Metabolism of Styrene by *Rhodococcus rhodochrous* NCIMB 13259

A. MICHAEL WARHURST,1 KENNETH F. CLARKE,2 ROBERT A. HILL,3 ROBERT A. HOLT,4 AND CHARLES A. FEWSON1*  

Departments of Biochemistry1 and Chemistry, 3 University of Glasgow, Glasgow G12 8QQ, Scotland, and BCRA  
Scientific and Technical Services Ltd., Wingerworth, Chesterfield, Derbyshire S42 6NG, 2 and  
Zeneca Bio Products, Billingham, Cleveland TS23 1YN, 4 United Kingdom  

Received 12 October 1993/Accepted 21 January 1994

*Corresponding author. Phone: 041 330 4835. Fax: 041 330 4620.  
Electronic mail address: c.a.fewson@udcf.gla.ac.uk.

*Rhodococcus rhodochrous* NCIMB 13259 grows on styrene, toluene, ethylbenzene, and benzene as sole carbon sources. Simultaneous induction tests with cells grown on styrene or toluene showed high rates of oxygen consumption with toluene cis-glycol and 3-methylcatechol, suggesting the involvement of a cis-glycol pathway. 3-Vinylcatechol accumulated when intact cells were incubated with styrene in the presence of 3-fluorocatechol to inhibit catechol dioxygenase activity. Experiments with 18O2 showed that 3-vinylcatechol was produced following a dioxygenase ring attack. Extracts contained a NAD-dependent cis-glycol dehydrogenase, which converted styrene cis-glycol to 3-vinylcatechol. Both catechol 1,2- and 2,3-dioxygenase activities were present, and these were separated from each other and from the activities of cis-glycol dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase by ion-exchange chromatography of extracts. 2-Vinylmuconate accumulated in the growth medium when cells were grown on styrene, apparently as a dead-end product, and extracts contained no detectable muconate cycloisomerase activity. 3-Vinylcatechol was cleaved by catechol 2,3-dioxygenase to give a yellow compound, tentatively identified as 2-hydroxy-6-oxoocta-2,4,7-trienoic acid, and the action of 2-hydroxymuconic acid semialdehyde hydrolase on this produced acrylic acid. A compound with the spectral characteristics of 2-hydroxyxypenta-2,4-dienoate was produced by the action of 2-hydroxymuconic acid semialdehyde hydrolase on the 2,3-cleavage product of 3-methylcatechol. Extracts were able to transform 2-hydroxyxypenta-2,4-dienoate and 4-hydroxy-2-oxopentanoate into acetaldehyde and pyruvate. These results show that *R. rhodochrous* NCIMB 13259 metabolizes styrene by dioxygenation to give styrene cis-glycol, followed by dehydration to form 3-vinylcatechol, which undergoes nonproductive ortho cleavage and complete metabolism by a meta-cleavage pathway.

Styrene (phenylethene, phenylethylene, or vinylbenzene) is a major industrial chemical. It has many uses, such as the manufacture of polystyrene, plastics, and styrene-butadiene rubbers. It is one of the most important aromatic chemicals produced by industry, with 3.64 × 109 kg manufactured in 1990 in the United States alone (16). It is a colorless, volatile, strong-smelling, aromatic compound which is only slightly soluble in water. The release of man-made styrene into the environment can occur by a variety of routes, including factory wastewater, evaporation, and the pyrolysis of polystyrene. In nature, styrene is usually produced by the decarboxylation of cinnamic acid, a common plant acid (22). *Penicillium caseiolum* can synthesize styrene, though the route of synthesis is not known (30). Substituted styrenes are found in wine, beer, soy sauce, and blueberries (2).

The fate of styrene in the environment has been studied by Fu and Alexander (16), who detected rapid mineralization of styrene in sewage, neutral mineral soil, and organic soil. The lack of any mineralization in sterile soil showed that styrene breakdown was undertaken by microorganisms. Initial attempts to isolate organisms that could grow on styrene were unsuccessful, probably because the concentrations of styrene used were generally so high that they would be toxic. More recently, it has been shown that it is possible to isolate large numbers of styrene-utilizing organisms from soil if the substrate concentration is kept low; for example, Hartmans et al. (21) isolated 14 strains of aerobic bacteria and 2 fungal strains from normal soils by using a styrene concentration of about 0.5 mM. Styrene can also be degraded anaerobically, by microbial consortia (18).

Aerobic breakdown of styrene must initially proceed via an attack on either the aromatic ring or the side chain. No pathway involving initial ring attack has yet been proven, although (−)-1,2-dihydroxy-3-ethyl-3-cyclohexene has been isolated from the medium of *Pseudomonas putida* MST growing on styrene (6). Ring attack has also been suggested in other studies, for example, in *Xanthobacter* strain 124X (20).

There are several possible methods for attacking the vinyl side chain. The initial reaction could be a monooxygenation to give styrene oxide, as in the human liver (25), which could then be reduced to either 1- or 2-phenylethanol, hydrolyzed to 1,2-phenylethanaldehyde, or isomerized to phenylacetaldehyde (phenylethanol). Alternatively, water could be added across the vinyl group to give, again, either 1- or 2-phenylethanol. The side chain could also be reduced, to give ethylbenzene. *P. putida* R1 has been found to produce phenylethanaldeoi and mandelic acid from styrene (27). However, the majority of organisms investigated so far have been found to use a pathway that converts styrene to 2-phenylethanol or phenylacetaldehyde, probably via styrene oxide, followed by further oxidation to give phenylacetic acid (phenylethanoic acid). The most detailed enzymological investigation of styrene breakdown through phenylacetic acid has been undertaken by Hartmans et al. (20, 21). This group found that *Xanthobacter* strain 124X was able to degrade styrene oxide and 2-phenylethanol via phenylacetaldehyde and phenylactic acid, though there was no evidence of styrene itself being degraded by this route. Styrene oxide was isomerized to phenylacetaldehyde by a novel enzyme, styrene oxide isomerase, which required no cofactors (20). Hartmans et al. (21) later isolated 14 strains of bacteria...
which could utilize styrene, 4 of which were gram negative and the rest of which were gram positive. Extracts of 11 of these strains had a flavin adenine dinucleotide-requiring styrene monooxygenase activity, which transformed styrene to styrene oxide. One of the isolates, strain S5, was characterized further and was found also to possess styrene oxide isomerase. The yeast Exophiala jeanselmei also seems to use a pathway through styrene oxide and phenylacetic acid to metabolize styrene (11). There appear to be no reports of bacteria which can degrade styrene through side chain attack to 1-phenylethanol, though some bacteria are known to be able to metabolize this substrate. Cripps et al. (12) established two pathways of 1-phenylethanol oxidation, one through an initial oxidation of the side chain to give acetoephone and the other through an initial attack on the ring.

