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Metabolism of *p*-Cresol by the Fungus *Aspergillus fumigatus*

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The fungus *Aspergillus fumigatus* ATCC 28282 was shown to grow on *p*-cresol as its sole source of carbon and energy. A pathway for metabolism of this compound was proposed. This has protocatechuate as the ring-fission substrate with cleavage and metabolism by an *ortho*-fission pathway. The protocatechuate was formed by two alternative routes, either by initial attack on the methyl group, which is oxidized to carboxyl, followed by ring-hydroxylation, or by ring-hydroxylation as the first step with subsequent oxidation of 4-methylcatechol to the acid. The pathway was elucidated from several pieces of evidence. A number of compounds, including 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, protocatechuic acid, protocatechualdehyde, and 4-methylcatechol, appeared transiently in the medium during growth on *p*-cresol. These compounds were oxidized without lag by *p*-cresol-grown cells but not by succinate-grown cells. Enzyme activities for most of the proposed steps were demonstrated in cell extracts after growth on *p*-cresol, and the products of these activities were identified. None of the activities were found in succinate-grown cells.

Cresols are among those phenolic compounds generally regarded as industrial pollutants, and, as such, their removal by microbial metabolic processes is of importance. It should be noted, however, that *p*-cresol at least is a naturally occurring metabolic product, being formed from tyrosine by bacteria under anaerobic conditions (6). Such conditions exist in the rumen or in the digestive tract of nonruminants, and substantial amounts of *p*-cresol are excreted by animals (13). It is not, therefore, surprising that other microbes that will grow on this compound can be readily isolated. The bacterial degradation of *p*-cresol has been intensively studied, and two pathways in aerobic organisms have been proposed. In one, the ring is hydroxylated to give 4-methylcatechol as the substrate for a ring-fission dioxygenase (2). In the other, initial attack takes place on the methyl group, which is oxidized to carboxyl. The 4-hydroxybenzoic acid formed is then ring-hydroxylated, giving protocatechuic acid as the ring-fission substrate (5, 9). A novel feature of this latter pathway is that the initial methylhydroxylase, in a number of bacterial species, is a flavocytochrome *c* that acts by dehydrogenation of the substrate to form a quinone methide. This is then hydrated to give the 4-hydroxybenzyl alcohol (8). Thus, in contrast to the monooxygenase type of hydroxylases, the enzyme has no direct requirement for oxygen or for an electron donor, such as NAD(P)H. Such a system allows for the anaerobic oxidation of the methyl group of *p*-cresol, and there have been several reports of the anaerobic degradation of *p*-cresol by bacteria. In those in which the pathway has been studied, the methyl is oxidized to carboxyl with further metabolism of the ring by a reductive pathway (3).

Less is known of the fungal pathways for metabolism of *p*-cresol, although its degradation by several species has been reported (12). Perhaps the most thorough study was that by Powlowski and Dagley (16), who described the pathway used by the yeast *Trichosporon cutaneum*. In this organism, an NADPH-dependent hydroxylase converts *p*-cresol into 4-methylcatechol, the ring-fission substrate, which is cleaved by an *ortho*-fission enzyme. The product is converted into central metabolites by a modified 3-oxoadipate (β-ketoadipate) pathway (16). Another genus of fungi, known to metabolize aromatic compounds, is *Aspergillus* (4). For example, *Aspergillus fumigatus* ATCC 28282 has been shown to metabolize both phenylacetic and homogentisic acids (20, 21). In this paper, we show that this same strain of the organism is also capable of growth on *p*-cresol as its sole carbon source and describe the route it uses for the breakdown of this compound.

MATERIALS AND METHODS

Maintenance and growth of the organism. Stock cultures of *A. fumigatus* ATCC 28282 were grown at 30°C on glucose agar plates until sporulation occurred (4 to 5 days) and then were stored at 4°C. For growth in liquid medium, 10 discs (7 mm in diameter) were cut aseptically from a sporulated culture on a glucose agar plate and used to inoculate 100 ml of glucose medium in a 250-ml Erlenmeyer flask. The flask was incubated at 30°C with shaking for 24 h, and the mycelial pellets were then harvested by sterile vacuum filtration. The pellets were then transferred to 1 liter of *p*-cresol or succinate medium and incubated as before. Finally, mycelial pellets were harvested, resuspended in 42 mM Na-K phosphate buffer, pH 7.1, and either used immediately or stored at −20°C. The liquid medium contained (per liter) 4 g of Na_2HPO_4, 2 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, and 4 ml of a trace element solution (17) and a carbon source and was adjusted to pH 6.0 with 2 M HCl. Carbon sources were glucose (0.14 M), succinate (8 mM), and *p*-cresol (2.8 mM).

