37°C and above.

mentioned aromatic compounds but are induced when the cells are grown in the presence of mandelic acid. The mandelic acid-grown cells utilized catechol only after a lag period, whereas protocatechuic acid was readily oxidized. This indicates that protocatechuic acid, but not catechol, may be the intermediate of mandelic acid degradation by *P. convexa*.

Since *P. convexa* can grow better on glucose than on mandelic acid, the effect of glucose on mandelic acid metabolism in *P. convexa* was tested. Figure 3 shows the mandelic acid-metabolizing ability of *P. convexa* grown on mandelic acid alone and on mandelic acid with glucose. It is clear from Fig. 3 that glucose had no repressing effect on the enzymes of the mandelic acid pathway. In fact, there is a slight stimulation. Therefore, for the enzymatic studies, *P. convexa* was grown on a medium containing 0.5% glucose and 0.1% DL-mandelic acid as inducer.

Identification of products of mandelic acid metabolism. The earlier studies of oxygen uptake by *P. convexa* with various aromatic substrates showed that mandelic acid was utilized rapidly by the organism. After 18 to 20 h of growth of the organism on mandelic acid, the culture medium was collected and analyzed for the products of mandelic acid metabolism by extracting it with peroxide-free ether after acidification and chromatographing it on paper as well as on Silica Gel G plates. From the isolates, three compounds, 4-hydroxymandelic acid, 4-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid, were identified by comparing the chromatographic and spectral properties with those of authentic samples. The results are presented in Table 2. In replacement studies, the replacement medium was analyzed at different time intervals for the appearance of various products of mandelic acid metabolism by *P. convexa*. 4-Hydroxymandelic acid was the first product to appear in the medium (in about 2 h), whereas 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid could be detected in the medium only 5 to 6 h after placing the organism in the replacement medium. Only in the replacement studies was 4-hydroxybenzaldehyde detected as a metabolite, whereas not even a trace amount of 4-hydroxybenzoylformic acid, which is also an expected intermediate product, as shown in other microorganisms (11, 20), could be detected either in the replacement studies or in the analysis of the culture medium. The chromatographic analysis of replacement medium and culture medium did not show the presence of benzoylformic acid, benzoic acid, or catechol.

Enzymes of the mandelic acid pathway of *P. convexa*. Since both D- and L-isomers of mandelic acid were metabolized rapidly by *P.*

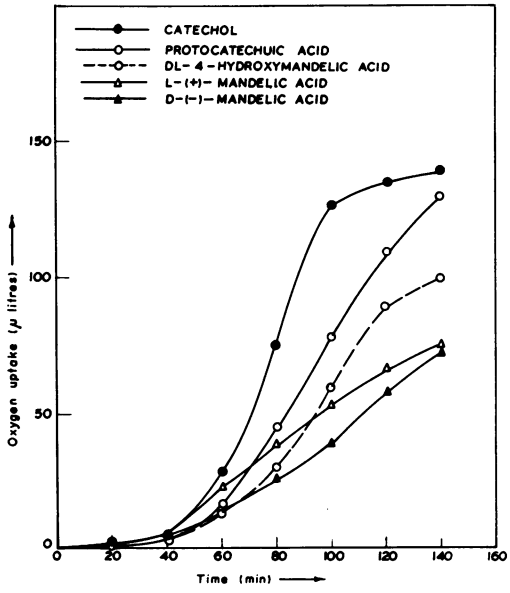

FIG. 1. *Oxidation of mandelic acid, 4-hydroxymandelic acid, protocatechuic acid, and catechol by P. convexa grown on glucose. Results are corrected for endogenous respiration.*

convexa, the 100,000 × *g* supernatant was tested for mandelate racemase activity. The supernatant fraction converted L-mandelic acid to D-mandelic acid, showing the presence of mandelate racemase.