In this paper, we describe what we believe to be a novel pathway for the metabolism of styrene by Rhodococcus rhodochrous NCIMB 13259, involving an initial oxidation of the aromatic ring followed by meta cleavage.

**MATERIALS AND METHODS**

**Isolation and growth of microorganism.** R. rhodochrous NCIMB 13259 was isolated from an industrial chemical dump by enrichment culture with approximately 1 mM styrene as the sole source of carbon and energy. Identification was performed by the National Collections of Industrial and Marine Bacteria Ltd. (Aberdeen, United Kingdom [U.K.]).

The standard salts medium consisted of 2 g of KH₂PO₄ and 1 g of (NH₄)₂SO₄ in 1 liter of distilled water, adjusted to pH 7.0. After autoclaving, 20 ml of a 2% (wt/vol) sterile MgSO₄ · 7H₂O solution was added per liter, unless an iron supplement was needed, when 20 ml of a solution containing 2% (wt/vol) MgSO₄ · 7H₂O and 0.22 g of FeSO₄ (this solution had been adjusted to pH 2.0 and autoclaved) was added per liter.

Cultures using volatile substrates were grown in gas-tight screw-top flasks (Scotlab, Bellshill, Scotland) in salts medium, with the substrate generally added at a final concentration of 1 mM, though 0.5 mM was also used for the growth tests. Nonvolatile substrates were usually added at 5 mM, though a concentration of 0.5 mM was also tested in growth experiments. The inoculum was usually 5 ml of a nutrient broth culture for each 100 ml of medium. For growth on substrate concentrations of more than 1 mM, an iron supplement was routinely added.

For larger cultures, R. rhodochrous NCIMB 13259 was grown in rice-tissue medium containing 50 mM MES (morpholineethanesulfonic acid) buffer, to control the pH, in a 10-liter Biostat V fermentor (B. Braun, Melsungen AG, Germany). All tubing exposed to styrene vapor was solvent-resistant Viton (BDH, Poole, Dorset, U.K.). Salts medium (9.5 liters) was autoclaved in the fermentor. A supplement containing 106.6 g of MES, 2 g of MgSO₄, and 20 ml of a chelated metals solution (5) was prepared in 500 ml of distilled water and adjusted to pH 7.0; this solution was membrane filtered and added to the fermentor after autoclaving. The inoculum was prepared by growing cells on 1 mM styrene in a 500-ml gas-tight flask with 100 ml of salts medium and a nutrient broth inoculum of 10 ml. After 2 days of growth, the contents of the flask, which had been shaken at 200 rpm at 30°C, were added to the fermentor. The medium was stirred, and air was bubbled through at 4 liters/min. In addition to the main air supply, 200 to 400 ml/min was bubbled through styrene before entering the fermentor, thus providing the styrene substrate in the vapor phase. The bacteria in the fermentor were usually harvested after 6 days of growth at 30°C.

In order to obtain higher yields of cells grown in the presence of styrene, the following medium was used: 130 g of nutrient broth, 20 g of KH₂PO₄, 10 g of (NH₄)₂SO₄, 4 g of MgSO₄ · 7H₂O, 104.4 g of MES buffer, 20 ml of chelated metals solution, and 0.5 ml of antifoam poly(propylene glycol) 2025, all in 10 liters of distilled water, with the pH adjusted to 7.0 with 5 M NaOH. The inoculum was 500 ml of a 3-day-old nutrient broth culture. After 48 h of growth on the nutrient broth-based medium, styrene was provided in the air supply as described above. After a further 24 h, the bacteria were harvested.

**Suspensions of washed cells and extracts.** Cells were harvested by centrifugation in an MSE (Crawley, U.K.) high-speed 18 centrifuge with a fixed-angle rotor (6 by 250 ml) at 8,000 x g for 20 min. For very large volumes, an MSE Mistral was used, with a 6-liter rotor, at 6,000 x g for 30 min. The pellet was then washed three times by resuspending it in ice-cold 50 mM phosphate buffer, pH 7.0. Pellets of bacteria were stored at −20°C.

For the preparation of extracts, the cells were resuspended in degassed PEG (pH 7.2), which contained 50 mM phosphate buffer, 10% glycerol, and 10% ethanol (33). The cell suspension was then pressed between three and five times in an Amino French pressure cell press (SLM Instruments Inc., American Instrument Co., Urbana, Ill.) at 117 MPa. The homogenate was centrifuged at 12,000 x g in a Beckman model J2-21 centrifuge for 10 min, and the supernatant was removed and centrifuged at 145,000 x g for 1 h in a Beckman LS-65 ultracentrifuge with a type 65 rotor. This supernatant was used as the extract and was stored at −20°C. When specified, extract was treated for 5 min at 55°C and any precipitated protein was centrifuged off, to give heat-treated extract.

Protein concentrations were estimated by a modification of the Bradford (8) method. Samples were digested with NaOH (final concentration, 0.67 M) overnight at 37°C before the protein analysis was performed.

