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Metabolism of Phenol and Cresols by *Bacillus stearothermophilus*

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An obligate thermophilic strain of *Bacillus stearothermophilus*, strain PH24, isolated from industrial sediment by elective culture, grew readily at 55 C on phenol or on one of the isomers of cresol as the major carbon source. Intact cells grown in the presence of phenol, *o*-cresol, *m*-cresol, or *p*-cresol were induced to oxidize, without lag, these substrates together with catechol, 3-methylcatechol, and 4-methylcatechol. Cell extracts prepared from *B. stearothermophilus* PH24 after growth in the presence of phenol converted phenol to catechol with a concomitant uptake of 1 mol of oxygen per mol of substrate in reaction mixtures supplemented with reduced nicotinamide adenine dinucleotide. These preparations also catalyzed the oxidation of *o*-cresol to 3-methylcatechol and of *m*-cresol and *p*-cresol to 4-methylcatechol. Enzyme activity was inhibited by 1 mM *p*-chloromercuribenzoate and by 0.1 mM *o*-phenanthroline. Catechol and the corresponding methylcatechol intermediates were further dissimilated by cell extracts of phenol-grown cells via the *meta*-cleavage route to yield 2-hydroxymuconic semialdehyde and the respective methylated derivatives.

Phenol and the isomers of cresol have been widely reported to support the growth of mesophilic bacteria and yeasts. So far, much of the work on the metabolism of phenolic compounds by bacteria has involved species of *Pseudomonas*. Dagley and Patel (11) reported that the degradation of *p*-cresol by a nonfluorescent pseudomonad proceeded by an initial oxidative attack on the methyl group, whereas *P. putida* U metabolized phenol and cresols by converting them into catechol and methylcatechols, respectively (2). Ribbons (27) presented evidence consistent with a reaction sequence whereby *o*-cresol was oxidized to 3-methylcatechol in *P. aeruginosa* T1, and Nakagawa and Takeda (20) concluded that *o*-cresol was similarly metabolized in a strain of *Brevibacterium fuscum* isolated from soil. These latter authors further described the conversion of *m*- and *p*-cresol to 4-methylcatechol by this organism.

Earlier reports on the decomposition of phenolic compounds by yeasts concerned selected strains belonging to the genera *Oospora*, *Saccharomyces*, *Candida*, and *Debaryomyces* (14, 18). More recently Neujahr and Varga (24) reported the oxidation of phenol and some phenol derivatives by intact cells and cell extracts of *Trichosporon cutaneum* grown on phenol as the major carbon source. Purified phenol hydroxylase from this yeast converted phenol to catechol, which was subsequently cleaved to *cis,cis*-muconic acid via intradiol ring fission (22, 24). Neujahr et al. (23) and Hashimoto (15, 16) further demonstrated the oxidation of phenols by *C. tropicalis*. In the former case, extracts of phenol-grown cells exhibited phenol hydroxylase and catechol 1,2-oxygenase activity.

Experiments with enrichment cultures had earlier suggested that phenolic compounds could serve as growth substrates for thermophilic microorganisms (12, 13), and more recently the utilization of aromatic compounds by pure cultures of thermophilic bacteria was reported (6; J. A. Buswell and D. G. Twomey, Proc. Soc. Gen. Microbiol. **1**:48, 1974). This communication describes the metabolism of phenol and the isomers of cresol to catechol and methylcatechol intermediates by whole cells and cell extracts of *Bacillus stearothermophilus* PH24 and the subsequent *meta* cleavage of the benzenoid nucleus.

MATERIALS AND METHODS

Isolation, growth, and identification of the microorganism. The organism used throughout this investigation was isolated from industrial sediment at Ravenscraig Steel Works near Motherwell, Scotland, by enrichment, with phenol as the major carbon source, in a mineral salts medium at 55 C (5). It was previously identified (6) from morphological and biochemical characteristics as a strain of *B. stearothermophilus* by the classification of Smith et al. (29)

and will be referred to hereafter as strain PH24. *B. stearothermophilus* PH24 was routinely grown in 2-liter Erlenmeyer flasks that contained 1 liter of a medium consisting of (per liter): K_2HPO_4, 0.5 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; yeast extract (Oxoid), 0.2 g; Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.1 g; trace elements (1), 1 ml; and phenolic substrate, 0.5 g, adjusted to pH 7.4 with 2 N H_2SO_4. For noninduced cells, sodium succinate or sodium fumarate (2 g/liter) was substituted for the aromatic compound. Cultures were incubated at 55 C in an orbital incubator shaker operating at 160 to 180 rpm. Cells in late exponential phase (14 to 16 h with phenol as growth substrate) were harvested by centrifugation at room temperature, washed once with 0.1 M KH_2PO_4 buffer (pH 7.5), and either used immediately or stored at −20 C until required. Stock cultures were maintained by monthly subculture on slopes of phenol medium solidified with 2% (wt/vol) agar.

