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Catabolism of L-Tyrosine in *Trichosporon cutaneum*

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Protocatechuic acid was a catabolite in the degradation of L-tyrosine by *Trichosporon cutaneum*. Intact cells oxidized to completion various compounds proposed as intermediates in this conversion, but they did not readily oxidize catabolites of the homogentisate and homoprotocatechuate metabolic pathways, which are known to function in other organisms. Cell extracts converted tyrosine first to 4-hydroxycinnamic acid and then to 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid. The proposed hydration product of 4-hydroxycinnamic acid, namely, β-(4-hydroxyphenyl)-hydracrylic acid, was synthesized chemically, and its enzymatic degradation to 4-hydroxybenzaldehyde was shown to be dependent upon additions of adenosine triphosphate and coenzyme A. The hydroxylase that attacked 4-hydroxybenzoate showed a specific requirement for reduced nicotinamide adenine dinucleotide phosphate. Protocatechuate, the product of this reaction, was oxidized by cell extracts supplemented with reduced nicotinamide adenine dinucleotide or, less effectively, with reduced nicotinamide adenine dinucleotide phosphate, but these extracts contained no ring fission dioxygenase for protocatechuate. Evidence is presented that the principal hydroxylation product of protocatechuate was hydroxyquinol, the benzene nucleus of which was cleaved oxidatively to give maleylacetic acid.

Two reaction sequences have been described for utilizing L-tyrosine as a carbon source to support the growth of aerobic bacteria. The first of these pathways was found in gram-negative organisms (22) and is similar to that used by mammalian systems. Homogentisic acid is the substrate for oxidative fission of the benzene nucleus (1, 10) and is catabolized by way of fumarate and acetoacetate (3). The second pathway is that used by several, but not all, of the gram-positive strains investigated by Sparnins and Chapman (18). In this case, the ring fission substrate formed from tyrosine is homoprotocatechuic acid, which is then degraded to succinate and pyruvate before entry into the tricarboxylic acid cycle. In this paper we report that the phenol-utilizing yeast *Trichosporon cutaneum* catabolizes L-tyrosine aerobically by a third route, involving initial attack by an ammonia lyase to form 4-hydroxycinnamic acid. After hydration and aldol cleavage, 4-hydroxybenzaldehyde is given, so that degradation then proceeds by way of 4-hydroxybenzoate, protocatechuate, and hydroxyquinol. The production of 4-hydroxybenzoate, after removing two carbons from the side chain of 4-hydroxycinnamate, has been observed in higher plants and fungi. In principle, this could be achieved by forming the coenzyme A (CoA) ester of 4-hydroxycinnamic acid (16), followed by reactions of β-oxidation, but French et al. (8), using enzymes from potato tubers and also from a basidiomycete, presented evidence that the conversion did not involve thioesters. Instead, acetic acid was released, presumably by aldol cleavage of the side chain, after hydration of 4-hydroxycinnamic acid. A similar reaction was suggested for the degradation of ferulic acid by *Pseudomonas acidovorans* (23). However, investigations by these authors were hampered because the proposed hydration product of 4-hydroxycinnamic acid, β-(4-hydroxyphenyl)-hydracrylic acid (HPH), was not available. We have synthesized this compound and find that it is metabolized by cell extracts of *T. cutaneum* only when ATP and CoA are added. No other reactions in the proposed sequence are CoA dependent.

MATERIALS AND METHODS

Organism and cell extracts. The organism, a strain of *T. cutaneum* used in previous work (17), is able to utilize L-tyrosine and certain other aromatic compounds, such as phenol and 4-hydroxybenzoic, phenylacetic, and 4-hydroxyphenylacetic acids, as main carbon sources. Shake cultures were grown at 30°C in a medium that contained (per liter): Na_2HPO_4, 1.97 g; KH_2PO_4, 4.92 g; NH_4Cl, 2.0 g; Casamino Acids, 0.5 g; yeast extract, 0.5 g; $MgSO_4$, 0.1 g; and L-tyrosine

(or succinic acid), 0.35 g. The pH of the medium was 6.4. Cells were frozen for storage and, before being broken in a Hughes bacterial press, were kept for 1 h on dry ice. Broken cells were taken up in an equal weight of 0.1 M K^+-Na^+ phosphate buffer, pH 6.5, and clear cell extracts were prepared by centrifugation.