**Oxygen consumption.** Simultaneous induction experiments were carried out with a Clark-type oxygen electrode obtained from Rank Brothers (Cambridge, U.K.). The electrode was kept at 30°C. The well of the oxygen electrode was filled with 2.8 ml of aerated 50 mM phosphate (pH 7.0) buffer and 100 μl of cell suspension (in 50 mM phosphate buffer [pH 7.0] with a protein concentration between 0.5 and 5.0 mg/ml), and the rate of oxygen consumption without substrate (the endogenous consumption) was then measured over at least 5 min. The substrate, either 100 μl of a 10 mM solution (final concentration, 0.33 mM) or, for sparingly soluble substrates, 100 μl of a saturated solution, was then added. The rate of oxygen consumption was then measured over at least 5 min. The difference between this rate and the endogenous rate was taken to be the increase in oxygen consumption due to the substrate. The oxygen electrode was calibrated by use of the oxidation of known concentrations of NADH by N-methylphenazonium methosulfate (26).

**Production, extraction, and analysis of intermediates.** The production of styrene cis-glycol was done with P. putida UV4, a mutant strain containing tolulene dioxygenase but lacking toluene cis-glycol dehydrogenase. P. putida UV4 was grown in a pyruvate-salts medium; cells were harvested, resuspended in phosphate buffer with ethanol as a source of reducing equivalents, and placed in a center-well baffled flask. Styrene was placed in the center well. The transformation was done at 28°C on an orbital shaker. Styrene cis-glycol was extracted with ethyl
acetate and then crystallized from cold n-hexane. The structure of the styrene cis-glycol was confirmed by both $^1$H and $^13$C nuclear magnetic resonance (NMR) analyses (Fig. 1a), with the results in close agreement with those obtained for styrene cis-glycol produced by P. putida 39D (23). Although these NMR data could not distinguish between styrene cis-glycol and styrene trans-glycol, it is very unlikely that the trans isomer would be formed by P. putida UV4, since other workers have found that bacterial dioxygenases produce cis-glycols (34). Styrene cis-glycol was stored in ethyl acetate with 0.1% triethylamine at 4°C and was generally used as a solution in ethyl acetate. If larger quantities were used, then it was extracted from the ethyl acetate into 50 mM phosphate buffer, pH 7.6.

Production of vinylcatechol was done in Thunberg tubes, which contained 3 ml of buffer (50 mM phosphate, pH 7.6), NAD (12 μmol), and cis-glycol (approximately 3.6 μmol, as 100 μl of a 5-mg/ml solution in ethyl acetate) in the test tube portion and 1.2 ml of extract from cells grown on nutrient broth-styrene (4.7 mg of protein per ml) in the bulb. The tubes were repeatedly evacuated and then refilled with oxygen-free nitrogen. The contents were then mixed, and the tubes were incubated at 30°C for 30 min. The reaction was stopped with 300 μl of 1 M HCl, and the reaction mixture was then centrifuged to remove precipitated protein. The supernatant was then extracted with ethyl acetate or diethyl ether.

For the production of intermediates by whole cells in the presence of 3-fluorocatechol, intact frozen cells that had been grown on nutrient broth-styrene were suspended in phosphate buffer at 1 g (wet weight)/100 ml in a gas-tight shake flask. 3-Fluorocatechol was added at a final concentration of 2 mM, along with 20 μl of styrene; then the flask was incubated at 30°C on an orbital shaker for 2 h. The reaction was stopped by acidification, the cells were removed by centrifugation, and the medium was extracted with ethyl acetate.

**Use of $^{18}$O$_2$ to determine the nature of oxygenase attack.** Two 50-ml sidearm flasks had 19 ml of buffer (50 mM phosphate, pH 7.0) placed in them, with 0.4 g of cells which had been grown on nutrient broth-styrene. 3-Fluorocatechol was added to each flask, 0.5 mg in 1 ml of buffer per flask (final concentration, 2 mM). Suba seals (BDH) were used to seal both flasks, and they were evacuated and flushed with oxygen-free nitrogen. This was repeated three times, and the flasks were evacuated again. The control flask was injected through the Suba seal with 20 ml of $^{18}$O$_2$, and the remaining vacuum was filled by oxygen-free nitrogen gas. The other flask was injected with 20 ml of an approximately 50:50 mixture of $^{18}$O$_2$ and $^{16}$O$_2$; then oxygen-free nitrogen was allowed in to release the partial vacuum. Both flasks then had 10 μl of styrene added to them through the Suba seals, and they were placed on an orbital shaker at 30°C for 2 h, after which the flasks were acidified and chilled. The contents were centrifuged to remove the precipitated protein, and the supernatants were each extracted with ethyl acetate.

**Enzyme assays.** All assays were done at 30°C, using extracts prepared in PEG buffer from cells which had been grown on nutrient broth-styrene (induced extract) or on nutrient broth (uninduced extract).

The cis-glycol dehydrogenase activity was assayed by using a reaction mixture consisting of 2.85 ml of 50 mM phosphate buffer (pH 7.0), 50 μl of extract, and 50 μl of 7 mM tolune cis-glycol or 5 μl of approximately 9 mg of styrene cis-glycol per ml of ethyl acetate. The reaction was started with 100 μl of 50 mM NAD. The rate of production of NADH was measured at 340 nm, and rates were calculated by using $ε = 6.3 \text{ mM}^{-1} \text{ cm}^{-1}$ (7).

Catechol 1,2-dioxygenase activity was measured by using a reaction mixture consisting of 2.85 ml of 50 mM phosphate buffer (pH 7.0) and 50 μl of extract, and the reaction was started by the addition of 100 μl of a 3 mM solution of the substrate in buffer, giving a final concentration of 0.1 mM (17). EDTA was added, when specified, at a final concentration of 1.3 mM. The reaction was monitored by measuring the accumulation of the 1,2-cleavage products at 260 nm. Absorption coefficients of cleavage products used in calculating rates were as follows: catechol, $ε = 16.8 \text{ mM}^{-1} \text{ cm}^{-1}$; 3-methylcatechol, $ε = 18.0 \text{ mM}^{-1} \text{ cm}^{-1}$; 4-methylcatechol, $ε = 13.9 \text{ mM}^{-1} \text{ cm}^{-1}$ (13).

