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Coexistence of Different Pathways in the Metabolism of *n*-Propylbenzene by *Pseudomonas* sp.

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Pseudomonas desmolytica S449B1 and *Pseudomonas convexa* S107B1 grown on *n*-propylbenzene oxidized *n*-propylbenzene to β-phenylpropionic acid and benzoic acid by initial oxidation of the *n*-propyl side chain and the following β-oxidation, respectively. The same strains also oxidized *n*-propylbenzene to 3-*n*-propylcatechol by initial oxidation of positions 2 and 3 of the aromatic nucleus. A ring fission product, 2-hydroxy-6-oxononanoic acid, was also isolated from the culture broth. Together with the results of oxygen uptake experiments, the data obtained suggested not only the existence of a reductive step to form 2-hydroxy-6-oxononanoic acid, but also the coexistence of two different pathways in the metabolism of *n*-propylbenzene by the strains used.

In a previous report on the oxidation of α- and β-methylstyrenes by *Pseudomonas convexa* S107B1 (15), the authors described how the oxidation of the α-isomer (isopropenylbenzene) was initiated by oxygenation at positions 2 and 3 of the aromatic nucleus and not by oxygenation of the *iso*-alkenyl side chain, whereas initial oxidation of the β-isomer (*n*-propylbenzene) occurred at the methyl group of the *n*-alkenyl side chain and not in the aromatic nucleus. From these results, the possibility was suggested that the initial oxidation of various hydrocarbons may depend upon whether the aromatic nucleus is substituted with normal- or iso-type alkenyl groups.

Further studies on the oxidation of *n*-butylbenzene (12), isobutylbenzene, and isopropylbenzene (13), which were found to be utilized by the same strain of *P. convexa* and also by *P. desmolytica* S449B1, revealed that the initial oxidation by both strains occurred on the benzene nucleus (2 and 3 positions) and not on the *n*-alkyl or iso-alkyl side chain. Since the results obtained seemed to be inconsistent with the presumptions mentioned above, the metabolism of *n*-propylbenzene, an isomer of isopropylbenzene, was further investigated to confirm the initial oxidation of these aromatic hydrocarbons.

As the results presented here show, the metabolism of *n*-propylbenzene by these strains involves at least two different pathways, one through oxygenation of the methyl group of the *n*-propyl side chain and the other through oxygenation of the benzene nucleus (2 and 3 positions); we also describe here the coexistence of two different metabolic pathways in organisms growing with short *n*-alkyl-substituted aromatic hydrocarbons.

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MATERIALS AND METHODS

Cultivation methods. *P. desmolytica* S449B1 and *P. convexa* S107B1, which could grow on *n*-propylbenzene and were characterized in a previous paper (16), were used for study. The composition of the medium used for the isolation of products was the same as reported previously (11), i.e.: NH_4NO_3, 4.0 g; KH_2PO_4, 1.5 g; $Na_2HPO_4 \cdot 12H_2O$, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; yeast extract, 0.5 g; deionized water to make 1 liter. The initial pH was adjusted to 7.0. A liquid culture medium was autoclaved at 115°C for 10 min before cultivation, and *n*-propylbenzene (1.0%, vol/vol) was added as a source of carbon and energy without sterilization. A loopful of cells grown on an *n*-propylbenzene agar slant for 3 to 4 days was inoculated and incubated at 30°C on a reciprocal shaker with a 500-ml flask containing 100 ml of medium. This seed culture was transferred to a 5-liter Erlenmeyer flask containing 1 liter of medium and grown for 2 days on a rotary shaker. For the isolation of products, a 30-liter jar fermentor containing 20 liters of medium (0.5% [vol/vol] substrate concentration) was used under the conditions of 1-volume of air/volume of medium per min aeration and 350-rpm agitation. After 2 days, 25 ml of *n*-propylbenzene was added to replace the hydrocarbon loss by volatilization; the cells were harvested 1 day later. During cultivation, the pH of the medium was not controlled, unless otherwise mentioned. For cultures where pH was controlled, 5 N NH_4OH was used to counter the drop in pH.