P. putida (NCIB 10015), used as a source of metapyrocatechase (catechol 2,3-dioxygenase, EC 1.13.11.2), was kindly provided by P. A. Willaims, Department of Biochemistry and Soil Science, University College of North Wales. The growth and preparation of cell extracts were described previously (2).

Preparation of cell extracts. *B. stearothermophilus* PH24 was grown with phenol as the major carbon source, and cells were harvested towards the end of log phase (about 14 h). After being washed once with 0.1 M KH_2PO_4 buffer (pH 7.5), the cells were suspended in 2 volumes of the same buffer containing 10 mM dithiothreitol, 2 μM flavine adenine dinucleotide and 0.1 mM ethylenediaminetetraacetic acid, and broken by four passages through the X-press (AB Biox, Nacka, Sweden) previously cooled to −20 C. After treatment with bovine pancreas deoxyribonuclease (10 μg/ml) for 15 min at room temperature, unbroken cells and cell debris were removed by centrifugation at 30,000 × *g* for 20 min. The supernatant material was further centrifuged at 150,000 × *g* for 80 min, and the clear supernatant solution was used for enzyme assays.

Protein was measured by the method of Lowry et al. (19). Bovine serum albumin was used as standard.

Analytical methods. The uptake of oxygen by whole cells was measured in a Warburg apparatus at 50 C. The oxygen consumption by cell extracts was measured polarographically with a Clark oxygen electrode (Rank Bros., Bottisham, United Kingdom). Ultraviolet and visible spectra were determined on a Pye Unicam SP1800 recording spectrophotometer. Reaction products were identified by chromatography on a thin layer of silica gel with a fluorescent indicator (Eastman Chromagram Sheets, type 13181). The plates were developed in benzene-ethanol (10:1 vol/vol) and were visualized by viewing under ultraviolet light and by spraying with either $FeCl_3$-$K_3Fe(CN)_6$ (equal parts of 0.3% solutions) or 2,6-dichloroquinone-4-chloroimide (2% in ethanol).

Enzyme assays. Phenol hydroxylase was assayed by measuring the consumption of oxygen polarographically at 50 C with a Clark oxygen electrode or by following the oxidation of reduced nicotinamide adenine dinucleotide (NADH) at 340 nm. The assay of catechol 2,3-dioxygenase was the same as that described by Sala-Trepat and Evans (28).

Chemicals. Phenol and the isomers of cresol were of the highest purity commercially available and were used without further purification. Catechol, 3-, and 4-methylcatechol were purified by vacuum sublimation. NADH, NADPH, NADase, dithiothreitol, and flavine adenine dinucleotide were from Sigma Chemical Co., St. Louis, Mo. Deoxyribonuclease was obtained from BHD Chemicals Ltd., Poole, England.

RESULTS

Oxidation of aromatic substrates by whole cells. Washed-cell suspensions of *B. stearothermophilus* PH24 grown in the presence of phenol rapidly oxidized phenol, *o*-, *m*-, and *p*-cresol, catechol, 3-methylcatechol, and 4-methylcatechol (Fig. 1). The total amounts of oxygen consumed were considerably less than theoretical and indicated negligible oxidation of the products of ring cleavage. The explanation for this is not clear. Benzoic acid, *o*-, *m*-, and *p*-hydroxybenzoic acids, and protocatechuic acid were not oxidized. Suspensions of intact cells grown in the presence of *o*-, *m*-, or *p*-cresol oxidized phenol and the isomers of cresol at almost equal rates (Table 1). Oxygen uptake on catechol, 3-, and 4-methylcatechol was accompanied by the transient appearance of yellow