Catechol 2,3-dioxygenase activity was measured by using a reaction mixture consisting of 2.85 ml of 50 mM phosphate buffer (pH 7.0) and 50 μl of heat-treated extract. The reaction was started by the addition of 100 μl of a 10 mM solution of the substrate in buffer, giving a final concentration of 0.3 mM (17). The reaction was monitored by measuring the accumulation of the 2,3-cleavage products of the catechols. Absorption coefficients used for calculating rates were as follows: catechol, $ε = 48.4 \text{ mM}^{-1} \text{ cm}^{-1}$ at 375 nm; 3-methylcatechol, $ε = 19.8 \text{ mM}^{-1} \text{ cm}^{-1}$ at 390 nm; 4-methylcatechol, $ε = 33.2 \text{ mM}^{-1} \text{ cm}^{-1}$ at 380 nm (31).

2-Hydroxymuconic acid semialdehyde hydrolyase activity was determined by using a reaction mixture consisting of 2.85 ml of 50 mM phosphate buffer (pH 7.0) and 2-hydroxymuconic acid semialdehyde, which was prepared from heat-treated extract and catechol and was added to a final concentration of 15 μM. The reaction mixture was preincubated for 5 min to allow the $A_{375}$ to stabilize before the reaction was started by the addition of 50 μl of extract. The reaction was monitored by observing the removal of 2-hydroxymuconic acid semialdehyde at 375 nm, using $ε_{375} = 48.4 \text{ mM}^{-1} \text{ cm}^{-1}$ (31).

**Ion-exchange chromatography.** All procedures were carried out at 4°C. Cells which had been grown in the fermentor on nutrient broth-styrene (7.7 g) were suspended in 23 ml of TEGD buffer containing 20 mM Tris (pH 7.5), 10% ethanol, 10% glycerol, and 1 mM diithiothreitol before being broken by three passes in a French press and centrifuged as usual to obtain 17 ml of high-spin supernatant. Extract (15 ml) was added at 20 ml/h to a column packed with DEAE-Sephadex superfine (7 by 2.6 cm; volume, 20 ml) which had been preequilibrated with TEGD buffer. The column was washed
with approximately 2 column volumes of TEGD buffer at 20 ml/h and then with approximately 2 column volumes of 0.15 M NaCl-TEGD buffer. A linear 0.15 to 1 M NaCl gradient in TEGD buffer was used to elute enzyme activities. The gradient volume was 300 ml, and the flow rate was 20 ml/h. Enzyme activities in fractions were assayed by essentially the same procedures as described above.

**Analytical techniques.** Thin-layer chromatography (TLC) was done on Merck silica gel 60 F-254 plates (BDH), which included a UV indicator. The solvent system used was 180 ml of chloroform-20 ml of glacial acetic acid. Samples were usually dissolved in methanol. Components on plates were visualized by using UV light and iodine vapor.

Gas chromatography-mass spectrometry (GC-MS) analysis was performed on samples derivatized with bis-trimethylsilylacetamide and heated at 80°C for 4 min, producing trimethylsilyl ether derivatives of phenolic compounds. The samples were analyzed in a Hewlett-Packard 5890 series 2 GC coupled to a Hewlett-Packard 5791 quadrupole MS (Hewlett-Packard, Bracknell, U.K.). The GC column was 25 m by 0.22 mm (internal diameter) and was a fused silica capillary column. The stationary phase was phase-bonded methyl silicone BPI (Scientific and Glass Engineering, Milton Keynes, U.K.). The carrier gas was He, and the inlet pressure was 10 lb/in².

NMR analysis was carried out on a Bruker WP-200 SY 200 spectrometer at 200 MHz for 1H NMR and 50 MHz for 13C NMR, with tetramethysilane as an internal standard. Samples were dissolved in CDC, or CD,CO. Values are quoted in parts per million.

Analysis of the accumulation of acrylic acid in solutions was done by high-performance liquid chromatography (HPLC) analysis, using a Gilson HPLC system (Anachem Ltd., Luton, U.K.) with an Aminex HPX-87H organic acids column (300 by 7.8 mm; Bio-Rad, Richmond, Calif.) protected by a Microguard Cation H guard column (Bio-Rad). The buffer was 25 mM H,SO, at a flow rate of 1 ml/min. Components were detected with a UV detector at 210 nm.

The production of pyruvate was measured by HPLC and confirmed by using a reaction mixture containing NADH and lactate dehydrogenase.

The production of acetaldehyde was measured by coupling to NADH oxidation and to NAD reduction in the presence of alcohol dehydrogenase and aldehyde dehydrogenase, respectively.

Total organic carbon analysis was done with a Shimadzu TOC-500 analyzer, using an autosampler and a sample size of 5 µl. The low inorganic carbon and organic carbon standards were both distilled water. The high inorganic carbon standard was 400 ppm of sodium carbonate, and the high organic carbon standard was 400 ppm of sodium phthalate. Each sample was analyzed five times.