Preparation of cell extracts. Cell extracts were prepared by ultrasonic treatment of mycelial suspensions with a Soniprobe 150 (MSE, Crawley, United Kingdom) set at a probe amplitude of 18 μm for a total of 2 min in half-minute bursts separated by periods of cooling. Cell debris was removed by centrifugation at 48,000 × *g* for 20 min at 4°C.

Extraction of products. Products were extracted from growth media or reaction mixtures with an equal volume of diethyl ether, after acidification to pH 1 with HCl. The ether extract was dried over anhydrous sodium sulfate and then evaporated to dryness.

TLC. Thin-layer chromatography (TLC) was performed on 0.20-mm-thick precoated plastic-backed silica gel (60 F_{254}) plates. The following solvents were used and are

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referred to in the text by their letters: A, toluene-1,4-dioxan-acetic acid (90:20:4); B, ethyl formate-petroleum ether (40 to 60°C)-propionic acid (30:70:15.4); and C, *n*-butanol–ethanol–ammonia (specific gravity, 0.88)–water (35:5:0.5:10). Aromatic compounds were detected by their quenching of the background fluorescence when the plates were viewed under UV light. Phenolic compounds were visualized by being sprayed with Folin-Ciocalteu's phenol reagent which gave a blue color, immediately with *o*- or *p*-diphenols and after exposure to ammonia with monophenols. *o*-Diphenolic compounds were also detected with 100 mM ferric chloride. This gives a blue-green color which turns red on exposure to ammonia (19). Aldehydes were detected as orange spots after being sprayed with a solution of 2,4-dinitrophenylhydrazine (0.1%) in 2 M HCl.

GC-MS. Gas chromatography-mass spectrometry (GC-MS) samples were examined with a Hewlett-Packard 5890 instrument with a 5971 mass selective detector. An HP5 (cross-linked 5% phenyl methyl silicone) column (25 m by 0.2 mm by 0.33 μm film) with helium as the carrier gas and a temperature program of 4 min at 70°C rising at 20°C/min to 275°C were used. The trimethylsilyl derivatives of samples were prepared by incubation with *N*,*O*-bis(trimethylsilyl) trifluoroacetamide at room temperature overnight. Compounds were identified by comparing the retention times and fragmentation patterns with those of standards and with entries in the Wiley library of mass spectra.

Measurement of oxygen consumption. Oxidations by intact mycelia were monitored with conventional Warburg respirometers at 30°C. Each flask contained 10 mg (dry weight) of mycelia suspended in 1.6 ml of 42 mM phosphate buffer, pH 6.0, 0.1 ml of 3.6 M KOH in the center well, and 0.3 ml of 10 mM substrate tipped from the side arm. Oxidations by cell extracts were monitored with an oxygen electrode in 3.0 ml of 42 mM sodium-potassium phosphate buffer (pH 7.1) at 30°C.