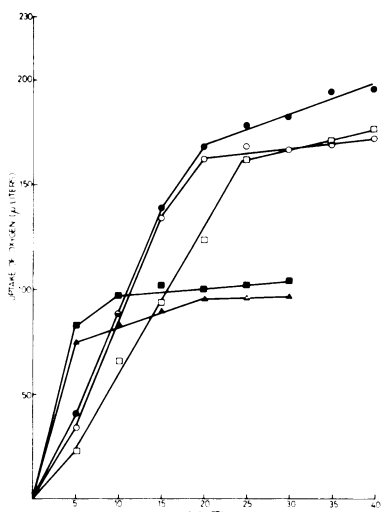

FIG. 1. *Oxidation of aromatic substrates by whole cells of B. stearothermophilus PH24 grown in the presence of phenol. Warburg vessels at 50 C contained in 3 ml: phosphate buffer (pH 7.0), 250 μmol; cell suspension, 14.2 mg (dry weight); and substrate, 3.0 μmol. Oxygen uptakes are corrected for endogenous respiration (equivalent to 35 μl of O_2/30 min). Symbols: ○, phenol; □, o-cresol; ●, m- and p-cresol; ■, catechol, 4-methylcatechol; ▲, 3-methylcatechol.*

TABLE 1. *Oxidation of aromatic compounds by washed cells of B. stearothermophilus PH24*[a]

Substrate	Rate of oxygen consumption[b] by *B. stearothermophilus* PH24 grown in the presence of:		
	o-Cresol	*m*-Cresol	*p*-Cresol
Phenol	0.19	0.27	0.38
o-Cresol	0.17	0.27	0.36
m-Cresol	0.19	0.28	0.34
p-Cresol	0.17	0.27	0.38
Catechol	0.30	0.68	1.34
3-Methylcatechol	0.17	0.59	1.16
4-Methylcatechol	0.35	0.78	1.12
Benzoic acid	0.01	0.02	0.02
Protocatechuic acid	0.01	0.01	0.02

[a] Warburg vessels at 50 C contained in 3 ml; phosphate buffer (pH 7.0), 250 μmol; cells, 18 to 25 mg (dry weight); and substrate, 3.0 μmol.

[b] Rates are expressed as microliters of O_2 consumed per minute per milligram (dry weight) of cells. Oxygen consumption in the absence of substrate (equivalent to 0.14, 0.06, and 0.28 μl of O_2/min per mg [dry weight] of *o*-, *m*-, and *p*-cresol-grown cells, respectively, has been subtracted.

intermediates that showed identical spectral characteristics with those of the respective *meta*-cleavage products, namely 2-hydroxymuconic semialdehyde, 2-hydroxy-6-oxohepta-2,4-dienoic acid, and 2-hydroxy-5-methylmuconic semialdehyde. Cells grown with succinate did not oxidize any of the aromatic substrates.

Oxidation of substrates by cell extracts. Freshly prepared, crude cell extracts oxidized phenol with an uptake of 2.5 mol of O_2 per mol of substrate in reaction mixtures supplemented with NADH (Table 2). NADPH did not serve as a cofactor in this reaction. Extracts that had been stored at 4 C for 3 days or treated with 45 mM H_2O_2 and that showed negligible catechol 2,3-dioxygenase activity catalyzed the conversion of phenol to catechol with a total uptake of 1 mol of O_2 per mol of substrate. The additional 0.5 mol of O_2 per mol of phenol over that required for hydroxylation by a monooxygenase system, followed by ring fission of the catechol intermediate, is accounted for by back oxidation of NADH generated by an NAD-dependent 2-hydroxymuconic semialdehyde dehydrogenase present in the extracts (5). Phenol hydroxylase activity was strictly proportional to protein concentration, except at high dilution (Fig. 2), and no activity was observed in extracts of cells grown with gluconate, fumarate, or succinate.

Cell-free extracts from phenol-grown *B. stearothermophilus* PH24 were found to metabolize not only phenol but also *o*-, *m*-, and *p*-cresol. *p*-Cresol was oxidized with equal efficiency to that observed with phenol as enzyme substrate. Oxidative activity toward the *ortho* and *meta* isomers was 74 and 88%, respectively, compared with the rate with phenol.