**Chemicals.** 1-Phenylethanol, 2-phenylethanol, acetaldehyde, benzene, benzaldehyde, benzyl alcohol, catechol, cinnamic acid, Coomassie brilliant blue G250, DL-mandelic acid, ethylbenzene, p-xylene, phenylacetic acid, poly(propylene glycol) 2025, styrene, toluene, triethylamine, and hexane were from BDH Chemicals. 3-Fluorocatechol, 3-methylcatechol, 4-methylcatechol, α-methylstyrene, β-methylstyrene, phenylethanol, phenylacetaldehyde, styrene oxide, and bis-trimethylsilylacetamide were from Aldrich Chemical Co., Gillingham, Dorset, U.K. MES buffer, oct-1-ene, and protocatechuic acid were from Sigma Chemical Co., Poole, Dorset, U.K. Tris buffer, NAD, NADH, and NADF were from Boehringer GmbH, Mannheim, Germany. Phenylglyoxylic acid was from Fluka, Buchs, Switzerland. m-Xylene and o-xylene were from Hopkin and Williams, Chadwell Heath, Essex, U.K. Toluene cis-glycol was from ICI Biological Products (now Zeneca Bio Products), Billingham, U.K. The cylinder of approximately 50% 18O2-50% 16O2 was obtained from IONC Services Ltd., Summit, N.J. Deuterochloroform was obtained from CEA, Gif-sur-Yvette, France. Bovine serum albumin (fraction V from bovine plasma) was obtained from Wilfrid Smith Ltd., London, U.K. Nutrient broth, nutrient agar, and bacteriological agar no. 1 were from Oxoid, Basingstoke, Hampshire, U.K. DEAE-Sephacel superfine was from Pharmacia, Uppsala, Sweden. 2-Hydroxyxypenta-2,4-dienoate was prepared from 1-allyl glycin by the action of l-aloeno acid oxidase and catalase (10). 4-Hydroxy-2-oxopentanoate was prepared by the mild alkaline hydrolysis of 4-methyl-2-oxobutyrolactone kindly provided by P. A. Williams (12). All other chemicals were of analytical grade.

**RESULTS AND DISCUSSION**

**Growth characteristics.** *R. rhodochrous NCIMB 13259* could grow on the following substrates as sole carbon sources: styrene, α-methylstyrene, toluene, ethylbenzene, benzene, cinnamic acid, 1-phenylethanol, phenylethanolamido, benzyl alcohol, benzaldehyde, benzoic acid, phenol, vinylactic acid, glucose, succinate, and acetate. It could also grow on 2-phenylethanol vapor. No growth occurred with phenylactic acid, DL-mandelic acid, phenylglyoxylic acid, β-methylstyrene, phenylacetalddehyde, acetophenone, m-xylene, o-xylene, p-xylene, oct-1-ene, octane, or acetic acid.

When bacteria were grown on styrene vapor in a 10-liter fermentor, the doubling time was approximately 10 h. However, this culture method was not reliable, since on several occasions the bacteria failed to grow, possibly because of the toxicity of the styrene. More reliable results were obtained by growth on a nutrient broth-based medium for 48 h, followed by 24-h growth with styrene vapor as part of the air supply, inducing the enzymes involved in styrene degradation and producing a yield of around 90 g (wet weight) per 10 liters.

**Oxygen consumption.** Rates of oxygen consumption by washed cell suspensions of *R. rhodochrous* NCIMB 13259 grown on styrene or toluene were monitored in the presence of various substrates (Table 1). Cells grown on styrene oxidized a range of aromatic substrates, including some of those on known pathways of styrene breakdown, including 2-phenylethanol, phenylethanolamido, and phenylacetalddehyde, though they did not oxidize phenylactic acid or mandelic acid, making styrene metabolism through these routes less likely. However, styrene-grown cells were able to oxidize other aromatic substrates such as toluene and benzene. Since pathways for metabolism of toluene are well known, and the intermediates are easily obtainable, these intermediates were tested on both styrene-grown and toluene-grown cells (Table 1). It is clear that there is very little difference in induction patterns between cells grown on styrene and those grown on toluene, after allowing for possible variation due to differences in growth stage and concentration of the growth substrates. The pattern of oxygen consumption compared with known pathways of toluene degradation suggests that toluene is probably degraded through toluene cis-glycol and 3-methylcatechol. All other routes seem to be ruled out because of low or very low rates of oxygen consumption with at least some of the potential intermediates. Cells grown in the fermentor on nutrient broth-styrene showed a pattern of responses similar to those of cells grown on styrene alone (results not shown). *R. rhodochrous* NCIMB 13259 grown on nutrient broth alone showed very little response to styrene, toluene, and o-xylene.
TABLE 1. Comparison of rates of oxygen consumption by washed cell suspensions of *R. rhodochrous* grown on styrene or toluene

<table>
<thead>
<tr>
<th>Assay substrate</th>
<th>Oxygen consumption (nmol of O$_2$ min$^{-1}$ mg of cell protein$^{-1}$)$^{a}$ for washed cells grown on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Styrene</td>
</tr>
<tr>
<td>Styrene oxide</td>
<td>40</td>
</tr>
<tr>
<td>Phenylacetaldehyde</td>
<td>40</td>
</tr>
<tr>
<td>Phenylethanolide</td>
<td>50</td>
</tr>
<tr>
<td>Phenylacetic acid</td>
<td>0</td>
</tr>
<tr>
<td>1-Phenylenol</td>
<td>90</td>
</tr>
<tr>
<td>2-Phenylenol</td>
<td>120</td>
</tr>
<tr>
<td>Dl-Mandelic acid</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Toluene</td>
<td>140</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>60</td>
</tr>
<tr>
<td>Benzoaldehyde</td>
<td>10</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>0</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>&gt;10</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>&lt;10</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>10</td>
</tr>
<tr>
<td>Catechol</td>
<td>360</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>1,500</td>
</tr>
<tr>
<td>4-Methylcatechol</td>
<td>140</td>
</tr>
<tr>
<td>Toluene cis-glycol</td>
<td>270</td>
</tr>
<tr>
<td>Acrylic acid</td>
<td>20</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>180</td>
</tr>
<tr>
<td>Glucose</td>
<td>40</td>
</tr>
</tbody>
</table>

$^{a}$ Corrected for endogenous oxygen uptake.

$^{b}$ ND, not determined.

Aromatic substrates (<10 nmol of O$_2$ min$^{-1}$ mg of cell protein$^{-1}$), though there was some response to catechol and 4-methylcatechol (80 and 30 nmol of O$_2$ min$^{-1}$ mg of cell protein$^{-1}$, respectively; results not shown). Cells grown on a glucose-salts medium did not oxidize any of the aromatic substrates listed in Table 1 (<20 nmol of O$_2$ min$^{-1}$ mg of cell protein$^{-1}$; results not shown). These results suggested that styrene was degraded by an inducible system involving the formation of styrene cis-glycol, probably followed by dehydrogenation of the cis-glycol.