Enzyme assays and chemical analyses. Rates of oxidation were usually measured by means of an oxygen electrode as described previously (20); in some experiments, where stated, a Warburg respirometer was used (19). Reaction mixtures for assay of 4-hydroxybenzoate or protocatechuate hydroxylases contained, in a total volume of 1.5 ml of 0.1 M phosphate buffer (pH 7.0), 20 μmol of substrate, 0.22 μmol of either NADH or NADPH, and 0.25 to 0.6 mg of cell extract protein. Rates of O_2 uptake were recorded, and, in calculating specific activities, these rates were corrected for the oxidation of reduced coenzymes, catalyzed by cell extracts in the absence of substrates. Hydroxyquinol dioxygenase was assayed by a similar procedure, except that reduced pyridine nucleotides were omitted. The procedures described by Chapman and Ribbons (4) were employed to prepare solutions of maleylacetic acid from hydroxyquinol, using extracts of *Pseudomonas putida* ORC grown with resorcinol, and also to assay maleylacetate reductase present in extracts of *T. cutaneum*. Acetate kinase was used to assay acetic acid in reaction mixtures (12), and yeast citrate synthase was used in experiments to trap acetyl-CoA as citric acid. These enzymes were obtained from Sigma Chemical Co., St. Louis, Mo.

Protocatechuic acid was detected in reaction mixtures by observing O_2 uptake after small additions of solutions of either crystalline protocatechuate 3,4-dioxygenase, the gift of John D. Lipscomb, or partially purified protocatechuate 4,5-dioxygenase from *Pseudomonas testosteroni* (7). β-Ketoadipic acid was detected by the Rothera test, in which solutions are saturated with $(NH_4)_2SO_4$, precipitated protein is removed, and additions are made of 1 drop each of 5% sodium nitroprusside and 0.88 ammonia: a maroon coloration is given by β-ketoadipic and acetoacetic acids. Compounds were chromatographed as described by Sparnins et al. (19), using alumina sheets precoated with silica gel gel.

Synthesis of *dl*-HPH (III). Compound III was obtained by reducing 4-benzyloxyphenylhydracrylic acid by means of H_2/Pd on carbon as described by LaManna et al. (14); Armstrong and Shaw used similar reactions to prepare the *m*-hydroxy isomer of this acid (2). Ethyl 4-benzyloxyphenylhydracrylate, which upon saponification and acidification gave the free acid, was obtained by refluxing ethyl bromoacetate in benzene solution with zinc powder and 4-benzyloxybenzaldehyde (2). The last named compound is now available commercially (Aldrich Chemical Co., Milwaukee, Wis.), but samples in early experiments were synthesized from 4-hydroxybenzaldehyde and benzyl chloride (2). During the preparation of 4-benzyloxyphenylhydracrylic acid from its ethyl ester, we found it necessary to add the required alkali and acid dropwise, with stirring. When this precaution was ignored the elements of water were released from the side chain of the compound, giving 4-benzyloxycinnamic acid; thus, on removing the benzyl blocking group by reduction, the resulting product was 4-hydroxyphenylpropionic acid instead of 4-hydroxyphenylhydracrylic acid. Accordingly, samples were routinely examined by chromatography on silica plates in a solvent of hexane-ethyl formate-propionic acid (70:30:18.4), and spots were located by spraying with a reagent for phenolic compounds, 2,6-dibromoquinonechlorimide (13). A blue spot (R_f, 0.13) was given by 4-hydroxyphenylhydracrylic acid, whereas 4-hydroxyphenylpropionic acid was gray (R_f, 0.5). Mass spectrometry (19) readily distinguishes between these two compounds since the former combines with three trimethylsilyl groups and the latter with two. Furthermore, 4-hydroxyphenylhydracrylic acid, when dissolved in 5 N HCl, is rapidly converted into 4-hydroxycinnamic acid, which is easily distinguished by its UV spectrum and chromatographic properties. Neither 4-hydroxyphenyllactic acid, the isomer, nor 4-hydroxyphenylpropionic acid undergoes this conversion with these conditions. When chromatography showed that 4-hydroxyphenylpropionic acid or any other contaminant was present, the final product was submitted to preparative chromatography; 4-hydroxyphenylhydracrylic acid was then eluted with ethanol, which was removed under reduced pressure.