The requirements for mandelic acid hydroxylation by the cell-free extract of *P. convexa* were substrate, Fe^{2+}, tetrahydropteridine, NADPH, and oxygen (Table 3). All preparations of the enzyme showed an absolute requirement for Fe^{2+}. The optimum pH for hydroxylation was found to be 5.4. Mandelate-4-hydroxylase activity was found to be in the supernatant fraction. The enzymatic product of mandelate-4-hydroxylase was identified as 4-hydroxymandelic acid by comparing its chromatographic mobilities and spectral characteristics with those of an authentic sample (1). A crude cell-free extract of *P. convexa* converted both D- and L-isomers of mandelic acid to 4-hydroxymandelic acid more or less to the same

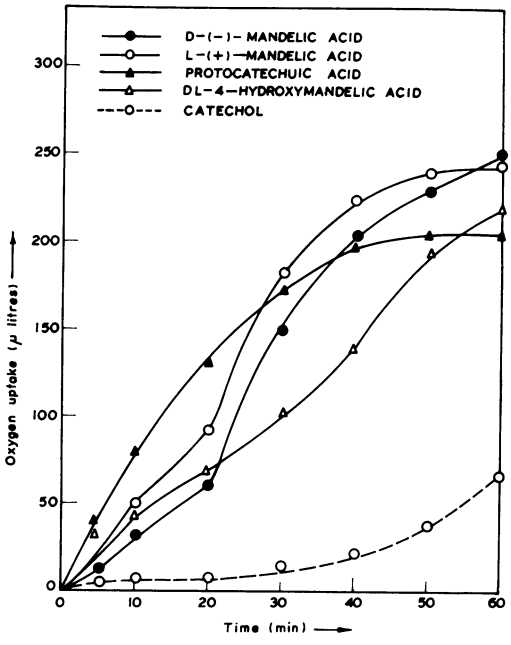

FIG. 2. *Oxidation of mandelic acid, 4-hydroxymandelic acid, catechol, and protocatechuic acid by P. convexa grown on mandelic acid. Results are corrected for endogenous respiration.*

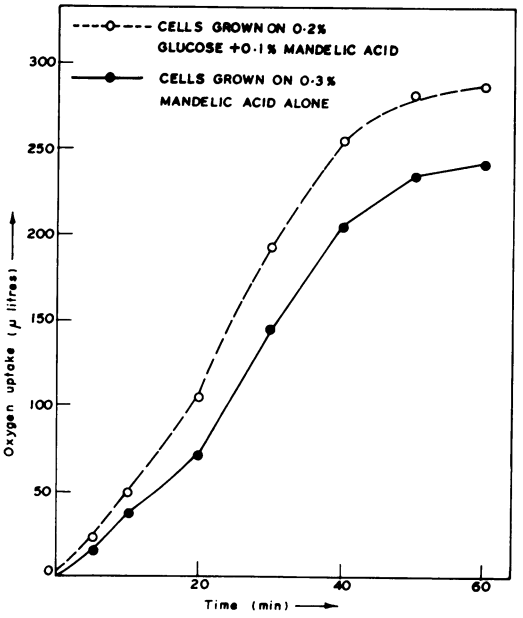

FIG. 3. *Oxidation of DL-mandelic acid by P. convexa grown on mandelic acid alone and on mandelic acid and glucose. Results are corrected for endogenous respiration.*

TABLE 2. *R_f values and λ_{max} of authentic compounds and isolated products*[a]

Compound	R_f values								λ_{max} (nm)	
	Paper chromatogram				Thin-layer chromatogram					
	A		B		C		D		a	i
	a[b]	i[c]	a	i	a	i	a	i		
4-Hydroxymandelate	0.91	0.90	0.07	0.07	0.26	0.26	0.15	0.15	276	276
									282	282
4-Hydroxybenzaldehyde	0.63	0.63	0.47	0.46	0.46	0.45	0.38	0.37	268	268
4-Hydroxybenzoate	0.69	0.70	0.40	0.40	0.42	0.42	0.30	0.30	254	254
Protocatechuate	0.58	0.59	0.41	0.40	0.31	0.31	0.23	0.22	259	259
									292	292

[a] Solvent systems used: A, formic acid-water (2:98, vol/vol); B, benzene-acetic acid-water (4:4:1, vol/vol/vol organic phase); C, benzene-methylisobutyl ketone-formic acid (60:37:3, vol/vol/vol; D, benzene-ethylmethyl ketone-formic acid (80:18:2, vol/vol/vol).