Effect of inhibitors on phenol hydroxylase activity. *p*-Chloromercuribenzoate at a concentration of 1 mM depressed hydroxylase activity in crude extracts by about 80%. This inactivation was virtually fully reversed by the addition of stoichiometric amounts of reduced glutathionine. *o*-Phenanthroline (0.1 mM) completely

TABLE 2. *NADH requirement for phenol oxidation by cell-free extracts of B. stearothermophilus*[a]

Pyridine nucleotide added	Rate of oxygen uptake[b]	Total oxygen uptake[c]
None	0	0
NADH	0.58	2.40
NADPH	0	0

[a] Warburg flasks at 50 C contained in a total volume of 3 ml: phosphate buffer (pH 7.6), 180 μmol; reduced pyridine nucleotide, 6.0 μmol; and phenol, 3.0 μmol. Protein (4.6 mg, freshly prepared extract) was added from the side arm.

[b] Expressed as micromoles of oxygen per minute. Oxygen consumption in reaction mixtures without phenol has been subtracted.

[c] Expressed as micromoles of oxygen per micromole of phenol.

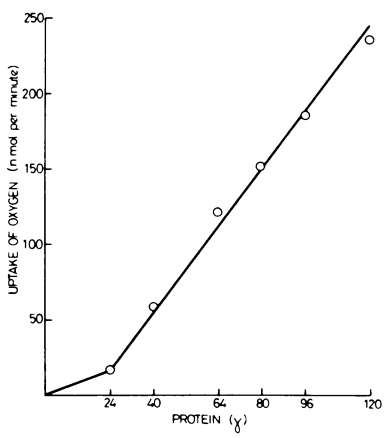

FIG. 2. *Effect of protein concentration on phenol oxidation by cell-free extracts of B. stearothermophilus. The reaction mixture at 50 C contained in 3 ml: phosphate buffer (pH 7.6), 250 μmol; NADH, 1.0 μmol; phenol, 100 nmol; and extract, as indicated. Uptake of oxygen in the absence of phenol has been subtracted.*

abolished the hydroxylation reaction, and slight inhibition was observed with the copper chelator diethyldithiocarbamate (0.1 to 1 mM). Tiron, ethylenediaminetetraacetic acid, azide, arsenite, and KCN at 1 mM concentrations had no significant effect.

Formation of catechol intermediates. The products of phenol hydroxylase activity on phenol and the isomers of cresol were conveniently demonstrated in reaction mixtures containing cell extract (10 mg of protein/ml) previously exposed to 45 mM H_2O_2 for 5 min. This treatment resulted in almost complete inhibition of the ring-fission enzyme (21) but had little effect on the hydroxylase enzyme. Oxygen uptake on phenol and each cresol isomer was followed to completion at 50 C in Warburg vessels containing (in a total volume of 3 ml): 250 μmol of phosphate buffer (pH 7.6), 5 μmol of substrate, 25 μmol of NADH, and H_2O_2-treated extract (3.2 mg of protein). Vessel contents were deproteinized with 10% metaphosphoric acid and extracted three times with equal volumes of ether. After being dried with sodium sulfate and concentrated to 0.5 ml, the fractions were applied to thin-layer plates and developed, as described above. Single spots were observed with the same R_f values as those for catechol, 3-methylcatechol, and 4-methylcatechol from reaction mixtures that originally contained phenol, *o*-cresol, and *m*- and *p*-cresol, respectively.

Further evidence in support of the identification of the catechol and methylcatechol intermediates was obtained from the spectral properties of the compounds produced on the conversion of the catechols into the products of ring fission. Decrease in absorbancy at 340 nm was measured at 50 C in reaction mixtures containing 240 μmol of phosphate buffer (pH 7.6), H_2O_2-treated extract (48 μg of protein), 1.0 μmol of NADH, and 100 nmol of substrate (phenol or one of the isomers of cresol). After no further decrease occurred, the cuvette was cooled to 20 C and, upon the addition of 0.1 ml of a heat-treated extract (55 C for 10 min) of *P. putida* NCIB 10015 grown on phenol, the reaction mixtures turned yellow. The spectral pattern in alkaline, neutral, and acidic conditions of the derivative produced from phenol was identical to that reported for 2-hydroxymuconic semialdehyde, the *meta*-cleavage product of catechol (2). The ring-fission products from *o*-cresol and from *m*- and *p*-cresol showed identical spectral properties to those described for 2-hydroxy-6-oxo-hepta-2,4-dienoic acid and 2-hydroxy-5-methylmuconic semialdehyde, respectively (2).