Materials. Other chemicals were purchased from the commercial sources specified by Hareland et al. (10) and Sparnins and Chapman (18).

RESULTS AND DISCUSSION

Oxidation of metabolites by intact cells. Washed-cell suspensions of *T. cutaneum* grown with L-tyrosine readily oxidized various compounds in the sequence of Fig. 1, namely, L-tyrosine (I), *trans*-4-hydroxycinnamic acid (*p*-coumaric acid, II), *dl*-HPH (III), 4-hydroxybenzaldehyde (V), 4-hydroxybenzoic acid (VI), and protocatechuic acid (VII). By contrast, metabolites of the two established catabolic routes for tyrosine were oxidized only slowly; these included 4-hydroxyphenylpyruvic, homogentisic, 4-hydroxyphenylacetic, and homoprotocatechuic acids. Moreover, an isomer of compound III, *dl*-β-(4-hydroxyphenyl)-lactic acid, was not attacked (Table 1). When grown at the expense of succinic acid, cells failed to oxidize the aromatic compounds of Fig. 1.

Induction of enzymes. Two distinctive features of the proposed catabolic pathway (Fig. 1) may be noted: (i) before the benzene nucleus is opened, all three of the side chain carbon atoms are removed, giving rise to hydroxyquinol (VIII) as substrate for benzene ring fission; and (ii) protocatechuate, although a common ring fission substrate in other catabolic sequences (5, 6), was not attacked by cell extracts of *T. cutaneum* unless they were supplemented with NAD(P)H. The hydroxylation of protocatechuate by enzyme g of Fig. 1 is accompanied by simultaneous decarboxylation. Since they are characteristic of the proposed pathway, enzymes f, g, and h,

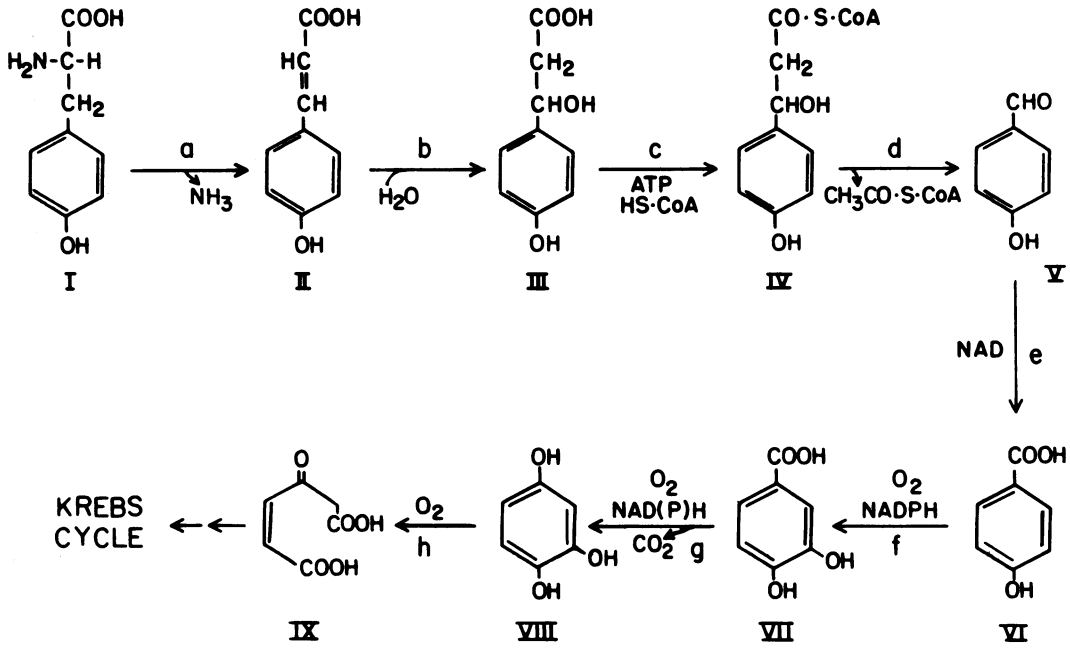

FIG. 1. *Proposed reaction sequence for the degradation of L-tyrosine by T. cutaneum.*