**Production of vinylcatechol from styrene cis-glycol.** It would be expected that the product of the postulated cis-glycol dehydrogenase reaction of *R. rhodochrous* NCIMB 13259 would be vinylcatechol. It is then likely that the aromatic ring of vinylcatechol would be split oxygenatively, in either the 1,2 or the 2,3 position. Accordingly, extract was incubated anaerobically with styrene cis-glycol and NAD as described in Materials and Methods. When the products were separated by TLC, a few spots were visible under UV, and only one spot showed characteristics similar to those of 3-methylcatechol: a permanent stain when exposed to iodine vapor, an $R_f$ (approximately 0.5) close to that of 3-methylcatechol, and browning on prolonged exposure to air. This spot was probably vinylcatechol. Another sample of the putative vinylcatechol was analyzed by GC-MS. A component which produced a fragmentation pattern consistent with a trimethylsilyl ether derivative of vinylcatechol was present, with $m/z$ of 280 (M$^+$, 12%), 265 (M-15, 3%), and 73 {[Si(CH$_3$)$_3$]}$^{+}$, 100%).

Accumulation of catechols by whole cells in the presence of 3-fluorocatechol. Several reports in the literature have documented the inhibitory effects of 3-fluorocatechol on catechol dioxygenases (3, 13). Preliminary tests on extracts of *R. rhodochrous* NCIMB 13259 grown on nutrient broth-styrene showed substantial inhibition (>95%) of 3-methylcatechol 2,3-dioxygenase activity and partial inhibition of 3-methylcatechol 1,2-dioxygenase activity in the presence of 3-fluorocatechol. It was therefore hypothesized that 3-fluorocatechol could be used as a catechol dioxygenase inhibitor with whole cells to produce an accumulation of catechol intermediates. The accumulation was done as described in Materials and Methods; then as a first identification step, the entire extract was derivatized and subjected to GC-MS. A component that had a retention time and fragmentation pattern identical to those identified as the trimethylsilyl ether derivative of vinylcatechol described above was identified, with $m/z$ of 280 (M$^+$, 13%), 265 (M-15, 3%), and 73 {[Si(CH$_3$)$_3$]}$^{+}$, 100%). No vinylcatechol was detectable when 3-fluorocatechol was omitted from the reaction mixture.

In order to obtain a firm identification of the vinylcatechol, including the ring position of the vinyl group, it was necessary to use $^1$H NMR. A much larger quantity of cells (6 g) was used to produce enough material, and the TLC system already described was used to separate the vinylcatechol. The sample was then analyzed by $^1$H NMR (Fig. 1b), confirming that the isolated substance was 3-vinylcatechol.

**Identification of the muconic acid accumulating in the growth medium.** It had been noticed that there was frequently an absorbance between 250 and 300 nm in growth medium after *R. rhodochrous* NCIMB 13259 had been grown on styrene and other aromatic substances. When cells were grown on styrene in a fermentor, the medium showed an absorption in the UV band ($\lambda_{max} = 283$ nm). In addition, a thick band was visible on TLC of an extract from a reaction medium in which intact cells had been incubated with styrene but with no fluorocatechol. It was thought that this band could be a muconate, accumulated when cells were incubated with styrene. A batch of cells (20 g) was suspended in buffer, and styrene was added. After 4 h on a shaker at 30°C, the ensuing suspension was acidified, centrifuged, and separated by TLC. The major UV-absorbing band was eluted with ethyl acetate, which was then evaporated off. This produced 12 mg of a yellow liquid, which was analyzed by $^1$H and $^{13}$C NMR, and the analysis (Fig. 1c) confirmed the structure to be 2-vinylmuconic acid (2-ethenylhexa-2,4-dienedioic acid). An absorbance spectrum was taken of a sample from an identical experiment, which showed a major peak in the UV band, varying little with pH: pH 1.5, $\lambda_{max} = 287.2$ nm; pH 6.4, $\lambda_{max} = 283.6$ nm; pH 12.9, $\lambda_{max} = 282.8$ nm.

To test whether the muconic acid detected in the growth medium was simply a transient product, being formed more quickly than it was utilized, or whether it was a dead-end product, cells were grown on styrene and the approximate concentration of muconic acids was determined by monitoring the absorbance spectrum of the medium. It was found that the apparent concentration of muconic acid increased approximately in parallel with the growth of *R. rhodochrous* NCIMB 13259. The concentration of the putative muconate plateaued at the end of growth, and the fact that it never fell suggests that it was not metabolized. A total carbon analysis of substrates, cells, and products suggested that as much as 40% of the styrene added as growth substrate was accumulated as muconic acid.

**Use of $^{18}$O$_2$ to distinguish between an initial monoxygenase or dioxygenase ring attack.** The involvement of styrene cis-glycol in the metabolism of styrene strongly indicates that the initial attack on the aromatic ring of styrene is by a dioxygenase. In order to confirm this, cells were incubated with styrene and 3-fluorocatechol under an atmosphere of either 100% $^{18}$O$_2$ or a mixture of approximately 50% $^{16}$O$_2$ and 50% $^{18}$O$_2$, and the 3-vinylcatechol produced was extracted and analyzed by GC-MS. The molecular ion of the trimethylsilyl derivative
FIG. 2. Comparison of fragmentation patterns in the region of the molecular ion of 3-vinylcatechol produced from styrene by intact cells in an atmosphere of approximately 50% $^{18}$O$_2$-50% $^{16}$O$_2$ (a) or 100% $^{16}$O$_2$ (b).