The stoichiometry of ring-fission product formation is shown in Table 3. Hydroxylase activity was followed in reaction mixtures as described above and, after no further decrease in absorbance occurred at 340 nm, the cuvette was cooled to 20 C to reduce further metabolism of the ring-fission products. Three separate additions of heat-treated extract of *P. putida* (1.2 mg of protein in 0.1 ml) were made over a period of 45, and the absorbance E_{max} was determined. The rates of enzymatic conversion of the products of right fission were extrapolated back to zero time and the amounts formed were calculated from the molar absorption coefficients reported by Bayly et al. (2).

Oxidation of catechol by cell extracts. Crude extracts of *B. stearothermophilus* PH24 grown in the presence of phenol rapidly oxidized catechol via *meta* cleavage with the transient accumulation of a yellow compound with the absorption spectrum of 2-hydroxymuconic semialdehyde. Partially purified catechol 2,3-oxygenase was previously reported to catalyze the stoichiometric conversion of catechol to 2-hydroxymuconic semialdehyde together with a simultaneous uptake of an equimolar amount of oxygen (5). However, crude extracts, supplemented with NAD, required an additional 0.5 mol of oxygen per mol of substrate in addition to that necessary for ring fission (Table 4). The same extracts treated with NADase and without NAD supplementation consumed only 1 mol of oxygen per mol of catechol, and the yellow compound disappeared less rapidly. Further metabolism of 2-hydroxymuconic semialdehyde by a NAD-dependent dehydrogenase and a non-NAD-dependent enzyme present in extracts of *B. stearothermophilus* PH24 was reported earlier (5). Reaction products formed as a result of 2-hydroxymuconic semialdehyde dehydrogen-

TABLE 3. *Formation of ring-fission products from phenol and the isomers of cresol by cell-free extracts of B. stearothermophilus*[a]

Substrate metabolized	Ring-fission product formed[b]
Phenol	0.84
o-Cresol	0.90
m-Cresol	0.86
p-Cresol	0.82

[a] The reaction mixture at 50 C contained in a total volume of 2.7 ml: tris(hydroxymethyl)aminomethane buffer (pH 7.6), 240 μmol; protein, 48 μg; NADH, 1.0 μmol; H_2O_2, 0.33 mM; and substrate, 100, 150, or 200 nmol.

[b] Average of three substrate concentrations expressed as moles per mole of substrate.

TABLE 4. *Total oxygen consumption by untreated and NADase-treated cell-free extracts of B. stearothermophilus in the presence of catechol*[a]

Catechol (nmol)	Oxygen uptake (nmol)	
	Untreated[b]	NADase treatment[c]
300	404	302
200	282	198

[a] The reaction mixture contained in a total volume of 3 ml: phosphate buffer (pH 8.0), 250 μmol; protein (NADase-treated or untreated incubated under the same conditions), 1.5 mg; and catechol. NAD (1.0 μmol) was added to reaction mixtures containing untreated extract.

[b] An initial rapid uptake of 315 nmol and 212 nmol of oxygen was observed followed by a slower rate of consumption to the final value.

[c] One milliliter of cell extract (15 mg of protein) was incubated with 1 ml of NADase (containing 1 U of NADase activity) for 2 h at 37 C.

ase activity were determined spectrophotometrically as γ-oxalocrotonate and NADH (5). Additional oxygen consumption is consistent with back oxidation of the NADH produced by NADH oxidase present in the same extracts. Ring-fission activity was virtually absent from succinate- or fumarate-grown cells, but high levels of enzyme (about a 400-fold increase) were detected in extracts of cells induced by growth on phenol.

The catechol 2,3-oxygenase preparation from mesophilic bacteria is known to be very unstable in the presence of air although low concentrations of organic solvents have been reported to stabilize this enzyme (25, 28). Activity of the ring-fission enzyme in extracts of *B. stearothermophilus* similarly decreased by about 90% after three days at 4 C unless 10% acetone was added to the buffer for protection (Fig. 3).