Enzyme assays. All enzyme units are expressed as nanomoles of substrate converted per minute. Anaerobic *p*-cresol methylhydroxylase (EC 1.17.99.1) was assayed as described by McIntire et al. (14). All other assays were performed at 30°C in 42 mM sodium-potassium phosphate buffer (pH 7.1). Aerobic *p*-cresol hydroxylase was assayed spectrophotometrically by monitoring the decrease in A_{340} due to the oxidation of NADPH in a reaction mixture containing 0.15 mM NADPH, 1.0 mM *p*-cresol, and cell extract. *p*-Cresol was replaced by 4-methylcatechol for assay of 4-methylcatechol methylhydroxylase. For *p*-hydroxybenzoate hydroxylase, *p*-cresol was replaced by *p*-hydroxybenzoate and 25 mM flavin adenine dinucleotide (FAD) was included in the reaction mixture. A value of 6.22 for the ε_{mM} of NAD(P)H was used. Alcohol and aldehyde dehydrogenases were assayed by monitoring the increase in absorbance due to reduction of NAD^+ in reaction mixtures containing 0.2 mM NAD^+, 1.0 mM substrate, and cell extract. For 4-hydroxybenzyl-alcohol dehydrogenase, the reaction was monitored at 340 nm, and a reaction millimolar absorbance coefficient of 15.06 was used, which also takes into account the absorbance due to the product 4-hydroxybenzaldehyde. For 4-hydroxybenzaldehyde dehydrogenase, the reaction was monitored at 370 nm and a reaction millimolar absorbance coefficient of 1.7 was used. Protocatechuate and catechol dioxygenases were assayed by measuring the consumption of oxygen with an oxygen monitor. Reaction mixtures of 3.0 ml contained 0.33 mM substrate and extract.

Other assays. Protein was assayed by the biuret method of Gornall et al. (7) with bovine serum albumin as a standard.

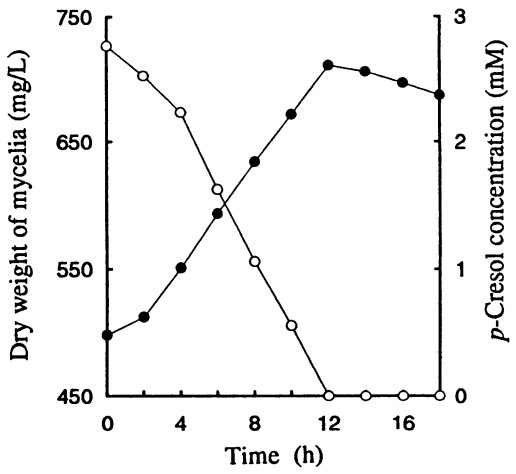

FIG. 1. Growth of *A. fumigatus* on *p*-cresol (●) and disappearance of growth substrate from the medium (○).

p-Cresol in growth medium was estimated from the blue color produced with Folin-Ciocalteu's phenol reagent. To 1.0 ml of test solution were added 0.5 ml of reagent and 2.0 ml of (10%, wt/vol) sodium carbonate solution. After 10 min, 1 ml of the solution was diluted to 5 ml with water and the A_{620} was recorded. 3-Oxoadipic acid was detected in reaction mixtures by the Rothera reaction (18).

2,4-Dinitrophenylhydrazones. 3-Oxoadipic acid was chromatographed as its 2,4-dinitrophenylhydrazone. This was prepared by incubating reaction mixtures with equal volumes of 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl at 30°C for 30 min. The reaction mixture was extracted with ethyl acetate and the acidic derivatives isolated by extraction of the ethyl acetate with 10% (wt/vol) sodium carbonate solution. This was then acidified with 2 M HCl and the products extracted into ethyl acetate for chromatography.

Chemicals. *p*-Cresol, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, catechol, protocatechuic acid, and protocatechualdehyde were obtained from BDH Chemicals (Poole, Dorset, United Kingdom). 4-Methylcatechol was from Aldrich Chemical Company (Gillingham, Dorset, United Kingdom). 4-Hydroxybenzyl alcohol was from ICN Biomedicals (High Wycombe, Buckinghamshire, United Kingdom).

RESULTS

Growth of *A. fumigatus* on *p*-cresol. Incubation of *p*-cresol medium inoculated with *A. fumigatus* resulted in a visible increase in mycelial mass with time, and a growth curve was constructed by measuring the increase in dry weight of the mycelia at intervals (Fig. 1). Growth was concomitant with disappearance of phenolic material from the medium, demonstrating the ability of this organism to use *p*-cresol as a growth substrate. The initial *p*-cresol concentration in this experiment was 2.8 mM; higher concentrations increased the lag before germination of spores, and at a concentration of 4.6 mM there was no germination over a 7-day observation period.

