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# Bacterial Metabolism of *para*- and *meta*-Xylene: Oxidation of a Methyl Substituent

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*Pseudomonas* Pxy was isolated on *p*-xylene as sole source of carbon and energy. Substrates that supported growth were toluene, *p*-methylbenzyl alcohol, *p*-tolualdehyde, *p*-toluic acid, and the analogous *m*-methyl derivatives, including *m*-xylene. Cell extracts prepared from *Pseudomonas* Pxy after growth with either *p*-xylene or *m*-xylene oxidized the *p*- and *m*-isomers of tolualdehyde as well as *p*-methylbenzyl alcohol. The same cell extracts also catalyzed a "meta" fission of both 3- and 4-methylcatechol. Treatment of *Pseudomonas* Pxy with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine led to the isolation of two mutant strains. *Pseudomonas* Pxy-40, when grown on succinate in the presence of *p*-xylene, accumulated *p*-toluic acid in the culture medium. Under the same conditions *Pseudomonas* Pxy-82 accumulated *p*-toluic acid and also 4-methylcatechol. When *Pseudomonas* Pxy-82 was grown on succinate in the presence of *m*-xylene, 3-methylcatechol and 3-methylsalicylic acid were excreted into the culture medium. A pathway is proposed for the initial reactions utilized by *Pseudomonas* Pxy to oxidize *p*- and *m*-xylene.

Bacteria known to grow with *p*- or *m*-xylene as sole carbon source have been shown to oxidize these substrates through the corresponding toluic acids (2, 8, 9). In addition to *p*-toluic acid, *p*-methylbenzyl alcohol was also shown to be an intermediate in the oxidation of *p*-xylene by cell extracts of *Pseudomonas aeruginosa* grown with *p*-xylene as sole carbon source (7).

The identities of the ring-fission substrates formed during bacterial growth with *p*- or *m*-xylene are not well established. Omori and Yamada (11) concluded from simultaneous adaptation data and chromatographic evidence that *p*-xylene is further metabolized via *p*-cresol, *p*-hydroxybenzoic acid, and protocatechuic acid. *P. aeruginosa* S668B2 produced and also oxidized 4-methylcatechol when grown on *p*-xylene, which suggested the presence of an alternative pathway for *p*-xylene degradation (10, 11). A different pathway apparently was utilized by this organism to metabolize *m*-xylene. Although the major product formed from the oxidation of *m*-xylene and *m*-toluic acid was 3-methylsalicylic acid, this metabolite was not further oxidized by *P. aeruginosa* S668B2 (10). Davis et al. (2) showed that *p*-xylene-grown cells of *Pseudomonas* PX oxidized both *p*-xylene and 4-methylcatechol to a compound whose spectral characteristics were identical to those reported for 2-hydroxy-5-methylmuconic semialdehyde. The authors were unable to decide between 3- or 4-methylcatechol as an intermediate in *m*-xylene metabolism since *m*-xylene-grown cells rapidly oxidized both of these compounds to ring-fission products (2).

This communication describes the isolation and identification of 3- and 4-methylcatechol as intermediates in the bacterial degradation of *m*- and *p*-xylene, respectively.

## MATERIALS AND METHODS

**Isolation, growth, and identification of the microorganism.** *Pseudomonas* Pxy was isolated with *p*-xylene as sole carbon source from soil taken from the edge of a polluted creek at Austin, Tex. The composition of the medium and the procedures used to introduce the hydrocarbon growth substrates were as previously described (3). *Pseudomonas* Pxy was characterized by the procedure described by Stanier et al. (15).

**Isolation of mutant strains.** The procedure used was that of Ornston (12). Organisms that would not utilize either *p*- or *m*-xylene as growth substrates were selected as potential mutants. Each mutant strain was grown on mineral salts medium containing 0.2% (wt/vol) succinate. The xylene isomers were introduced in the vapor phase as described previously (3). Products accumulating in the culture filtrates were detected by thin-layer chromatography (see below).

Two mutant strains, *Pseudomonas* Pxy-40 and *Pseudomonas* Pxy-82, were chosen for further study.