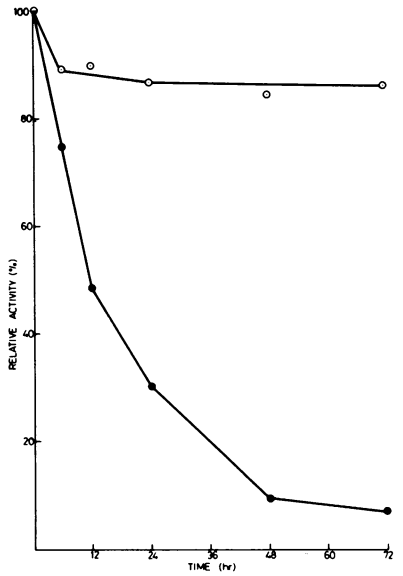

FIG. 3. *Stability of catechol 2,3-oxygenase in extracts of B. stearothermophilus. Cell extracts were prepared as described in the text, and one fraction was supplemented with 10% acetone immediately after cell breakage. The extracts were kept at 4 C, and the activity was measured polarographically at the indicated intervals. Symbols: ●, unsupplemented; ○, supplemented with 10% acetone.*

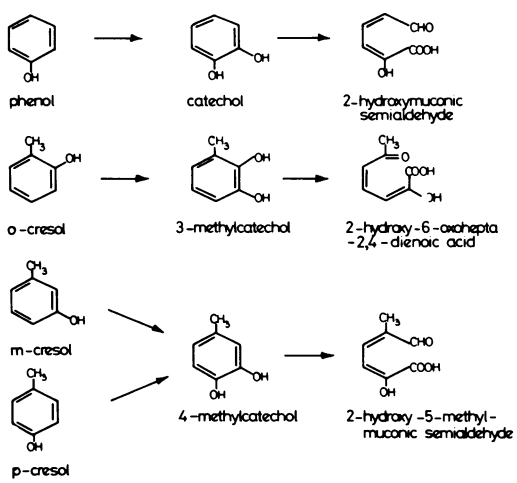

FIG. 4. *Proposed reaction sequence for the metabolism of phenol and the isomers of cresol by B. stearothermophilus.*

DISCUSSION

The initial reactions in the catabolism of phenol and the isomers of cresol by *B. stearothermophilus* depicted in Fig. 4 are consistent with observations made with crude extracts of cells grown in the presence of phenol. The first step in the reaction sequence is an NADH-dependent hydroxylation of the aromatic ring. Thus, phenol is converted into catechol, *ortho*-cresol to 3-methylcatechol, and *meta*- and *para*-cresol to 4-methylcatechol.

To my knowledge, phenol hydroxylase has not been described hitherto in thermophilic bacteria or in members of the genus *Bacillus* although the degradation of aromatics appears to be widespread in this group (8; 30; A. J. Willetts and R. B. Cain, Biochem. J., **120**:28p, 1970). Nakagawa and Takeda (20) have reported that a crude extract of *B. fuscum* carried out an NADPH- or NADH-dependent oxidation of phenol and the isomers of cresol. Activity was inhibited by diethyldithiocarbamate and potas-

sium ethylxanthate, suggesting that cupric ion participated in the reaction. Phenol hydroxylases, strictly dependent on the presence of NADPH, have also been described in extracts of *T. cutaneum* (22) and *C. tropicalis* (23). The enzyme purified from *T. cutaneum* contained a flavine adenine dinucleotide component and required sulfhydryl groups for activity (22). No inhibition by iron and copper chelators was reported, but enzyme activity was highly sensitive to the presence of chloride ion. Phenol hydroxylase from *B. stearothermophilus* was inhibited by 1 mM *p*-chloromercuribenzoate even though dithiothreitol was incorporated into the buffer system used in the preparation of cell extracts. Enzyme activity was also abolished by 0.1 mM *o*-phenanthroline and was reduced to a lesser extent by diethyldithiocarbamate, but chloride ions had no effect. Thus, on the basis of the results presented here, phenol hydroxylase from *B. stearothermophilus* appears to differ in several respects from those previously described.