Analytical methods. To examine the products of *n*-propylbenzene, 1 liter of culture broth of strains S449B1 and S107B1 grown on *n*-propylbenzene for 2 days was extracted with ether after acidification to pH 2.0. The ether extract was fractionated into strongly acidic, weakly acidic, and neutral fractions as follows: the partition of the ether extract between ether and 5% (wt/vol) $NaHCO_3$ solution (pH 8.6) resulted in the separation of the strongly acidic fraction, which was further extracted with ether after acidification to pH 2.0. The weakly acidic and neutral fractions involved in the residual ether phase were separated by use of 5% (wt/vol) NaOH solution (pH 12.0). The former fraction was transferred to the water phase, which was acidified to pH 2.0 with concentrated HCl. The extraction of the acidified water phase with ether gave the weakly acidic fraction. Throughout the procedure, the neutral fraction was left in the ether phase. The respective ethereal solutions thus obtained were evaporated in vacuo and subjected to thin-layer chromatography (TLC). TLC was performed with commercially available chromatogram sheets (silica gel, 0.25-cm thickness, types S195 and S073, Tokyo Kasei). The solvent system used was benzene-dioxane-acetic acid (90:25:4, vol/vol/vol) (17). Compounds located on chromatograms were observed by spraying the following reagents to detect functional groups: bromocresol green for acidic compounds, diazotized benzidine for phenolic compounds, 2,4-dinitrophenylhydrazine for carbonyl compounds, and potassium permanganate for unsaturated neutral compounds. The presence of *ortho*-dihydroxy compounds was revealed by use of the reagents described by Evans (6). Column chromatography on silicic acid (Silica Gel 60; particle size, 0.063 to 0.200 mm; E. Merck AG, Darmstadt, West Germany) was used without activation before use to separate products, and details are given later. Volatile compounds were also analyzed by gas-liquid chromatography, using a Hitachi K53 gas chromatograph. The operating conditions were as follows: column, Chromosorb 101 (100 cm by 3 mm); oven temperature, 170°C; injection temperature, 230°C; carrier gas, nitrogen (30 ml/min); detector, flame ionization. The instruments used for the analysis of the isolated products (infrared and mass spectra, nuclear magnetic resonance) and the measurement conditions were as reported in previous work (13).

Manometric methods. The amount of oxygen uptake by 0.5 ml of cell suspension (optical density at 660 nm = 1.50; 1.2 mg of dry cells per ml) was determined by conventional constant-volume respirometric procedures at 30°C. Substrate (5 μM) and sufficient 0.06 M potassium phosphate buffer (pH 7.0) were placed in the main compartment of the Warburg flask to give a final volume of 2.0 ml, with cell suspension in the side arm. The center cup contained 0.2 ml of 20% (wt/vol) KOH. Cell suspensions were prepared as follows: the *n*-propylbenzene-grown cultures were centrifuged at 10,000 × *g* for 10 min and washed twice with phosphate buffer to remove hydrocarbon as completely as possible. The washed cells, which still contained a detectable odor of hydrocarbon, were resuspended in phosphate buffer to the indicated cell density (optical density = 1.50). Hydrocarbon was added to the main compartment as 10 μl of an acetone solution (12.0 mg/100 ml), and the acetone was allowed to evaporate before cell suspension was added.

RESULTS

TLC of the ether extract from 1 liter of culture broth revealed mainly one spot in the weakly acidic fraction and several spots in the strongly acidic fraction. No remarkable products were detected in the neutral fraction. Of these, that from the weakly acidic fraction (product 1, R_f = 0.54) and a few components of the strongly acidic fraction (product 2, R_f = 0.75; product 3, R_f = 0.73; product 4, R_f = 0.34; product 5, R_f = 0.90) appeared to be the major products formed. Both strains showed similar spots on TLC, but the amount of product 1 of strain S449B1 seemed to be larger than that of strain S107B1 as judged from the dark brown color development obtained by spraying diazotized benzidine. Thus, strain S449B1 was used for further study.