TABLE 1. *Rates of oxidation by washed cells of T. cutaneum grown with L-tyrosine*

Compound oxidized[a]	Oxygen uptake (μl/min)[b]
L-Tyrosine (I)	3.2
4-Hydroxycinnamic acid (II)	3.3
dl-HPH (III)	5.3
4-Hydroxybenzaldehyde (V)	3.1
4-Hydroxybenzoic acid (VI)	8.7
Protocatechuic acid (VII)	4.6
4-Hydroxyphenylpyruvic acid	<0.6
Homogentisic acid	<0.6
Homoprotocatechuic acid	<0.6
4-Hydroxyphenylacetic acid	<0.6
dl-β-(4-Hydroxyphenyl)-lactic acid	<0.6

[a] Roman numerals refer to compounds shown in Fig. 1.

[b] Measured after subtracting endogenous respiration (1.0 μl of O_2 per min). Warburg cups contained, in 2.8 ml of 0.1 M phosphate buffer (pH 6.5), 4 mg (dry weight) of cells and 4 μmol of substrate.

TABLE 2. *Induction of enzymes in T. cutaneum*

Enzyme assayed[a]	Sp act (nmol/min per mg of protein)	
	L-Tyrosine[b]	Succinate[b]
4-Hydroxybenzoate hydroxylase (f)	198	18
Protocatechuate hydroxylase (g)	420	17
Hydroxyquinol dioxygenase (h)[c]	286	48
Maleylacetate reductase	1,240	67

[a] The letters in parentheses denote enzymes identified in Fig. 1.

[b] Growth substrate.

[c] Specific activity corrected for autoxidation rate of 45 nmol of oxygen per min.

catalyzing formation and oxidation of protocatechuate and hydroxyquinol, were chosen for induction studies. These enzymes, and also the reductase for maleylacetate (IX), were assayed in extracts of cells grown, respectively, with L-tyrosine and succinate as carbon sources. Levels were strongly elevated on growth with L-tyrosine (Table 2).

Formation of 4-hydroxycinnamic acid (II). A solution of 10 μmol of L-tyrosine in 1.0 ml of phosphate buffer, pH 7.0, was incubated at 30°C for 2 h with 6 mg of cell extract protein from L-tyrosine-grown cells. The solution was then acidified to pH 2 with HCl, extracted with ether, and dried over Na_2SO_4. The reaction product showed the same absorption spectrum in ether as authentic 4-hydroxycinnamic acid (λ_{max} at 310 nm), the mass spectrum using a direct probe showed the same parent ion (m/e = 164), and the product also had the same R_f value (0.13) in light petroleum ether (bp, 60 to 80°C)-acetone-acetic acid (75:25:1).

Formation of 4-hydroxybenzoic acid (VI).

Freshly prepared cell extracts of *Trichosporon* converted 4-hydroxycinnamic acid into 4-hydroxybenzoic acid. A solution containing 1.0 mmol of $MgCl_2$ and 40 μmol of 4-hydroxycinnamic acid in 100 ml of phosphate buffer, pH 8.0, was incubated with 36 mg of cell extract protein for 5 h at 20°C. After acidification and extraction into ether, trimethylsilyl derivatives were formed, and, when examined by gas chromatography and mass spectrometry, the derivative of 4-hydroxybenzoic acid was identified (parent ion $m/e = 282$). It may be noted that no NAD was added to the incubation mixture, although this coenzyme is involved in the oxidation of 4-hydroxybenzaldehyde to 4-hydroxybenzoic acid (Fig. 1). Similar observations were made by French et al. (8) when investigating the conversion of 4-hydroxycinnamic acid into 4-hydroxybenzoic acid by extracts from potato tubers. They attributed lack of NAD dependence to close association of 4-hydroxybenzaldehyde dehydrogenase with endogenous cofactor. The experiment using an extract of *T. cutaneum* and 4-hydroxycinnamic acid was repeated, with 5 mM semicarbazide hydrochloride present in the reaction mixture as a trapping agent for 4-hydroxybenzaldehyde. After acidification and addition of 0.1% 2,4-dinitrophenylhydrazine, a nonacidic derivative was extracted into ethyl acetate and identified by chromatography as the 2,4-dinitrophenylhydrazone of 4-hydroxybenzaldehyde.