of 3-vinylcatechol produced in an atmosphere of $^{18}$O$_2$-$^{16}$O$_2$ is illustrated in Fig. 2a, and that of the 3-vinylcatechol produced in an atmosphere of 100% $^{16}$O$_2$ is shown in Fig. 2b. These results clearly show that the molecular ion, m/z of 280, in the control sample is also present in the $^{18}$O$_2$-$^{16}$O$_2$ sample, but this sample has an additional ion, m/z of 284, 4 mass units higher. The ions at m/z of 280 and 284 are the molecular ions of the trimethylsilyl derivatives of 3-vinylcatechol containing $^{16}$O$_2$ and $^{18}$O$_2$ respectively. The smaller fragments at M+1 and M+2 are present in both samples and are due to isotopes of silicon and carbon. The relative abundance of the peak at m/z = 282.3 is no higher in the sample from $^{18}$O$_2$-$^{16}$O$_2$ than in the sample produced in an atmosphere of 100% $^{16}$O$_2$. Therefore, apart from these isotope effects, there is no additional contribution to the ion at m/z of 282.3 from $^{16}$O. Also, there is no evidence of ions at m/z of 283, which would be expected if the mixed isotope derivative $^{16}$O,$^{16}$O were present as a result of successive monooxygenase reactions. The production of ions at m/z of 284 but no ions at m/z of 283 when intact cells were exposed to styrene in the presence of 3-fluorocatechol and in an atmosphere of approximately 50% $^{16}$O$_2$ strongly supports the hypothesis that the initial ring oxygenation of styrene is carried out by a dioxygenase.

Identification of enzyme activities in extracts. A NAD-linked styrene cis-glycol dehydrogenase activity was found in extracts of R. rhodochrous NCIMB 13259 which had been grown on nutrient broth-styrene but not when the cells had been grown on nutrient broth only (Table 2). No activity was found when NADP was used as the cofactor. A similar NAD-linked activity was found when toluene cis-glycol instead of styrene cis-glycol was used as the substrate, with rates of up to 130 nmol min$^{-1}$ mg of protein$^{-1}$ in some extracts. During incubation of induced extract and styrene cis-glycol, a yellow color accumulated in the reaction mixture ($A_{	ext{max}} = 334$ and 423 nm), but only when NAD was present. This color, which later disappeared, was probably due to the transient accumulation of the product of meta cleavage of 3-vinylcatechol.

Catechol 1,2-dioxygenase activity was found in extracts of induced cells and to a very much smaller extent in noninduced extracts (Table 2). With induced extract, the rate of catechol oxygenation was higher than that of 3-methylcatechol, with oxidation of 4-methylcatechol substantially slower. The standard catechol 1,2-dioxygenase assay as described in the literature (14) includes EDTA, which inactivates the next enzyme, muconate cycloisomerase, and thus permits the accumulation of muconate, which otherwise would be further metabolized. The discovery of an accumulation of muconic acids in the medium suggested that the cycloisomerase might not be present in R. rhodochrous NCIMB 13259, and indeed EDTA had little effect on the apparent activity of catechol 1,2-dioxygenase (Table 2), suggesting that this organism has little or no cycloisomerase activity or (much less likely) that it has a muconate cycloisomerase that is unaffected by EDTA. Another way of testing for cycloisomerase activity is to monitor a catechol 1,2-dioxygenase reaction mixture over a longer period of time in order to see whether there is any decrease in $A_{260\text{nm}}$ corresponding to further metabolism of the cis,cis-muconate formed by the dioxygenase. When an assay mixture containing

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay substrate</th>
<th>Enzyme activity (nmol$^{-1}$ min$^{-1}$ mg of protein$^{-1}$)</th>
<th>Nutrient broth with styrene</th>
<th>Nutrient broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-Glycol dehydrogenase</td>
<td>Styrene cis-glycol</td>
<td>44</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Toluene cis-glycol</td>
<td>50</td>
<td>&lt;1.4</td>
<td></td>
</tr>
<tr>
<td>1,2-Dioxygenase</td>
<td>Catechol</td>
<td>58</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-Methylcatechol</td>
<td>35</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-Methylcatechol</td>
<td>18</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Catechol + EDTA</td>
<td>62</td>
<td>ND*</td>
<td></td>
</tr>
<tr>
<td>2,3-Dioxygenase</td>
<td>Catechol</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-Methylcatechol</td>
<td>16</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-Methylcatechol</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hydrolase</td>
<td>2-Hydroxymuconic acid semialdehyde</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* ND, not determined.
catechol and extract from cells grown on nutrient broth-styrene was monitored for 30 min, it was observed that the $A_{260}$ increased rapidly at first (because of the formation of cis,cis-muconate), then very briefly decreased rapidly, and then decreased extremely slowly. The same sequence of events was found with a reaction mixture containing extract from cells grown on benzyl alcohol and with catechol as the substrate. These results suggest that little, if any, cycloisomerase activity is present in cells grown on either styrene or benzyl alcohol. Unfortunately, the 3-vinylcatalase produced from styrene cis-glycol was not sufficiently pure to be used in assays of this type, since it contained material that absorbed in the 260-nm region.

Preliminary catechol 2,3-dioxygenase assays were not reproducible, because of varying activities of the next enzyme, 2-hydroxyxmuconic acid semialdehyde hydrolase. Previous workers have found that this enzyme in other organisms is inactivated by heating treatment ($4^\circ$), and it was found that optimum and reproducible rates of catechol 2,3-dioxygenase could be obtained by treatment of the extract at $55^\circ$C for 5 min. Uninduced extract had no detectable activity (Table 2). Induced extract had a very low 4-methylcatechol 2,3-dioxygenase activity and a higher catechol 2,3-dioxygenase activity, with 3-methylcatechol 2,3-dioxygenase giving the highest rate. The 2,3-cleavage of 3-vinylcatechol was monitored by using a modification of the above method. The reaction was performed at pH 7.6, and the absorbance spectrum of the reaction mixture was repeatedly scanned. The reaction mixture turned intense yellow in about 10 min, with two absorbance peaks appearing, the higher at 429 nm and the other at 345 nm, indicating that 3-vinylcatechol is susceptible to 2,3-cleavage.

2-Hydroxymuconic acid semialdehyde hydrolase activity was found only in extracts of induced cells (Table 2).