[b] a, Authentic sample.

[c] i, Isolated sample.

TABLE 3. *Requirement for mandelic acid hydroxylation by cell-free extracts of P. convexa*

Component	*p*-Hydroxymandelic acid formed (nmol)
Complete system	89.0
Without enzyme	7.0
Without Fe^{2+}	0.0
Without mandelic acid	0.0
Without NADPH	5.0
Without tetrahydropteridine	8.0
Complete but boiled enzyme	5.0

extent, whereas partially purified mandelate-4-hydroxylase hydroxylated only the L-isomer of mandelic acid (unpublished observation).

L-4-Hydroxymandelate oxidase (decarboxylating) requires FAD and Mn^{2+} for its activity (Table 4). FAD served as a better cofactor than riboflavine 5'-phosphate. With riboflavine 5'-phosphate, enzyme activity was about 74% of that obtained with FAD. All preparations of the enzyme required Mn^{2+} for activity. Addition of riboflavine, NAD^+, $NADP^+$, and thiamine pyrophosphate with or without Mn^{2+} into the assay system had no effect. This enzyme is present in the particulate fraction (Table 5). The product of the L-4-hydroxymandelate oxidase reaction was identified as 4-hydroxy-benzaldehyde by comparing its properties with those of the authentic samples. The R_f values of 4-hydroxybenzaldehyde and the enzymatic product were identical (Table 2). The ultraviolet and infrared spectra of the isolated compound were indistinguishable from that of authentic 4-hydroxybenzaldehyde. The 2,4-dinitrophenylhydrazone of the enzymatic product melted at 273°C. The derivatives of the authentic 4-hydroxybenzaldehyde melted at 273 to 275°C. The mixed melting point was found to be 273 to 275°C. (All the values were uncorrected.)

To determine the stereospecificity of L-4-hydroxymandelate oxidase, an experiment was conducted as follows. To a thick suspension of particulate fraction in 0.2 M sodium phosphate buffer, pH 6.6 (2 ml, 4.6 mg of protein per ml), 36 mg of DL-4-hydroxymandelic acid (2 ml of neutralized solution), 8 mg of FAD, and 1 mg of $MnSO_4$ were added (final volume, 5 ml), and the reaction mixture was divided into two portions of 2.5 ml each. The enzyme reaction of one portion was stopped immediately by adding 0.5 ml of 30% trichloroacetic acid; this portion served as the control. The other portion of the reaction mixture was incubated at 30°C for 4 h. At the end of the incubation period, the reaction of the second portion was stopped by adding 0.5 ml of 30% trichloroacetic acid. Precipitated proteins of both portions were removed by centrifugation. The optical rotation of the clear supernatant was measured in a polarimeter using a 0.5-dm tube of 1.5-ml capacity. The difference in the optical rotation between the control and the experimental portion was −0.20. From the specific rotation of D- and L-4-hydroxymandelic acid ($[\alpha]_D^{20} = \pm 144.4$), it can be calculated that the experiment contained an 8.34-mg excess of the levorotatory isomer of DL-4-hydroxymandelic acid. Therefore, the experiment indicates that the oxidizable isomer is dextrorotatory. Although the absolute configuration of 4-hydroxymandelic acid is unknown, it is probable that by analogy with mandelic acid the dextrorotatory isomer has the L-configuration. The results of this experiment indicate that the 4-hydroxymandelate oxidase is specific for the L-isomer of 4-hydroxymandelic acid.

P. convexa possesses two 4-hydroxybenzaldehyde dehydrogenases: (i) NAD^+ dependent and (ii) $NADP^+$ dependent. When both nucleotides were added to the reaction mixture containing crude enzyme preparation, they had an additive effect on the enzyme reaction. Both the enzymes were heat labile. These two activities were separated from each other and partially purified by using the procedure outlined below.