**Preparation of cell extracts.** *Pseudomonas* Pxy was grown with either *p*- or *m*-xylene as sole carbon source. Cells were harvested at the end of the log phase of growth. After two washes with 0.02 M $KH_2PO_4$ buffer, pH 7.2, the cells were suspended in 2 volumes of the same buffer and subjected to sonication for 1 min in a Branson sonifier. Unbroken cells and cell debris were removed by centrifugation at 38,000 × *g* for 30 min. The clear supernatant solution was taken as a source of crude cell extract.

Protein was measured according to Lowry et al. (5). Bovine serum albumin was used as a standard.

**Enzyme assays.** 3- and 4-methylcatechol oxygenases were assayed as previously described (14). *p*-Tolualdehyde and *p*-methylbenzyl alcohol dehydrogenases were assayed by measuring the reduction of nicotinamide adenine dinucleotide ($NAD^+$) at 340 nm. A typical reaction mixture (3.0 ml total volume) contained (in micromoles): $KH_2PO_4$ buffer, pH 7.2, 145; $NAD^+$, 1.0; substrate, 0.1 to 1.0 (in ethanol); and cell extract (0.1 to 0.2 mg of protein). Enzymatic activity due to the oxidation of ethanol or NADH was negligible.

**Detection and isolation of metabolic products.** Samples (2.0 ml) of culture filtrate were extracted with 2 volumes of ethyl acetate under both basic and acidic conditions. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed in vacuo at 30 C. The residue was taken up in 0.1 ml of acetone, and 10 μliters was chromatographed on a thin layer of silica gel (Eastman Chromatogram Sheets, type K130R, silicia gel with fluorescent indicator). Solvents used for chromatography were chloroform-acetone (80:20, vol/vol; solvent A) and benzene-acetic acid-water (80:20:saturated, vol/vol; soluent B). Compounds were located on chromatograms by viewing under ultraviolet light and by spraying with either 2,6-dichloroquinone-4-chloroimide (2% in ethanol; Gibb's reagent) or bromocresol green.

When a compound was produced in sufficient quantities for isolation, 10 liters of culture filtrate was extracted with ethyl acetate. After removal of the solvent the residue was either crystallized directly or further purified by sublimation or silica gel column chromatography.

**Analytical methods.** Oxygen consumption was measured polarographically with a Clark oxygen electrode. Ultraviolet spectra were recorded on a Cary model 14 recording spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer model 137 spectrophotometer. Melting points were obtained with a Büchi melting-point apparatus and are uncorrected.

**Chemicals.** *p*-Xylene (99.95%) and *m*-xylene (99.92%) were obtained from Phillips Petroleum Co. 2-Hydroxy-3-methylbenzoic acid (3-methyl-salicylic acid) was a generous gift from P. J. Chapman, Department of Biochemistry, University of Minnesota. *p*-Toluic acid was synthesized according to Vogel (16). Attempts to synthesize *m*-methylbenzyl alcohol by the reduction of *m*-toluic acid were unsuccessful. 3- and 4-methylcatechol were purified by vacuum sublimation before use. All other chemicals were of the highest purity commerically available and were used without further purification.

## RESULTS

**Description of isolate.** The isolated organism was a gram-negative, nonsporeforming, polarly monoflagellated rod that produced a fluorescent pigment and did not liquify gelatin. This organism was considered to be a member of the genus *Pseudomonas* and was designated *Pseudomonas* Pxy.

**Growth of Pseudomonas Pxy on aromatic substrates.** Although *Pseudomonas* Pxy was isolated with *p*-xylene, it grew equally well with *m*-xylene. Other aromatic compounds which supported growth were toluene, *p*-methylbenzyl alcohol, *p*-tolualdehyde, *m*-tolualdehyde, *p*-toluic acid, *m*-toluic acid, benzoic acid, and *p*-hydroxybenzoic acid. Aromatic compounds that did not support growth were benzene, *o*-xylene, *o*-, *m*-, and *p*-cresol, naphthalene, and 1-methylnaphthalene.

**Oxidation of aromatic substrates by whole cells.** Washed-cell suspensions of *Pseudomonas* Pxy grown with *p*-xylene, *m*-xylene, or *p*-methylbenzyl alcohol rapidly oxidized all three growth substrates. The following substrates that are potential intermediates in the degradation of *p*- and *m*-xylene were also oxidized: *p*- and *m*-tolualdehyde, *p*- and *m*-toluic acid, and 3- and 4-methylcatechol (Table 1). Benzene, *o*-xylene, and the three cresol isomers were not oxidized. Benzoic acid and protocatechuic acid were oxidized at a very slow rate. Cells grown on succinate did not oxidize any of the substrates listed in Table 1, with the exception of 3- and 4-methylcatechol; however, the latter two compounds were only oxidized at approximately 10% of the rate observed when *p*- or *m*-xylene was the growth substrate.