To obtain more product, strain S449B1 was cultivated in a 30-liter jar fermenter containing 20 liters of medium, and the culture broth was subjected to the following procedure to isolate products. After centrifugation (10,000 × *g* for 10 min) to remove the cells and concentration in vacuo, the concentrated broth (5 liters) was extracted with ether and fractionated to leave 0.20 g of a weakly acidic residue and 3.15 g of a strongly acidic one. The weakly acidic residue (0.12 g) was applied to the silicic acid column (2.5 by 20 cm) and eluted with 300 g of benzene. Fractions of 10 g each were collected with a Toyo SF-200A fractionator. The strongly acidic residue (3.15 g) was also subjected to column chromatography on silicic acid (2.5 by 20 cm) and eluted first with 500 g of benzene, followed by 500 g of $CHCl_3$. Fractions of 10 g each were collected and subjected to TLC to identify those which contained the same product and these were combined. Further purification was achieved by TLC, and the zone corresponding to each product was scraped off. Ether extraction of the scraped zone and evaporation of the solvent gave each product, which was further purified by chromatography on a second silicic acid column (1.0 by 20 cm) with elution, using 300 g of $CHCl_3$. Isolation of product 5, a volatile component, was not possible by this procedure as this component was lost during the in vacuo concentration of the culture broth. An alternative procedure was used to obtain products for gas-liquid chromatographic analysis by extraction of the culture broth (1 liter) with ether after acidification to pH 2.0.

The weakly acidic product 1 isolated from fractions 13 to 26 of the first column chromatography was obtained as colorless crystals (60 mg)

after the second column chromatography. It gave a dark-brown coloration with diazotized benzidine, suggesting the presence of di-phenolic groups. Furthermore, its positive reaction with Evans reagents (6) also supported the presence of dihydroxy phenolic groups in an *ortho* pattern. Its mass spectrum showed ion peaks at *m/e* 152 (M^+), 123 ($M - C_2H_5$), 95 (C_6H_7O), 94 (C_6H_5OH), and 77. Elementary analysis agreed with the formula of $C_9H_{12}O_2$ (found: C, 70.89 and H, 7.98; calculated: C, 71.05 and H, 7.89). These properties are consistent with an *n*-propyl-substituted catechol structure. Its infrared spectrum (Fig. 1) showed a phenolic hydroxy band at 3.0 μm and aromatic CH bands at 12.0, 12.8, and 13.6 μm, which closely resembled that of 3-methyl- or 3-isopropyl-substituted catechol and not that of 4-methyl-substituted catechol. Furthermore, the nuclear magnetic resonance data confirmed the structure of product 1 and established the substitution pattern from the pattern of aromatic proton resonances. Comparison of the aromatic protons (3H) of product 1 with those of 3-methyl- and 4-methylcatechol showed similarity of the chemical shift and spectral pattern (6.65 ppm, singlet) to those of 3-methylcatechol. The aromatic protons of 4-methylcatechol were distinctly different (6.40 to 6.80 ppm, multiplet). Thus, product 1 was identified as 3-*n*-propylcatechol.

Products 2 and 3 were eluted together in fractions 15 to 30 of the benzene eluate of the first column; they gave almost identical R_f values on TLC (0.75 and 0.73, respectively) and were difficult to separate. Separation was achieved, however, by chromatography on the second column using $CHCl_3$. Product 2 (85 mg) was eluted in fractions 3 to 6, whereas product 3 (8 mg) emerged in fractions 12 and 13. Product 2 gave a positive test for acids with bromocresol green but was negative with diazotized benzidine and 2,4-dinitrophenylhydrazine. Its infrared spectrum showed the presence of a mono-substituted aromatic ring (5.9, 13.2, and 14.3 μm). These data suggested that product 2 was an aromatic carboxylic acid, such as β-phenylpropionic acid. Furthermore, the mass spectrum of the methylated product 2 showed a fragmentation completely identical to that of β-phenylpropionic acid methyl ester, indicating ion peaks at *m/e* 164 (M^+), 133 ($M - OCH_3$), 105 ($M - COOCH_3$), 91 ($C_6H_5CH_2$), and 77. Product 3 showed reactions similar to those of product 2 with spray reagents, and its mass spectrum gave ion peaks at *m/e* 122 (M^+), 105 ($M - OH$), and 77 (C_6H_5), which were essentially identical to the mass spectrum of benzoic acid. Confirmation of the structure was shown by its infrared spectrum being identical to that of benzoic acid.