Reactions of HPH (III). When cell extracts were stored, either frozen or at 4°C, for more than a few days, the reactions described above were no longer catalyzed. This could have been due, in part, to the lability of these enzymes, but the following experiment showed that lack of activity was probably due to depletion, during storage, of the coenzymes required in step c of Fig. 1. To 300 nmol of HPH in 1.0 ml of phosphate buffer, pH 6.5, was added cell extract (0.6 mg of protein) that had been frozen for 4 days and then thawed; disappearance of HPH was monitored at maximum extinction, 283 nm, over a period of 5 min, and an initial rate was obtained for the reaction. The experiment was repeated, with additions of 20 μmol each of ATP and CoA. Without these additions the initial rate was 45 nmol of HPH per min, but when the two cofactors were present the rate was >1,500 nmol of HPH per min.

French et al. (8) found that the conversion of 4-hydroxycinnamate into 4-hydroxybenzoate by extracts of potato tubers was unaffected by additions of CoA or ATP, and they also identified acetic acid as a reaction product. We were unable to detect any acetic acid formed when 4-hydroxycinnamate or HPH was degraded, although the method of assay used (12) gave quantitative recoveries of acetic acid either in the presence or absence of cell extracts of *T. cutaneum*. This suggests that in our system the CoA ester of HPH, and not the free acid, undergoes aldol fission and gives rise to acetyl-CoA rather than acetic acid. Such an aldolase would resemble ATP citrate-lyase (EC 4.1.3.8) in its cofactor requirements. Formation of acetyl-CoA was demonstrated by trapping the compound as citric acid, which was determined chemically by the pentabromoacetone method (21). Yields of citrate were low, being dependent upon activities of all the enzymes in a sequence catalyzing, successively, HPH-CoA formation, aldol fission, and citric acid synthesis; moreover, yeast citrate synthase is inhibited to some extent by ATP (11), which was needed to activate HPH. Reaction mixtures contained, in 1.0 ml of phosphate buffer (pH 6.5), 500 nmol of HPH, 600 nmol each of ATP and CoA, 3 mg of cell extract, and 18 U of citrate synthase. After incubation for 1 h at 30°C, 1,500 nmol of oxaloacetic acid was added, and incubation was continued for a further 1.5 h, when mixtures were deproteinized by adding 0.5 ml of 0.5 M trichloroacetic acid followed by centrifugation. Amounts of citric acid formed in two determinations were 98 and 102 nmol, respectively; no citrate could be detected in controls when HPH was replaced with buffer.

Formation and utilization of protocatechuic acid (VII). Protocatechuic acid was not attacked by cell extracts without additions of NADH or NADPH, the former being the more effective (Table 3). By contrast, NADPH was required for oxidation of 4-hydroxybenzoic acid by cell extracts, and this cofactor could not be replaced with NADH. When the latter reaction was monitored in the oxygen electrode, the transient accumulation of protocatechuate from 4-hydroxybenzoate was demonstrated by making additions (5 μl) of either protocatechuate 3,4-

TABLE 3. *Dependence of protocatechuate oxidation upon the presence of reduced pyridine nucleotides*

Cell extract (μl)	NADH (μmol)	NADPH (μmol)	O_2 uptake[a] (μmol/min)
25	0.2		0.121
50	0.2		0.257
25		0.2	0.068
50		0.2	0.141
50			<0.005

[a] Measured with an oxygen electrode for reactions in 1.5 ml of 0.1 M phosphate buffer, pH 7.0, each containing 0.2 μmol of protocatechuate, with the additions indicated.