Suspensions of *p*-xylene-grown cells (25 mg [dry weight] in 5 ml of 20 mM $KH_2PO_4$, pH 7.2) were incubated with 10 μliters of *p*-xylene. The reaction mixture turned yellow in 10 to 15 min. The cells were removed by centrifugation, and the absorption maxima of the clear supernatant solution were determined under acid and alkaline conditions between 500 to 230 nm. The values obtained (382 nm [pH 11.0] and 320 nm [pH 2.5]) were identical to those reported for 2-hydroxy-5-methylmuconic semialdehyde (1). The latter compound is the product of "meta" fission of 4-methylcatechol. In an analogous experiment, *m*-xylene-grown cells oxidized *m*-xylene to an acidic compound whose absorption

TABLE 1. *Oxidation of aromatic compounds by washed cells of Pseudomonas Pxy*[a]

| Substrate | Rate of oxygen consumption[b] by *Pseudomonas* Pxy grown on: | | |
|---|---|---|---|
| | *m*-Xylene | *p*-Xylene | *p*-Methylbenzyl alcohol |
| Endogenous | 0.11 | 0.09 | 0.06 |
| *m*-Xylene | 0.23 | 0.31 | 0.23 |
| *p*-Xylene | 0.22 | 0.27 | 0.18 |
| *p*-Methylbenzyl alcohol | 0.24 | 0.20 | 0.40 |
| *m*-Tolualdehyde | 0.22 | 0.27 | 0.40 |
| *p*-Tolualdehyde | 0.18 | 0.27 | 0.43 |
| *m*-Toluic acid | 0.13 | 0.24 | 0.40 |
| *p*-Toluic acid | 0.10 | 0.08 | 0.18 |
| 3-Methylcatechol | 1.10 | 1.05 | 0.73 |
| 4-Methylcatechol | 1.45 | 1.06 | 1.13 |
| Benzoic acid | 0.07 | 0.06 | ND[c] |
| Protocatechuic acid | ND | 0.01 | ND |

[a] Oxygen consumption was measured polarographically with a Clark oxygen electrode. Results are corrected for endogenous oxygen consumption in the absence of substrate.

[b] Rates are expressed as micromoles of $O_2$ consumed per minute per milligram (dry weight) of cells.

[c] ND, Not determined.

maxima (388 nm [pH 11.0] and 315 nm [pH 2.5]) were identical to those reported for 2-hydroxy-6-oxoheptadienoic acid, which is the product of "meta" fission of 3-methylcatechol (1).

**Oxidation of aromatic substrates by cell extracts.** Table 2 shows the results obtained in the $NAD^+$-dependent oxidation of *p*-methylbenzyl alcohol and *p*- and *m*-tolualdehyde by cell extracts prepared from cells of *Pseudomonas* Pxy that had been grown on either *p*- or *m*-xylene. Approximately 2 mol of $NAD^+$ was reduced per mol of *p*-methylbenzyl alcohol. When either *p*- or *m*-tolualdehyde was the substrate, 1 mol of $NAD^+$ was reduced per mol of substrate present in the reaction mixture. It was noticed that there was a substrate-dependent lag, in the case of the tolualdehyde isomers, before $NAD^+$ reduction was observed. The results obtained with *p*-tolualdehyde are shown in Fig. 1. Cell extracts, prepared from cells grown with succinate, did not oxidize any of the compounds listed in Table 2.

The components of the reaction mixture described in Table 2 were increased fivefold. After 15 min, 1.0 ml of 5 N $H_2SO_4$ was added to each flask. The precipitated protein was removed by centrifugation, and the clear supernatant solutions were extracted with ethyl acetate. Each organic extract was evaporated to a small volume and analyzed by thin-layer chromatography in solvent B. The results showed that *p*-methylbenzyl alcohol and *p*-tolualdehyde were converted to *p*-toluic acid. *m*-Tolualdehyde was converted to an acid that was chromatographically identical to *m*-toluic acid.