Product 4, eluted in chloroform from the first column, was obtained as a colorless oil (15 mg) by elution from the second column with 300 g of $CHCl_3$. On TLC it gave an acidic reaction with bromocresol green and a weak reaction with 2,4-dinitrophenylhydrazine, with an R_f of 0.34. These properties are similar to those of 2-hydroxy-6-oxodecanoic acid (compound I), a reduced ring fission product of *n*-butylbenzene formed by the same strains (12). Indeed, the mass spectrum of product 4 showed a fragmentation analogous to that of compound I. A molecular ion peak was not detected, and prominent ion peaks were observed at *m/e* 170 ($M - H_2O$), 150, 126, 99 ($M - H_2O - C_3H_7CO$), 91, and 71 (C_3H_7CO). The nuclear magnetic resonance spectrum of product 4 was also analogous to that of compound I, except for the diminution of 2H (methylene protons) at δ1.35 to 1.70. Signals were observed at $\delta^{CDCl_3}_{Me_4Si}$ 0.95 (3H, triplet, $-CH_2CH_3$), 1.35 to 1.70 (2H, multiplet, $-CH_2CH_2CH_3$), 1.80 (8H, multiplet, four methylenes), 4.46 [1H, triplet, $-CH_2CH(OH)-$], and 5.80 to 6.50 (1H, broad singlet, OH, shifted to a high field with an increase in measurement tem-

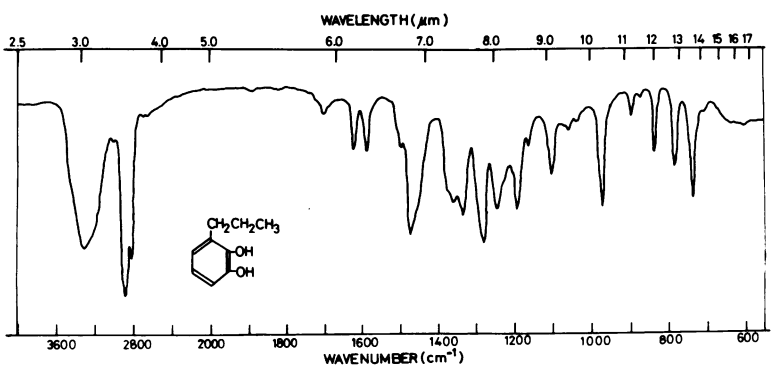

FIG. 1. *Infrared spectrum of product 1 (in Nujol).*

perature). A carboxylic proton could not be assigned in this spectrum. The nuclear magnetic resonance spectrum of the acetylated product 4 further confirmed the structure. As shown in Fig. 2 signals analogous to those of acetylated compound I were observed: $\delta^{CDCl_3}_{Me_4Si}$ 0.94 (3H, triplet, $-CH_2CH_3$), 1.60 (2 H, multiplet, $-CH_2CH_2CH_3$), 1.80 (4H, multiplet, two methylenes), 2.12 (3H, singlet, $-OCOCH_3$), 2.44 (4H, double triplet, $-CH_2CH_2COCH_2CH_2-$), 5.00 [1H, triplet, $-CH_2CH(OCOCH_3)$, J = 6 to 7 Hz], 8.30 (1H, broad singlet, exchangeable, COOH). Spin decoupling of the methylene protons at δ 1.80 changed the triplet at δ 5.00 due to a methine proton to a singlet. These properties are entirely consistent with the structure of product 4 as 2-hydroxy-6-oxononanoic acid.

Product 5, known to be a volatile compound with an unpleasant odor, gave an acidic reaction with bromocresol green on TLC. To obtain more of this product for the examination of the strongly acidic fraction of an ether extract, 1 liter of culture broth was examined by gas-liquid chromatography as described earlier. On gas-liquid chromatography, a major peak was observed with a retention time of 8.0 min, identical to that of authentic *n*-butyric acid. Under the same conditions, the following retention times were observed for related compounds: propionic acid, 3.2 min; isobutyric acid, 5.1 min; isovaleric acid, 10.0 min; and *n*-valeric acid, 13.2 min. Thus, product 5 was identified as *n*-butyric acid.

To examine the initial oxidation in the degradation of *n*-propylbenzene, oxygen uptake was measured with cells of strain S449B1 grown with *n*-propylbenzene. Products 1, 2, and 3 were used in this experiment, but insufficient amounts of product 4 were recovered to test. The results are shown in Fig. 3. The strain oxidized β-phenylpropionic acid (product 2) at a rate equal to that of the substrate (*n*-propylbenzene). 3-*n*-Propylcatechol (product 1) was also readily oxidized, and the total volume of the oxygen uptake was nearly equal to that of β-phenylpropionic acid. Furthermore, benzoic acid (product 3) was oxidized at a rate that increased with time, suggestive of enzyme induction, until its rate of oxidation was faster than that of *n*-propylbenzene. These results showed that products 1, 2, and 3 were oxidized by *n*-propylbenzene-grown cells.