Compounds in culture medium. Samples of the culture medium, taken every 2 h during growth on *p*-cresol, were acidified and extracted with diethyl ether, and the extracts were examined by TLC. This too showed the gradual utilization of *p*-cresol over the growth period, with the spot corresponding to this compound diminishing as growth pro-

TABLE 1. Identification by TLC of intermediates found in culture medium during growth on *p*-cresol

Compound[a] identified as	R_f in solvent:		Color with detection reagent		
	A	B	Folin-Ciocalteu/$^+NH_3$	$FeCl_3$/$^+NH_3$	2,4-Dinitrophenylhydrazine
p-Cresol	0.66	0.89	None/blue		
4-Hydroxybenzyl alcohol	0.23	0.29	None/blue		
4-Hydroxybenzaldehyde	0.41	0.48	None/blue		Orange
4-Hydroxybenzoic acid	0.32	0.61	None/blue		
Protocatechuic acid	0.13	0.36	Blue/deep blue	Blue/red	
4-Methylcatechol	0.40	0.60	Blue/deep blue	Blue/red	
Protocatechualdehyde	0.23	0.30	Blue/deep blue	Blue/red	Orange

[a] R_f values and reactions with color reagents are for both the isolated intermediate and the standard compound.

ceeded and disappearing altogether from samples taken after 12 h. The transient appearance of other phenolic compounds in the growth medium was also apparent from TLC, with spots showing for samples taken after 4 h. Most of these peaked in intensity at 8 h, with only trace amounts detected in samples taken after 12 h. The results of the TLC of the 8-h sample are given in Table 1. From the 8-h TLC, 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, protocatechuic acid, protocatechualdehyde, and 4-methylcatechol were tentatively identified as intermediates.

Confirmation of some of these identities came from examination of the 8-h sample by GC-MS. Compounds, other than *p*-cresol, were separated as their trimethylsilyl derivatives and identified either from the spectrum library or by comparison with authentic standards. *p*-Cresol was chromatographed underivatized and had a retention time of 8.03 min. The compounds identified by this system were 4-methylcatechol, 4-hydroxybenzoic acid, and protocatechuic acid, with retention times of 11.08, 12.74, and 13.94 min, respectively.

These findings suggest that the methyl group of *p*-cresol is oxidized to carboxyl at some stage before ring-fission with protocatechuic acid as the ring-fission substrate. Initial attack on the methyl group would account for the monohydroxylated intermediates observed, whereas the transient accumulation of 4-methylcatechol points to ring-hydroxylation as a possible first step. This latter compound is a ring-fission substrate in some organisms, but the appearance of protocatechualdehyde suggests oxidation of the methyl group. The ability of mycelia to oxidize the methyl group of 4-methylcatechol was demonstrated by incubating *p*-cresol-grown mycelia in medium containing 4-methylcatechol. Cycloheximide (0.36 mM), an inhibitor of protein synthesis, was included in the medium to show that the necessary enzymes were already present in the *p*-cresol-grown cells and not induced by exposure to the 4-methylcatechol. Neutral extraction of samples of the medium and examination of the products by TLC showed the transient accumulation of protocatechualdehyde.

In a similar experiment, the initial hydroxylation step was bypassed by incubating *p*-cresol-grown mycelia with 4-hydroxybenzyl alcohol. Although 4-hydroxybenzaldehyde accumulated transiently in the medium, no protocatechualdehyde was detected and protocatechuic acid was the only diphenolic compound that appeared.

Oxidations by intact mycelia. The putative pathway intermediates were well oxidized, without lag, in a Warburg apparatus by intact mycelial pellets of *A. fumigatus* grown on *p*-cresol (Fig. 2a). The pattern for protocatechualdehyde (not shown in Fig. 2) was similar to that for protocatechuic acid. In contrast, the oxidation of homoprotocatechuic acid, an aromatic compound unlikely to be an intermediate, occurred only after a lag. That this lag was due to a period of enzyme induction was shown by the total abolition of oxidation in the presence of 0.36 mM cycloheximide. A similar lag before rapid oxidation was also seen for the putative intermediates when incubated with succinate-grown mycelia (Fig. 2b).

After growth on *p*-cresol, the organism was also able to oxidize several other phenolic compounds, including *m*-cresol, *o*-cresol, phenol, and 4-ethylphenol, without lag, although not as rapidly as the growth substrate, suggesting broad specificity for the initial enzymes.