The presence in cell extracts of an enzyme which further oxidized *p*- or *m*-toluic acid was not detected, although several different assay procedures and cell extract preparation methods were tested. A rapid oxidation of both 3- and 4-methylcatechol was observed with cell extracts, irrespective of the xylene isomer used as growth substrate. In contrast, cell extracts

TABLE 2. *Oxidation of p-methylbenzyl alcohol, p-tolualdehyde, and m-tolualdehyde by cell extracts*[a]

| Substrate | Sp act[b] in extracts from cells grown with: | |
|---|---|---|
| | *m*-Xylene | *p*-Xylene |
| *p*-Methylbenzyl alcohol | 0.30 | 0.31 |
| *m*-Tolualdehyde | 0.23 | 0.25 |
| *p*-Tolualdehyde | 0.30 | 0.34 |

[a] Assay conditions were as described, except that 0.20 mg of extract protein was used and 150 nmol of each substrate was added to start the reaction.

[b] Specific activities are expressed as micromoles of $NAD^+$ formed per minute per milligram of protein.

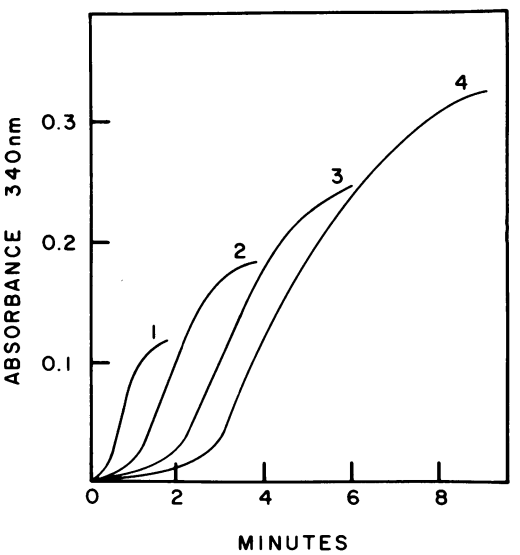


FIG. 1. *Effect of substrate concentration on the $NAD^+$-dependent oxidation of p-tolualdehyde by cell extracts. The assay conditions contained 0.20 mg of extract protein and the following substrate concentrations (in micromoles) added at zero time: 1, 0.05; 2, 0.10; 3, 0.15; 4, 0.20.*

prepared from cells grown with succinate oxidized both catechols at a much lower rate (Table 3).

**Isolation and identification of metabolic intermediates.** When *Pseudomonas* Pxy was grown on either *p*- or *m*-xylene, no metabolic intermediates were detected in the culture filtrate. Treatment of *Pseudomonas* Pxy with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine led to the isolation of two mutant strains, Pxy-40 and Pxy-82, which could no longer utilize either xylene isomer as a growth substrate. When *Pseudomonas* Pxy-40 was grown in 10 liters of succinate medium in the presence of *p*-xylene vapor, an acidic product that absorbed maximally at 234 nm was excreted into the culture medium (Fig. 2). Ethyl acetate extraction of the culture filtrate (pH 2.0) led to the isolation of 3.3 g of a brown solid. The crude product was recrystallized from acetone-water to yield white needles, mp 178 to 179 C. This melting point was not depressed when the bacterial product was mixed with a synthetic sample of *p*-toluic acid. The infrared spectrum of the *p*-xylene metabolite was identical to that given by a synthetic sample of *p*-toluic acid. Further evidence for the identity of the metabolite was provided by its ultraviolet spectrum ($\lambda_{max}^{\text{phosphate buffer, pH 7.5}}$ 234 nm; $\epsilon_{234}$ = 10,500) and chromatographic properties ($R_f$, 0.47 in solvent B). Identical properties were given by synthetic *p*-toluic acid.

When *Pseudomonas* Pxy-40 was grown on succinate in the presence of *m*-xylene, no metabolic products accumulated in the culture filtrate. It was observed that, although this mutant strain would not grow on *p*-xylene or *p*-toluic acid as sole source of carbon, it had not lost the ability to grow with *m*-xylene or *m*-toluic acid.

*Pseudomonas* Pxy-82 was selected for further study due to the observation that colonies, grown on succinate agar in the presence of *m*-xylene, developed brown zones that became more intense on further incubation. Also, *Pseudomonas* Pxy-82 would not utilize either *p*-xylene or *m*-xylene as a carbon source for growth.