Step I: preparation of crude extract. Crude

TABLE 4. *Cofactor requirement of L(+)-4-hydroxymandelate oxidase*

Change from complete system[a]	4-Hydroxybenzaldehyde formed (nmol)
Complete system	258
Omit substrate	0
Omit FAD	4
Omit Mn^{2+}	16
Omit enzyme	9
Omit FAD, add FMN	190
Omit FAD, add riboflavine	6
Omit FAD, add NAD^+	0
Omit FAD, add $NADP^+$	4
Omit FAD, add TPP	5
Complete system but boiled enzyme	8

[a] Concentration of FAD, riboflavine 5'-phosphate (FMN), and riboflavine tried as 0.1 μmol and that for NAD^+, $NADP^+$, and thiamine pyrophosphate (TPP) as 0.5 μmol.

TABLE 5. *Intracellular distribution of L-4-hydroxymandelate oxidase of P. convexa*

Fraction	4-Hydroxybenzaldehyde formed (nmol)
Crude extract	94
Particulate	265
Supernatant	8

extract was prepared as described in Materials and Methods.

Step II: protamine sulfate treatment. To 20 ml of crude extract, a 2% aqueous, neutralized solution of protamine sulfate was added dropwise with constant stirring to give a final concentration of 0.2% protamine sulfate. After stirring for 10 min more, the solution was centrifuged at 10,000 × *g* for 10 min and the precipitate was discarded.

Step III: ammonium sulfate fractionation. To 20 ml of protamine sulfate supernatant, finely powdered ammonium sulfate was added slowly over a period of 15 min to 0.45 saturation. The precipitate formed in 30 min was removed by centrifugation at 15,000 × *g* for 10 min. Ammonium sulfate was again added slowly to the supernatant to 0.65 saturation. The precipitate formed in 1 h was collected by centrifugation at 17,000 × *g* for 15 min and was dissolved in 2 ml of 0.01 M sodium phosphate buffer, pH 7.0. This was desalted by passing through a Sephadex G-25 column, using 0.01 M sodium phosphate buffer, pH 7.0, for elution.

Step IV: calcium phosphate gel treatment. The pH of the desalted preparation was adjusted to 6.0 by carefully adding dilute acetic acid. This preparation was used for adsorption on tricalcium phosphate gel. Tricalcium phosphate gel (10 ml, 10 mg/ml) was centrifuged, and the residue was suspended in 0.01 M sodium phosphate buffer, pH 6.0, and allowed to equilibrate for 30 min. Then the suspension was again centrifuged and gel residue was treated with enzyme solution. The mixture was stirred constantly for 20 min and centrifuged at 6,000 × *g* for 5 min. The gel supernatant contained most of the NAD^+-dependent 4-hydroxybenzaldehyde dehydrogenase activity and only very little $NADP^+$-dependent enzyme. The gel was washed twice with 20 ml of 0.025 M sodium phosphate buffer, pH 6.0; later $NADP^+$-dependent 4-hydroxybenzaldehyde dehydrogenase was eluted with 10 ml of 0.2 M sodium phosphate buffer, pH 7.4. The gel was removed by centrifugation and the eluate was used for the assay of $NADP^+$-dependent 4-hydroxybenzaldehyde dehydrogenase.

The separation of NAD^+- and $NADP^+$-dependent 4-hydroxybenzaldehyde dehydrogenases and their partial purification are given in Table 6. It can be noted from Table 6 that the ratio of NAD^+-dependent to $NADP^+$-dependent 4-hydroxybenzaldehyde dehydrogenase activity changes with the purification, thereby showing again that these are two different enzymes. The NAD^+-dependent enzyme was nearly three times more active than the $NADP^+$-dependent enzyme in the crude preparation. Both enzymes had a pH optimum of approximately 8.8 in tris(hydroxymethyl)aminomethane-hydrochloride buffer and both were equally active on 4-hydroxybenzaldehyde and benzaldehyde. The product of 4-hydroxybenzaldehyde dehydrogenase was identified as 4-hydroxybenzoic acid by comparing its chromatographic and spectral properties with those of authentic 4-hydroxybenzoic acid.

4-Hydroxybenzoate-3-hydroxylase activity has been demonstrated in the supernatant fraction of *P. convexa*. This enzyme is a monooxygenase and requires FAD and NADPH for its activity. The product of this enzyme reaction was identified as 3,4-dihydroxybenzoic acid by comparing its chromatographic and spectral properties with those of an authentic compound.