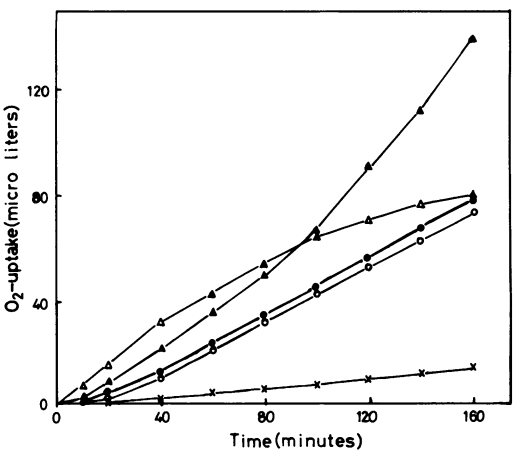

FIG. 3. *Oxygen uptake of n-propylbenzene-grown P. desmolytica S449B1. Symbols:* ○, *n-propylbenzene;* △, *3-n-propylcatechol;* ●, *β-phenylpropionic acid;* ▲, *benzoic acid;* ×, *endogenous respiration.*

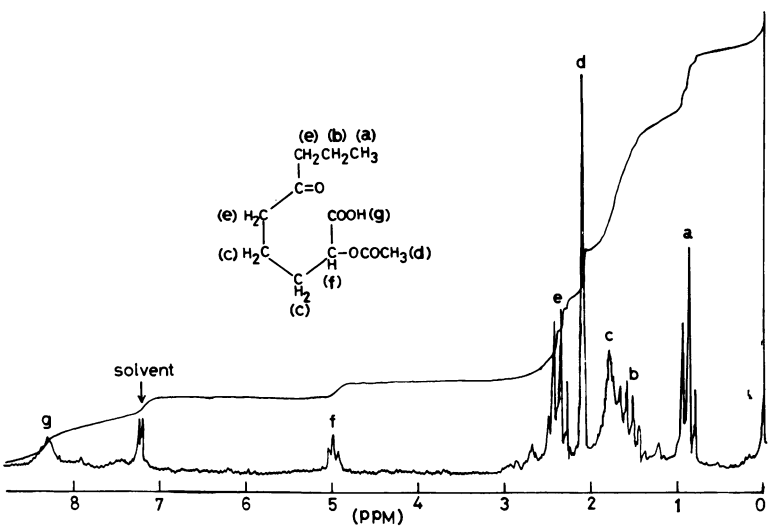

FIG. 2. *Nuclear magnetic resonance spectrum of the acetylated product 4 (in* $CDCl_3$*).*

The structures of the isolated products and the fact that they are readily oxidized are consistent with degradation of *n*-propylbenzene via at least two different pathways, each of which is initiated by a different oxidation of the alkylbenzene. One pathway is initiated by terminal oxidation of the *n*-propyl side chain to form β-phenylpropionic acid, which is further metabolized to benzoic acid (Fig. 4). The other is initiated by an attack at C-2 and C-3 of the aromatic nucleus to form 3-*n*-propylcatechol, which is further metabolized to form the ring fission product shown in brackets. This compound is assumed to be metabolized mainly by a hydrolytic cleavage to form *n*-butyric acid, which is detected on gas-liquid chromatography. The ring fission compound can also undergo reduction to 2-hydroxy-6-oxononanoic acid which is presumed not to be metabolized further (our unpublished data).

DISCUSSION

Information on the bacterial degradation of alkylbenzenes with short side chains having two to five carbon atoms is not extensive. Microorganisms able to grow with these hydrocarbons were not isolated until recently. A number of products derived from these hydrocarbons by so-called co-oxidation have been described, however. Ethylbenzene was oxidized to phenylacetic acid by *Nocardia salmonicolor* (5). *n*-Propylbenzene was also converted to cinnamic acid or benzoic acid by *Nocardia* sp. grown on hexadecane in the presence of *n*-propylbenzene (5). These compounds, however, were resistant to further degradation by the microorganisms used. Accordingly, experiments using microorganisms able to grow with these hydrocarbons were expected to reveal the entire catabolic pathways for these short *n*-alkyl-substituted aromatic hydrocarbons.