When *Pseudomonas* Pxy-82 was grown in 10 liters of succinate-mineral salts medium in the presence of *p*-xylene vapor, a neutral product ($R_f$, 0.43 in solvent A) and an acidic product ($R_f$, 0.47 in solvent B) were excreted into the culture medium. After 24 h the cells were removed by centrifugation, and the supernatant liquid (pH 7.5) was extracted with 3 liters of

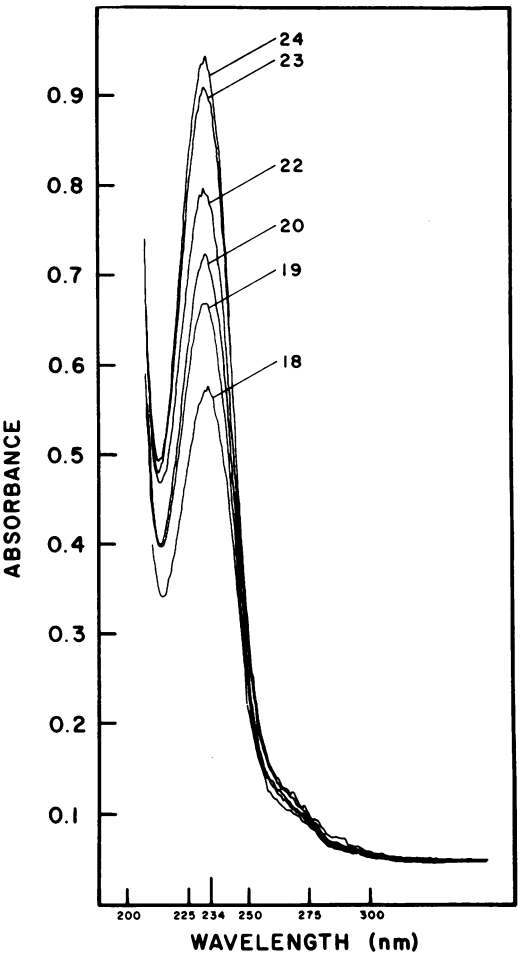


FIG. 2. *Production of p-toluic acid by Pseudomonas Pxy-40. Samples were taken from a 10-liter culture at the times indicated (hours), and, after removal of cells, 0.05 ml of the supernatent solution was diluted to 3.0 ml in 0.02 M $KH_2PO_4$, pH 7.2. Spectra were measured as described in Materials and Methods.*

TABLE 3. *Oxidation of 3- and 4-methylcatechol by cell extracts*[a]

| Substrate | Rate[b] of oxygen utilization by extracts from cells grown with: | | |
|---|---|---|---|
| | *m*-Xylene | *p*-Xylene | Succinate |
| 3-Methylcatechol | 1.80 | 2.41 | 0.034 |
| 4-Methylcatechol | 2.41 | 2.58 | 0.037 |

[a] Oxygen consumption was measured as described in Table 1. The reaction chamber contained in 0.02 M $KH_2PO_4$, pH 7.2 (2.0 ml final volume), 200 nmol of substrate, and 0.10 mg of extract protein.

[b] Rates are expressed as micromoles of $O_2$ consumed per minute per milligram of protein.

ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate, and the solvent was removed to yield 1.10 g of a brown oil. The oil was dissolved in a small volume of chloroform and applied to a silica gel column (16 by 1.0 cm). Fractions (10 ml) were collected by using chloroform as the eluting solvent. Fractions 4 through 12 were pooled, and the chloroform was removed to leave 600 mg of a pale brown oil. A portion of this oil was sublimed under vacuum at 45 C. Large white plates (mp 65 C) were obtained. This melting point was unchanged when the sublimed material was mixed with authentic 4-methylcatechol. The infrared and ultraviolet spectra of the *p*-xylene metabolite were identical to the spectra given by 4-methylcatechol.

The supernatant liquid was acidified to pH 2.0 with 5 N $H_2SO_4$ and extracted as described above. Removal of the ethyl acetate left 1.20 g of a solid residue that was recrystallized from water to give white needles, mp 178 C. This product gave identical infrared and ultraviolet absorption spectra to those given by authentic *p*-toluic acid.