Protocatechuate-3,4-oxygenase was present in the supernatant fraction. The enzyme activity is greatly stimulated by the addition of Fe^{2+} to the assay system. The product of protocatechuate-3,4-oxygenase was isolated from a large-scale reaction mixture. This isolated product melted at 120°C (uncorrected) and the melting

TABLE 6. *Separation of NAD^+- and $NADP^+$-dependent 4-hydroxybenzaldehyde dehydrogenases*

Step	Fraction	4-Hydroxybenzaldehyde dehydrogenase				Ratio of activity: NAD+/NADP+
		NAD+ dependent		NADP+ dependent		
		Total activity (milliunits)	Sp act (milliunits)	Total activity (milliunits)	Sp act (milliunits)	
I	Crude	576	2.4	199	0.83	2.9
II	Protamine sulfate	540	3.0	164	0.91	3.3
III	Ammonium sulfate (0.4–0.6 saturation)	430	8.6	115	2.30	3.7
IV	Tricalcium phosphate gel supernatant	382	10.6	7.5	0.21	50.5
V	Tricalcium phosphate gel eluate	6.5	0.9	62	8.60	0.1

point was not depressed by admixture with authentic 3-oxoadipic acid, which also melted at the same temperature with decomposition. Adding excess 2,4-dinitrophenylhydrazine in 2 N HCl to a solution of the isolated product and boiling the reaction mixture for 5 min to complete decarboxylation resulted in a material that, after recrystallization from ethanol, melted at 206°C (uncorrected). The melting point was not depressed by admixture with 2,4-dinitrophenyl hydrazone of authentic levulic acid, which also melted at the same temperature, again showing that the isolated enzymatic product was 3-oxoadipic acid.

Inducible nature of enzymes of the mandelate pathway in *P. convexa*. Glucose-grown cells of *P. convexa* oxidized both isomers of mandelic acid only after a lag period (Fig. 1), whereas mandelic acid-grown cells readily metabolized mandelic acid without any lag period (Fig. 2), thereby suggesting that the enzymes of the mandelate pathway of *P. convexa* are inducible. This is further confirmed by the failure to detect the enzymes of the mandelate pathway in the cell-free extract of *P. convexa* grown in the presence of glucose only. Table 7 shows the specific activities of the various enzymes of the mandelate pathway in the cell-free extracts of *P. convexa* grown in the presence of 0.1% different substrates and 0.5% glucose and in 0.5% glucose only. It is clear from the results that glucose-grown cells are completely devoid of the mandelate group of enzymes. Mandelate-4-hydroxylase is induced only by both isomers of mandelic acid and not by DL-4-hydroxymandelic acid; L-4-hydroxymandelate oxidase is induced by both isomers of mandelic acid and DL-4-hydroxymandelic acid. This suggests that these two enzymes may be controlled by different regulatory genes.

DISCUSSION

It has been reported that different groups of microorganisms that inhabit soil and surface water can utilize one or both isomers of mandelic acid (16). In addition, an exhaustive taxonomic study of the aerobic *Pseudomonas* by Stanier et al. (29) revealed that members of several groups within this genus can grow at the expense of one or both isomers of mandelic acid as sole source of carbon and energy. Although *P. convexa* can grow on mandelic acid as sole source of carbon, it grows better on glucose than on mandelic acid. Moreover, glucose had no repressing effect on the enzymes of the mandelate pathway (Fig. 3).