In this connection the report of the oxidation of ethylbenzene by *P. putida*, which can grow on this hydrocarbon, is noteworthy (8). This work described a pathway of ethylbenzene metabolism that was initiated by oxygenation at positions 2 and 3 of the benzene nucleus to form (+)-*cis*-3-ethyl-3,5-cyclohexadiene-1,2-diol and was converted subsequently to 3-ethylcatechol. A minor oxidation pathway was also described which involved benzylic hydroxylation of ethylbenzene to form (+)-*cis*-3-(1'-hydroxyethyl)-3,5-cyclohexadiene-1,2-diol.

We describe in this work the alternative pathways which operate in the catabolism of *n*-propylbenzene by *P. desmolytica*. One is initiated by oxidation of the methyl group of the *n*-propyl side chain, followed by β-oxidation to form benzoic acid in a manner analogous to that reported on the oxidation of long *n*-alkyl-substituted aromatic hydrocarbons (11, 20). The other is by oxidation of the 2 and 3 positions of the aromatic nucleus, followed by *meta* cleavage in a fashion similar to that reported for 2-phenylbutane and 3-phenylpentane degradation (1). The compound isolated from our cultures which most closely resembles the ring fission product is 2-hydroxy-6-oxononanoic acid, a compound presumed to arise by reduction of the immediate product of ring fission. The isolated compound resembles compounds previously reported as products of *n*-butylbenzene-isobutylbenzene and isopropylbenzene catabolism by the same strains (12, 13). These reduced products are presumably end products since no further metabolism could be shown with cell extracts or intact cells. The mechanism of formation of these compounds will be described elsewhere.

The benzylic oxidation of the methylene group of the ethyl substituent of ethylbenzene appears to be of minor importance as judged by oxygen consumption measurements (8). It was concluded by Gibson et al. (8) that the enzyme responsible for oxidation of the ring may have been responsible for oxidation of the adjacent methylene group. Our results suggest a different picture. Both pathways of *n*-propylbenzene degradation appear to be of equal importance in the microorganisms studied because the strains simultaneously oxidized the isolated intermediates of both pathways at almost equal rates. Furthermore, it was assumed that the enzymes responsible for the oxidation of the aromatic nucleus were not those responsible for the oxidation of the *n*-propyl side chain. The former system we presume to be of the di-oxygenase type, possibly forming a cyclic peroxide intermediate (7), whereas the latter system may be similar to the mono-oxygenase system, which converts terminal methyl groups to primary alcohols (14). The two enzyme systems are assumed to be different in properties and function.

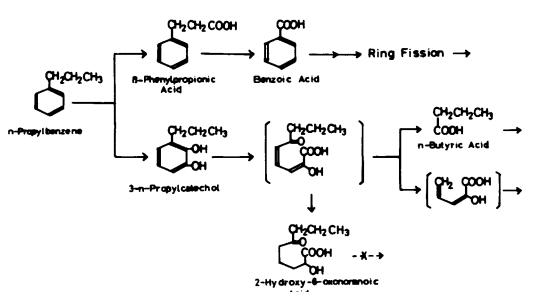

FIG. 4. *Proposed metabolic pathway of n-propylbenzene by P. desmolytica and P. convexa.*

It is useful to point out that a *p*-xylene oxidation reported by Raymond et al. (9, 10, 18, 19) also involves two different pathways. A species of *Nocardia* grown on *n*-hexadecane as a sole carbon source converted *p*-xylene to oxidized compounds which were resistant to further degradation. The nature of the oxidation products formed was a function of the pH of the medium. Thus, at pH 8.0 methyl group oxidation predominates and primarily *p*-toluic acid and dihydroxy-*p*-toluic acid are formed. At pH 6.5 direct ring hydroxylation becomes the more important route and a methyl-substituted muconic acid accumulates as a consequence of cleavage of the dihydroxylated ring.

Because the results referred to above may account for the oxidation routes of *n*-propylbenzene studied here, we examined the products formed at different culture pH values, namely, pH 7.5 and 6.0. Essentially the same pattern of product accumulation was found for the two sets of conditions. Thus, the two pathways of *n*-propylbenzene metabolism appear to be of equal importance in organisms growing at either pH 6.0 or 7.5.

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