*Pseudomonas* Pxy-82 was grown as described above with *m*-xylene replacing the *para*-isomer. The culture filtrate was acidified to pH 2.0 and extracted with ethyl acetate. Removal of the solvent left 800 mg of a solid residue. Part of the residue was sublimed under vacuum at 45 C. The white plates obtained had a melting point of 66 C, which was unchanged on admixture with an authentic sample of 3-methylcatechol. The $R_f$ value (0.43) in solvent A and the infrared and ultraviolet spectra of the isolated product were identical to those of 3-methylcatechol.

The residue from the sublimation experiment was dissolved in acetone and decolorized with active charcoal. The resulting pale-yellow acetone solution was evaporated to a small volume and allowed to crystallize at room temperature. The white needles that were obtained melted at 168 to 169 C. This melting point was unchanged upon admixture with an authentic sample of 3-methylsalicylic acid. The $R_f$ value (0.47) in solvent B and the infrared and ultraviolet spectra were in total agreement with those of authentic 3-methylsalicylic acid.

When *Pseudomonas* Pxy-82 was grown in the presence of *p*- or *m*-toluic acid, a purple coloration developed in the culture medium. Extraction with ethyl acetate at pH 7.5 and removal of the solvent left a brown, oily residue. The $R_f$ values in solvent A and the reaction with Gibb's reagent of the residues obtained from *p*- and *m*-toluic acid cultures corresponded to those of 4- and 3-methylcatechol, respectively.

## DISCUSSION

The initial reactions in the catabolism of *p*- and *m*-xylene by *Pseudomonas* Pxy are depicted as successive single-step oxidations of one methyl group to form the respective toluic acid (Fig. 3). Whole cells oxidized these substrates without lag after growth on either xylene isomer. Cell extracts catalyzed an $NAD^+$-dependent oxidation of *p*-methylbenzyl alcohol and *p*-tolualdehyde to *p*-toluic acid. Similarly, *m*-tolualdehyde was oxidized to *m*-toluic acid. Growth of the mutant strain, *Pseudomonas* Pxy-40, in the presence of *p*-xylene resulted in the isolation of *p*-toluic acid. All of these observations on the initial reactions in the oxidation of *p*- and *m*-xylene are in agreement with previous reports (2, 7–11). The inability to detect any metabolite when *Pseudomonas* Pxy-40 was grown in the presence of *m*-xylene suggests that different enzyme systems are responsible for the further oxidation of *p*- and *m*-toluic acid.

Whole cells and cell extracts of *p*- or *m*-xylene-grown *Pseudomonas* Pxy rapidly oxidized 3- and 4-methylcatechol. The isolation of 4-methylcatechol as a metabolite of *p*-xylene by *Pseudomonas* Pxy-82 provides strong evidence for its role as the ring-fission substrate in the wild-type organism. This is supported by the observation that *Pseudomonas* Pxy oxidizes both *p*-xylene and 4-methylcatechol to 2-hydroxy-5-methylmunconic semialdehyde. Analogous experiments imply that 3-methylcatechol is an intermediate in the degradation of *m*-xylene by this organism.

*Pseudomonas* Pxy-82 grown in the presence of *m*- and *p*-toluic acid formed products which were chromatographically similar to 3- and 4-methylcatechol, respectively. It has been shown by Murray et al. (6) that resting cells of *Pseudomonas arvilla* grown on *m*- or *p*-toluic acid oxidized these growth substrates to ring-fission products similar to those formed when 3- and 4-methylcatechol, respectively, were the oxidative substrates. A mutant strain of *Alcaligenes eutrophus*, when induced with benzoic acid, oxidized *m*-toluic acid to a product whose spectral characteristics were consistent with the structure 3-methylcyclohexa-3,5-diene-1,2-diol-1-carboxylic acid (13). In addition, an enzyme prepared from the wild-type organism catalyzed the conversion of the latter product to 3-methylcatechol (13). An analogy is drawn from these studies in proposing dihydrodiol intermediates in the conversion of *m*- and