Oxidation of compounds by cell extracts. When extracts of *p*-cresol-grown mycelia were tested for the anaerobic *p*-cresol–methylhydroxylase that has been found in several bacterial species, no activity was detected. However, when *p*-cresol was added to reaction mixtures containing cell extract and NADPH, there was immediate stimulation of oxygen consumption, measured with an oxygen electrode, and of NADPH oxidation, measured spectrophotometrically. This was indicative of a monooxygenase type of hydroxylase and, to demonstrate that the attack was on the methyl group, the products of a reaction mixture, scaled up to include 10 mmol of *p*-cresol, were extracted with diethyl

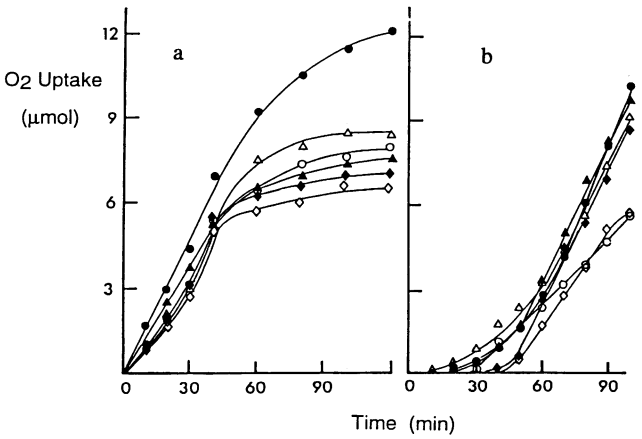

FIG. 2. Oxidation of various substrates by mycelia of *A. fumigatus* grown on *p*-cresol (a) or succinate (b). The following substrates (3 μmol) were tested: *p*-cresol (●), 4-methylcatechol (○), 4-hydroxybenzyl alcohol (▲), 4-hydroxybenzaldehyde (△), 4-hydroxybenzoic acid (◆), and protocatechuic acid (◇). Endogenous rates of 8 μmol of O_2 per h for the *p*-cresol-grown cells and 3.7 μmol of O_2 per h for the succinate-grown cells have been subtracted.

TABLE 2. Specific activities of enzymes in extracts of *p*-cresol-grown cells[a]

Enzyme	Sp act (nmol/min/mg of protein)
p-Cresol hydroxylase	10
p-Cresol methylhydroxylase (anaerobic)	ND[b]
4-Hydroxybenzyl-alcohol dehydrogenase	19
4-Hydroxybenzaldehyde dehydrogenase	40
4-Hydroxybenzoate hydroxylase	110
Protocatechuate dioxygenase	190
Catechol dioxygenase[c]	ND
4-Methylcatechol hydroxylase	7

[a] None of the activities was detected in extracts of succinate-grown cells.
[b] ND, not detected.
[c] Assayed with either catechol or 4-methylcatechol.

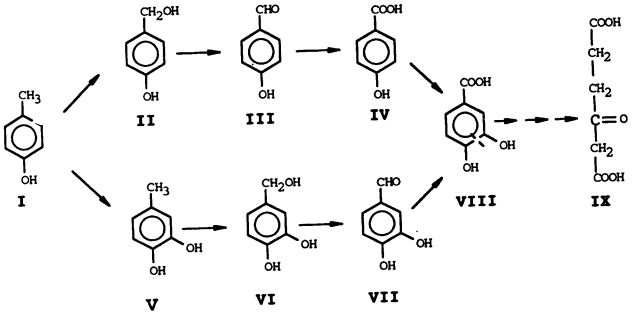

FIG. 3. Proposed pathway for the metabolism of *p*-cresol by *A. fumigatus*. The intermediates are as follows: I, *p*-cresol; II, 4-hydroxybenzyl alcohol; III, 4-hydroxybenzaldehyde; IV, 4-hydroxybenzoic acid; V, 4-methylcatechol; VI, 3,4-dihydroxybenzyl alcohol; VII, protocatechualdehyde; VIII, protocatechuic acid; and IX, 3-oxoadipic acid.

ether and examined by TLC. A minor spot of unchanged *p*-cresol was seen, but the major spot corresponded to 4-hydroxybenzaldehyde with a smaller amount of 4-hydroxybenzyl alcohol apparent and some 4-hydroxybenzoic acid when the reaction mixture was acidified before extraction. Neutral extraction also gave some 4-methylcatechol, indicative of ring hydroxylation, and some protocatechualdehyde. The hydroxylase was inactive when NADH replaced NADPH in the assay, and no activity was detected in extracts of succinate-grown mycelia (Table 2). Activity was not enhanced by addition of FAD or flavin mononucleotide to the assay mixtures.