P. convexa utilizes both isomers of mandelic acid by using mandelate racemase. However, the pathway for the degradation of mandelic acid by *P. convexa* is different from the reported pathways followed in other organisms. Whereas mandelate dehydrogenase is the first enzyme causing the oxidation of mandelic acid to benzoylformic acid in other bacteria, *P. convexa* uses L-mandelate-4-hydroxylase as the first enzyme of the pathway that hydroxylates L-mandelic acid to L-4-hydroxymandelic acid. Although many bacteria and fungi are reported to utilize mandelic acid, only a few of them, such as *P. putida* (10), *P. fluorescens* (16), *Azotobacter beijerinckii* (16), and *A. niger* (18), can use both isomers of mandelic acid. However, the mode of utilization of D- and L-isomers of mandelic acid by these organisms is different. In bacteria, L-mandelic acid is directly utilized by a stereospecific particulate L-mandelate de-

TABLE 7. *Levels of enzymes of mandelate pathway in P. convexa after growth on glucose alone and on glucose plus various intermediates (0.1%)*[a]

Growth on:	Sp act of enzymes of mandelate pathway (milliunits)						
	Mandelate racemase	Mandelate hydroxylase	4-Hydroxy mandelate oxidase	4-Hydroxybenzaldehyde dehydrogenases		4-Hydroxybenzoate hydroxylase	Protocatechuate oxygenase
				NAD+	NADP+		
Glucose	0	0	0	0	0	0	0
Glucose + D-mandelate	1.77	1.01	20.0	2.40	0.74	2.51	4.3
Glucose + L-mandelate	1.72	1.00	19.7	2.60	0.78	2.40	4.5
Glucose + DL-4-hydroxymandelate	1.65	0	21.2	2.48	0.81	2.38	4.7
Glucose + 4-hydroxybenzaldehyde	0	0	0	2.72	0.71	2.60	4.2
Glucose + 4-hydroxybenzoate	0	0	0	0	0	2.41	4.9
Glucose + protocatechuate	0	0	0	0	0	0.60	4.8

[a] Enzyme assays and definition of specific activity are described in Materials and Methods. Each experimental value is the average of three determinations.

hydrogenase, whereas D-mandelic acid is metabolized only after its isomerization to L-mandelic acid by the action of mandelate racemase (10, 16). In the case of the fungus *A. niger*, there exist two stereospecific oxidases that catalyze the oxidation of D- and L-mandelic acid separately (18).

The metabolism of 4-hydroxymandelic acid has been well documented (7, 11, 20). Whereas the mandelate pathway proceeds with the involvement of benzoylformic acid, benzaldehyde, and benzoic acid, that of the 4-hydroxymandelate pathway utilizes 4-hydroxybenzoylformic acid, 4-hydroxybenzaldehyde, and 4-hydroxybenzoic acid as the intermediates. It has been shown that the same set of enzymes as those responsible for the oxidation of mandelic acid to benzoic acid are involved in the oxidation of 4-hydroxymandelic acid to 4-hydroxybenzoic acid (20, 30). The results obtained in the case of *P. convexa* also support the formation of 4-hydroxybenzoic acid from 4-hydroxymandelic acid. However, in this micoorganism the only intermediate of 4-hydroxymandelic acid degradation that could be detected before the formation of 4-hydroxybenzoic acid is 4-hydroxybenzaldehyde. Even a trace amount of 4-hydroxybenzoylformic acid could not be detected in either in vivo or in vitro studies. Further, it has been shown in *P. convexa* (unpublished data) that the 4-hydroxymandelate oxidase oxidatively decarboxylates 4-hydroxymandelic acid to 4-hydroxybenzaldehyde. The 4-hydroxybenzoic acid is further converted to 3,4-dihydroxybenzoic acid, which in turn is oxygenatively cleaved, leading to the formation of 3-oxoadipic acid.

In *P. convexa*, the mandelate racemase is present in the soluble fraction. This enzyme resembles the racemase of *P. putida* in that the enzyme activity is stimulated by divalent metal ions such as Mg^{2+}, Mn^{2+}, and Co^{2+} (8).

L-Mandelate-4-hydroxylase has not been demonstrated in any other organism so far studied. This enzyme is present exclusively in the supernatant fraction. L-Mandelate-4-hydroxylase of *P. convexa* bears a notable resemblance to the inducible phenylalanine hydroxylase of *Pseudomonas* species (ATCC 11299a) (12). Most prominent among these is the requirement of both enzymes for tetrahydropteridine, NADH, and Fe^{2+}.