Degradation of methyl-substituted, aromatic compounds may proceed either by oxidation of the methyl group to carboxyl or by direct hydroxylation of the benzene nucleus to yield a catechol. Where the first reaction in the metabolism of a cresol is hydroxylation to give a methyl-substituted catechol, then *m*-cresol could give rise to either 3-methylcatechol or 4-methylcatechol. Bayly et al. (2) reported that in *P. putida*, strain U, the ring-fission product of catechol was produced from phenol, that of 3-methylcatechol was formed from *o*-cresol and *m*-cresol, and that of 4-methylcatechol was given from *p*-cresol. These observations were confirmed recently by Bayly and Wigmore (3) using mutant strains of *P. putida* deficient in enzymes of the degradative pathways of phenol and cresols. In this respect some substrate specificity was exhibited in the hydroxylation of a cresol insofar as *m*-cresol was metabolized through the ring-fission product of 3-methylcatechol and not of 4-methylcatechol. In this context the results obtained by Sala-Trepat and Evans (28) are important to note. These authors reported the presence in *Azotobacter* species of two different enzymatic activities about to attack 2-hydroxymuconic semialdehyde in crude extracts from benzoate-grown cells, one catalyzing a hydrolytic release of formate from the semialdehyde and the other a dehydrogenation of this compound to 4-oxalocrotonate. However, the low, noninducible levels of 2-hydroxymuconic semialdehyde hydrolase activity appeared negligible for metabolic purposes, and the semialdehyde seemed to be dissimilated almost exclusively via 4-oxalocrotonate by the action of a NAD-dependent dehydrogenase. If, as seemed to be the case, the hydrolytic step was the one functionally significant for the dissimilation of 3-methylcatechol and metabolic precursors, it is interesting to note that all of the *Azotobacter* strains studied were unable to grow on *m*-cresol.

Degradation of the isomers of cresol by cell extracts of *B. stearothermophilus* proceeded via 3-methylcatechol in the case of *o*-cresol, whereas 4-methylcatechol was formed from both *m*- and *p*-cresol. A yellow product, with identical spectral characteristics in both cases, was also formed after extradiol cleavage of the products formed after hydroxylation of *m*- and *p*-ethylphenol by the same extracts (unpublished data). The light absorption maxima at 380 to 381 nm (pH 7.6 and 12.0) were similar to those previously attributed to the *meta*-cleavage product of 4-ethylcatechol (4). Spectral characteristics of the corresponding ring-fission intermediate from *o*-ethylphenol were not obtained because of the very low hydroxylase activity towards this compound, possibly as a result of steric hindrance by the alkyl substituent. Similarly, Nakagawa and Takeda (20) concluded that, in *B. fuscum*, *o*-cresol was metabolized through methylcatechol and showed that the spectra of the ring-fission products of *m*- and *p*-cresol were consistent with that of the *meta*-cleavage product of 4-methylcatechol.

In yeasts, too, Hashimoto (16) presented data that supported the idea that in the case of *m*- and *p*-cresol oxidation by cells of *C. tropicalis* induced by growth on phenol, the formation of 4-methylcatechol by monohydroxylation was dominant or exclusive. Furthermore, hydroxylation of *m*-cresol and *p*-cresol by purified phenol hydroxylase from *T. cutaneum* gave, in both cases, 4-methylcatechol as the reaction product (22).

Recent speculations about the evolutionary origin of the pathways for aromatic catabolism have considered the possibility that the adoption of a particular dissimilative route might serve to illustrate evolutionary relationships (7, 9, 17, 26). Several clear-cut distinctions between taxonomic groups based on the operative mode of aromatic metabolism are already well documented in the literature. It is noteworthy that in reports of *m*-cresol degradation by gram-negative bacteria hydroxylation yields 3-methylcatechol, whereas catabolism in gram-positive species (*Brevibacterium*, *Bacillus*) and in yeasts proceeds via the 4-methyl derivative.

Compared with some other genera, little at-

tention has been given to the mechanisms of aromatic ring fission by *Bacillus* species although recent reports suggest that the ability to convert benzenoid compounds may be widely distributed among members of this group (5, 8, 30; Willetts and Cain, Biochem. J., **120**:28p, 1970). Willetts and Cain (30) reported that the aromatic nucleus obtained from undecylbenzene-*p*-sulfonate was further metabolized in an unidentified *Bacillus* sp. by an oxidation sequence involving the "*ortho*-cleavage" route. A novel reaction involving fission between C2 and C3 of the benzene ring was described recently in a strain of *B. circulans*, which degraded protocatechuic acid (8). In *B. stearothermophilus* PH24, ring fission of the corresponding *o*-diol compounds produced by hydroxylation of phenol and the isomers of cresol proceeded by the *meta*-cleavage route that was catalyzed by catechol 2,3-oxygenase.

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