Dehydrogenase activity for 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde in cell extracts was also detected (Table 2). In both cases, either NAD^+ or $NADP^+$ could serve as electron acceptor, but rates were faster with NAD^+. Again diethyl ether extracts of scaled-up reaction mixtures were examined by TLC to identify products. The alcohol gave rise to 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid. The aldehyde yielded 4-hydroxybenzoic acid and some of the alcohol, presumably by reversal of the alcohol dehydrogenase by using NADH produced by the forward reaction.

Oxidation of 4-hydroxybenzoic acid by cell extracts required the presence of both NADH and FAD, indicative of a flavoprotein-type of monooxygenase for hydroxylation of the ring. The FAD could not be replaced by flavin mononucleotide, but there was activity, albeit reduced, when NADPH was used instead of NADH. A study of the stoichiometry of the reaction showed that whereas 1 μmol of NADH per μmol of substrate was used, there was an uptake of 2 mmol of oxygen (Table 3), twice that expected for a monooxygenase. However, no aromatic compounds could be detected in a diethyl ether extract of the reaction mixture from such an incubation, and the additional oxygen consumption could be accounted for by the activity of the next predicted enzyme in the sequence, protocatechuate dioxygenase, which was detected in cell extracts (Table 2). No added cofactors were required for this activity, and the stoichiometry of the reaction (Table 3) was consistent with a ring-fission dioxygenase. The enzyme was completely inhibited by addition of 5 mM Tiron, a chelator of ferric ions, but not at all by 5 mM α,α′-bipyridyl, a ferrous ion chelator. This provided a means of studying the stoichiometry of the 4-hydroxybenzoic acid hydroxylase reaction and demonstrating its product. When Tiron was included in the reaction mixture for the oxidation of 4-hydroxybenzoic acid, the stoichiometry was as expected for a monooxygenase (Table 3) and the product was identified by TLC as protocatechuic acid. Similarly, protocatechuic acid was identified as the product when protocatechualdehyde was incubated with NAD^+ and cell extract in the presence of 5 mM Tiron.

The reaction mixtures for protocatechuate oxidation and for 4-hydroxybenzoate oxidation in the absence of Tiron both gave a positive reaction in the Rothera test, as expected for products of *ortho*-fission of the ring. The 2,4-dinitrophenylhydrazone of the product in each case corresponded to that of authentic 3-oxoadipic acid when examined by TLC, with R_f values of 0.73, 0.64, and 0.47 in solvent systems A, B, and C, respectively.

The specific activities of all the relevant enzymes in extracts of *p*-cresol-grown mycelia are shown in Table 2. None of the enzymes was detected in extract from succinate-grown mycelia.

TABLE 3. Stoichiometry of NADH oxidation and oxygen utilization for oxidation of 4-hydroxybenzoate and protocatechuate by cell extracts[a]

Substrate	Oxygen consumed (μmol/μmol of substrate)	NADH oxidized (μmol/μmol of substrate)
4-Hydroxybenzoate	2.2	0.97
4-Hydroxybenzoate + Tiron (5 mM)	1.09	0.95
Protocatechuate	0.89	
Protocatechuate + Tiron (5 mM)	0.00	

[a] Values are averages from three different amounts of substrate.