The process of enzymatic conversion of L-4-hydroxymandelic acid to 4-hydroxybenzaldehyde in *P. convexa* is entirely different from its counterparts reported in other bacteria. In *P. putida*, for example, L-4-hydroxymandelic acid is oxidized to 4-hydroxybenzoylformic acid by a particulate L-mandelate dehydrogenase. The 4-hydroxybenzoylformic acid thus formed undergoes nonoxidative decarboxylation to form 4-hydroxybenzaldehyde by the action of a soluble benzoylformate decarboxylase that requires thiamine pyrophosphate as a cofactor. In the case of *P. convexa*, both dehydrogenation decarboxylation reactions are carried out by particulate L-4-hydroxymandelate oxidase, which

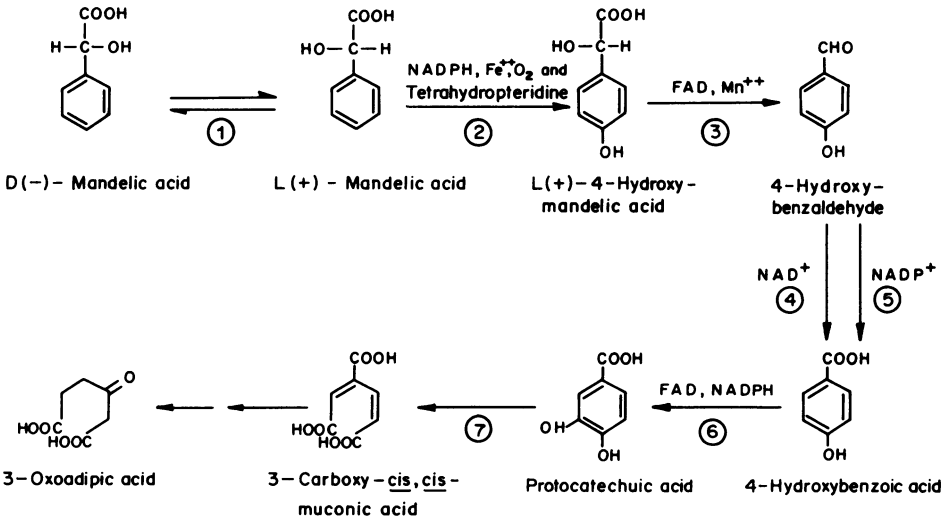

FIG. 4. *Pathway for the metabolism of mandelic acid by P. convexa. (1) Mandelate racemase; (2) L-mandelate-4-hydroxylase; (3) L-4-hydroxymandelate oxidase; (4) NAD+-dependent 4-hydroxybenzaldehyde dehydrogenase; (5) NADP+-dependent 4-hydroxybenzaldehyde dehydrogenase; (6) 4-hydroxybenzoate-3-hydroxylase; (7) protocatechuate-3,4-oxygenase.*

requires FAD and Mn^{2+} for its activity.

Analogous to *P. putida* (9) and *A. niger* (18), *P. convexa* also possesses two 4-hydroxybenzaldehyde dehydrogenases, one NAD^+ dependent and the other $NADP^+$ dependent. Both enzymes show equal activities towards benzaldehyde and 4-hydroxybenzaldehyde.

4-Hydroxybenzoate-3-hydroxylase of *P. convexa* is similar to that isolated from *P. putida* (17), *P. desmolytica* (32), and *P. fluorescens* (27) in the requirement for FAD and NADPH as cofactors for the reaction.

P. convexa, grown in the presence of mandelic acid, contained only the protocatechuate cleavage enzyme and not the catechol cleavage enzyme. 3-Oxoadipic acid was the product of protocatechuate cleavage, thereby suggesting that the protocatechuic acid is cleaved by the protocatechuate-3,4-oxygenase in this organism.

All these facts put together favor the operation of a new pathway for the metabolism of mandelic acid by *P. convexa*, as outlined in Fig. 4.

Although the biochemical steps for the conversion of mandelic acid to benzoic acid are the same in all the microorganisms studied earlier, they differ from each other with respect to both the enzyme complement and pattern of inductive control (13–15, 18, 19, 24; A. Livingston and C. A. Fewson, Biochem. J. 121:8–9P, 1971). In the case of *P. convexa*, even the biochemical events of the mandelate pathway are different.

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