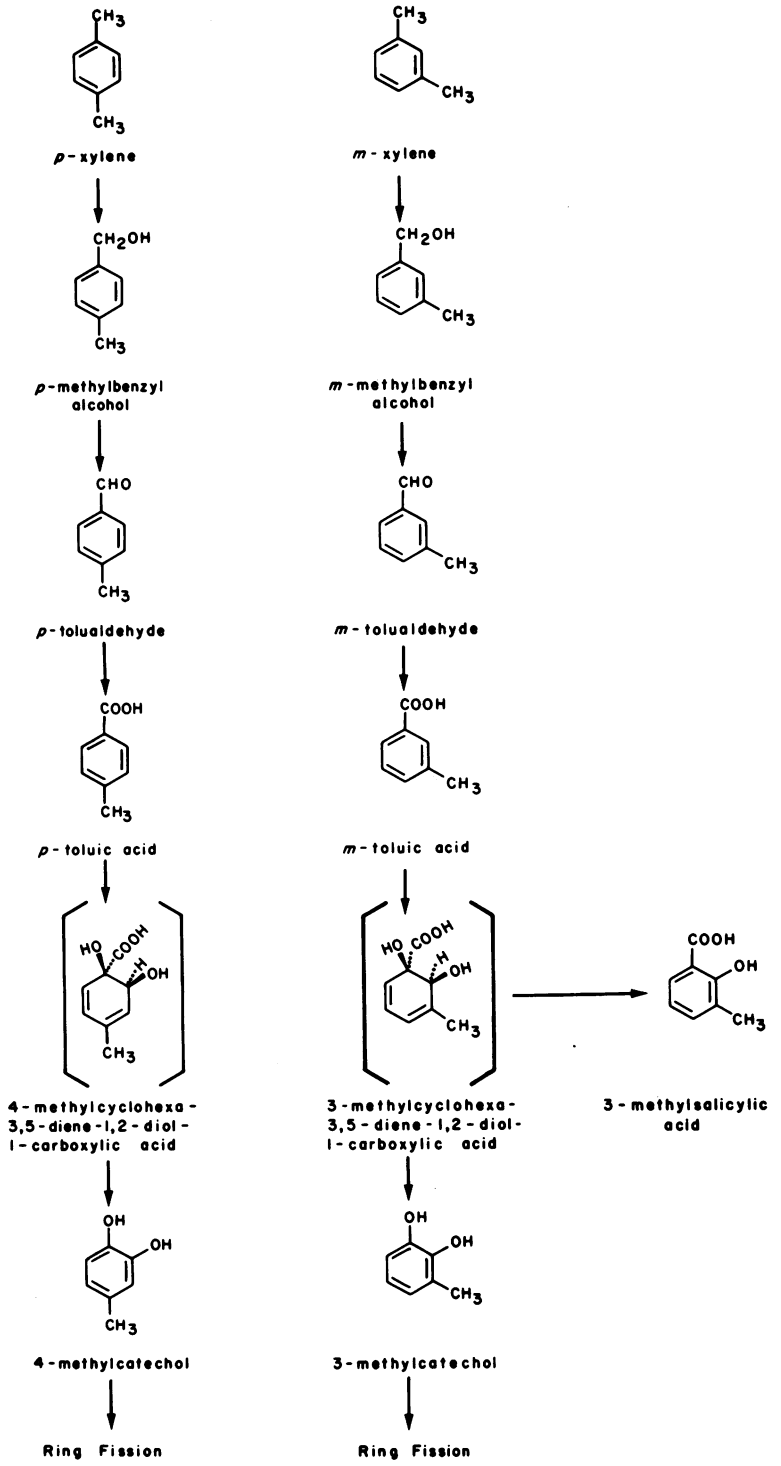


FIG. 3. *Proposed pathways for the oxidation of p- and m-xylene by Pseudomonas Pxy.*

*p*-toluic acid to 3- and 4-methylcatechol, respectively, by *Pseudomonas* Pxy (Fig. 3).

The isolation of 3-methylsalicylic acid as an intermediate in *m*-xylene oxidation could be the result of the acid-catalyzed dehydration of an intermediate such as 3-methylcyclohexa-3,5-diene-1,2-diol-1-carboxylic acid. Although 3-methylsalicylic acid was identified as a metabolite, it was not oxidized by whole cells or cell extracts of *Pseudomonas* Pxy. Similar observations were reported by Omori and Yamada (10), but they did not speculate on the possible origin of this product.

The bacterial metabolism of *p*- and *m*-xylene may also be initiated by direct enzymatic oxidation of the aromatic nucleus. This mode of oxidation is the subject of the following paper (4).

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