DISCUSSION

The growth of mycelial organisms is not always easy to measure, but, by taking dry weights, it was possible to demonstrate the ability of *A. fumigatus* to grow on *p*-cresol as its sole source of carbon (Fig. 1). The results of the metabolic studies suggested that methyl group oxidation to carboxyl and ring hydroxylation are involved in the catabolic pathway but that there is no strict order in which these processes occur. This gives rise to two routes converging at protocatechuic acid (Fig. 3). Preliminary evidence for this came from identification of a number of related aromatic compounds that appeared in the medium during growth. The

fact that their presence was transient suggested that they were intermediates in the degradative pathway and not dead-end side products. The identification of 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, and 4-hydroxybenzoic acid pointed to an initial attack on the methyl group, but the transient appearance of 4-methylcatechol in the medium suggested hydroxylation of the ring as an alternative first step. The proposed oxidation of the methyl group was supported by the immediate oxidation of the putative intermediates by intact *p*-cresol-grown mycelia, in contrast to the lag seen before they were oxidized by succinate-grown cells (Fig. 2). It was further strengthened by demonstrating the presence, in cell extracts, of the appropriate enzymatic activities induced by growth on *p*-cresol (Table 2) and identification of the proposed product at each step.

The requirement for NADPH and oxygen for the *p*-cresol methylhydroxylase activity in cell extracts is typical of a monooxygenase type of enzyme. This fungal activity contrasts with those of the bacterial, anaerobic *p*-cresol methylhydroxylases described in the introduction. Unlike the bacterial enzyme, which acts as a dehydrogenase, this monooxygenase would not be constrained in its specificity to those phenols capable of forming quinone methides, and this perhaps explains the ability of *p*-cresol-grown mycelia also to oxidize *m*-cresol.

The next step in metabolism of the 4-hydroxybenzoic acid formed from *p*-cresol was its hydroxylation to protocatechuic acid. Again, this was isolated from growth medium and identified and was oxidized by both intact mycelia and cell extracts. 4-Hydroxybenzoate 3-hydroxylase activity in cell extracts was demonstrated, and the product was identified as protocatechuic acid. The enzyme was NADH dependent, and the stoichiometry of NADH oxidized and oxygen utilized (Table 3) was as expected for a monooxygenase. The requirement for FAD is typical for this same ring-hydroxylase from bacterial species (10, 11) and for the fungal enzyme from the yeast *T. cutaneum*, which catalyzes a similar ring hydroxylation in the conversion of phenol into catechol (15). The possible oxidative decarboxylation of protocatechuate to give the ring-fission substrate hydroxyquinol, as occurs in *T. cutaneum* (1), was not observed here, and the evidence pointed towards *ortho*-fission of protocatechuate followed by metabolism via the 3-oxoadipate pathway. Such a route for the degradation of protocatechuate has previously been described for a number of fungi, including *Aspergillus* species (4).

Some of the *p*-cresol was converted into 4-methylcatechol, which is itself a ring-fission substrate, in the metabolism of *p*-cresol by *T. cutaneum* (16). However, there was no evidence for a ring-fission dioxygenase active towards this compound in *A. fumigatus* when grown on *p*-cresol. Instead, the evidence suggests that the methyl group of 4-methylcatechol is oxidized to carboxyl to give protocatechuic acid as the ring-fission substrate. Some protocatechualdehyde was found in medium during growth on *p*-cresol, and its production from 4-methylcatechol was demonstrated when mycelia from this medium were incubated with 4-methylcatechol in the presence of cycloheximide to prevent induction of any new enzymes. Dehydrogenase activity for the conversion of protocatechualdehyde into protocatechuic acid in cell extracts was demonstrated. The transient appearance of 4-hydroxybenzaldehyde but not protocatechualdehyde when *p*-cresol-grown mycelia were incubated with 4-hydroxybenzyl alcohol suggests that the two routes are not further interconnected by ring-hydroxylation of the alcohol or aldehyde, compounds II and III in Fig. 3, to give the corresponding diphenols, compounds VI and VII, respectively.

Although there are two routes, it is possible that some of the enzymes are common. This may be the case for the enzymes of methyl group oxidation but certainly is not for the hydroxylation of the ring. 4-Hydroxybenzoate 3-hydroxylase, which was NADH dependent, was inactive with *p*-cresol, whereas the formation of 4-methylcatechol from *p*-cresol by cell extracts required NADPH. The relative contributions of each pathway to *p*-cresol degradation have not been evaluated.

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