dioxygenase or protocatechuate 4,5-dioxygenase; these enzymes catalyzed rapid increases in O_2 uptake of short duration as protocatechuate was oxidized. When determined with the oxygen electrode, using an excess of NADH and with concentrations of protocatechuate lower than 50 nmol/ml, 2.0 nmol of O_2 was consumed per 1.0 nmol of protocatechuate. This ratio corresponds to the use of one molecule of O_2 for hydroxylation, with a second molecule required for ring fission. When the concentration of protocatechuate was progressively raised to 80 nmol/ml, the ratio diminished to 1.5; and in experiments using the Warburg respirometer, which require still higher concentrations (approximately 1.0 μmol/ml), a dialyzed extract in the presence of NADH catalyzed an uptake of 1.1 μmol of O_2 and an evolution of 0.7 μmol of CO_2 from 1.0 μmol of protocatechuate. An assay, using protocatechuate 3,4-dioxygenase, showed that 97% of the substrate had been metabolized. Ring fission, therefore, did not appear to be effectively catalyzed under these conditions; moreover, the amount of CO_2 measured suggests that a proportion of the protocatechuate might have undergone oxidation without being decarboxylated to hydroxyquinol (VIII). The possibility that protocatechuate might have undergone nonoxidative decarboxylation was eliminated by incubating reaction mixtures with purified catechol 2,3-dioxygenase (15): no trace of catechol could be detected. Hydroxyquinol is subject to autoxidation in aqueous solution (4), and evidence for the proposed hydroxylation-decarboxylation step (reaction g, Fig. 1) was obtained by taking advantage of the fact that cell extracts catalyzed an NADH-dependent oxidation of an analog of protocatechuic acid, namely, 5-methylprotocatechuic acid, at one-third of the rate for protocatechuate. The product, which is more stable than hydroxyquinol, was identified as 2,3,5-trihydroxytoluene as follows. 5-Methylprotocatechuic acid (5 μmol) was added dropwise to a stirred reaction mixture in 10 ml of 0.1 M phosphate buffer, pH 7.0, containing 9 mg of cell extract protein and 7 μmol of NADH. After stirring for 30 min, the mixture was acidified, the reaction product was extracted into ether and dried over Na_2SO_4, and the ether was removed. Mass spectrometry (19) showed a parent ion of *m/e* 356 for the trimethylsilyl derivative, and the metabolic product itself was also readily attacked by cell extracts of *P. putida* ORC grown at the expense of orcinol (4). It is therefore concluded that 2,3,5-trihydroxytoluene, a methyl analog of hydroxyquinol, was formed from 5-methylprotocatechuic acid by the cell extracts of *T. cutaneum*.

Hydroxyquinol was oxidized rapidly in respirometer experiments, with an uptake of approximately 1.0 μmol of O_2 per mol of substrate. The product showed the spectrum of maleylacetic acid (IX), with λ_{max} at 245 nm (pH 7.0), abolished on acidification (4). Cell extracts contained an active NADH-dependent reductase for maleylacetic acid. These observations support the reaction sequence of Fig. 1 by which protocatechuate is oxidized to hydroxyquinol; this compound might then be degraded through the Krebs cycle by way of β-ketoadipate, as proposed by Chapman and Ribbons for the catabolism of resorcinol in *P. putida* (4). We could detect only small amounts of β-ketoadipate, and traces of pyruvate were formed from protocatechuate in some experiments. However, Gaal and Neujahr (9) demonstrated recently that resorcinol is converted quantitatively into β-ketoadipate by extracts of another strain of *T. cutaneum* grown with vigorous aeration (24) at the expense of resorcinol. Since we sought to minimize the risk of contamination by widely distributed bacteria that grow readily with tyrosine (3, 18, 22), we used a rotatory shaker for aeration, but in other experiments using benzoic acid or resorcinol as sources of carbon, we found that oxygenase activities of *T. cutaneum* could be increased greatly by growing cultures with forced aeration rather than in shaken flasks. At low rates of enzymatic oxidation, enzyme inhibitors are more likely to accumulate from spontaneous oxidation of labile catabolites such as hydroxyquinol.

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