No styrene dioxygenase activity was detectable in cell extracts by using either the oxygen electrode or spectrophotometric detection of NAD(P)H oxidation.

Ion-exchange separation of enzyme activities. In order to examine the enzymes of the pathway more closely, an ion-exchange separation of the enzymes was developed. Both styrene and toluene cis-glycol dehydrogenase activities eluted in a single, symmetrical peak at 0.4 to 0.5 M NaCl. The rates of heat denaturation of the two activities at 34°C were almost identical, and both activities were slightly stimulated by heating for about 30 s. These results strongly indicate that toluene cis-glycol dehydrogenase and styrene cis-glycol dehydrogenase activities are the result of a single enzyme with a relaxed substrate specificity.

Catechol 2,3-dioxygenase activity eluted in a single peak at 0.3 to 0.35 M NaCl. Catechol 1,2-dioxygenase activity seemed to elute in two peaks, the first at 0.28 to 0.35 M NaCl and the second at 0.35 to 0.45 M NaCl. However, it was established that the first of these apparent 1,2-dioxygenase activities is the result of a single enzyme with a relaxed substrate specificity.

Identification of the 2,3-cleavage product of 3-vinylcatechol. A tentative identification of the meta-cleavage product of 3-vinylcatechol was made by using the anaerobic NAD-linked transformation of styrene cis-glycol to form 3-vinylcatechol, essentially as described above. Heat-treated ion-exchange column fractions containing catechol 2,3-dioxygenase activity were then added to the 3-vinylcatechol solution under aerobic conditions. The solution became yellow and turned red upon acidification. This product was then extracted with ethyl acetate, dried, evaporated down, and dried again; it was then extracted with 50% concentrated NH₃, producing a red solution. An absorbance scan showed two peaks, one at 284 nm and another at 412 nm. This extract was then left overnight at $30^\circ$C, after which the color was golden yellow and the $A_{412}$ had vanished, but there was a shoulder of absorbance at 260 to 290 nm. The changes in absorbance spectrum of the NH₃ extract are consistent with the formation of a picolinate, and this in turn is consistent with the formation of 2-hydroxy-6-oxoocta-2,4,7-trienoic acid from 3-vinylcatechol.

Hydrolysis of the 2,3-cleavage product of 3-methylcatechol. Earlier workers (10), using other organisms, have shown that catechol and 3-methylcatechol are both metabolized by catechol 2,3-dioxygenase and 2-hydroxymuconic acid semialdehyde hydrolase to produce 2-hydroxypenta-2,4-dienoate. 2-Hydroxymuconic acid semialdehydes and 2-hydroxypenta-2,4-dienoate have distinctive UV characteristics, enabling their reactions to be monitored. The 2,3-cleavage product of 3-methylcatechol, 2-hydroxy-6-oxo-2,4,7-trienoic acid, was produced by the action of $50 \mu l$ of the catechol 2,3-dioxygenase ion-exchange fraction on 3-methylcatechol, giving an absorbance peak at 320 nm, with a very small peak at 380 nm, similar to the peaks found by other workers (15). On addition of $100 \mu l$ of the hydrolase fraction, the peak at 319 nm rapidly disappeared, and simultaneously a new peak rapidly formed at 267.0 nm (at pH 6.0). The reaction was then acidified and scanned again, and a single peak was visible at 271.6 nm. Then the reaction mixture was made alkaline and scanned; a peak at 299.2 nm was visible on the first scan, which had vanished 2 min later. There was also a very small peak at 379.2 nm, presumably due to some remaining 2,3-cleavage product. These results are consistent with the accumulation of 2-hydroxypenta-2,4-dienoate. Other workers (10) have found this compound to have a melting point of 265 nm at neutral pH, one of 270 nm under acid conditions, and another of 305 nm under alkaline conditions. Under the last conditions, this compound rapidly tautomersizes to 2-oxopent-4-enoate, with no absorbance in this region (10).

Identification of acrylic acid as a product of the hydrolysis of 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid. The hydrolysis of 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid, the putative 2,3-cleavage product of 3-vinylcatechol, would be expected to produce 2-hydroxypenta-2,4-dienoate and acrylic acid. A reaction was started by the addition of $100 \mu l$ of an ion-exchange fraction containing catechol 2,3-dioxygenase activity to a 3-ml cuvette containing buffer and 3-vinylcatechol (produced anaerobically from 0.4 to 0.6 mg of styrene cis-glycol and extracted into diethyl ether), leading to a rapid accumulation of $A_{345.8}$ and $A_{424.9}$, with the peak at 255.5 nm in the initial reaction mixture due to 3-vinylcatechol no longer distinguishable. Upon addition of $100 \mu l$ of the fraction containing hydrolase activity, the peaks at 345 and 419 nm disappeared. Because of the absorbance of other peaks in the UV band, it was not possible to clearly distinguish any new peaks. However, a shoulder was visible at around 275 nm, which initially increased while all other peaks were diminishing or stable. Samples of $50 \mu l$ were removed at various times to determine the concentration of acrylic acid by HPLC. After addition of the 2,3-dioxygenase ion-exchange fraction, a very low concentration of acrylic acid
warhurst et al.

was present, approximately 0.01 mM, presumably due to contamination of the 2,3-dioxygenase activity by hydrolase activity (the peaks on the ion exchange slightly overlap). After addition of the hydrolase fraction, the acetic acid concentration increased to 0.07 to 0.09 mM. After the addition of a similar concentration of authentic acetic acid, a single, symmetrical peak was visible on the HPLC trace, an area corresponding to the sum of the two acetic acid concentrations. The ion-exchange fractions themselves did not contain detectable acetic acid.

*R. rhodochrous* could not grow on acetic acid under the conditions tested, though a small increase in oxygen consumption was found when acetic acid was added to intact cells in the oxygen electrode (Table 1). Samples from flasks growing on 1 mM styrene-salts medium showed that acrylic acid did not accumulate to any more than about 0.01 mM, and no acetic acid was detected at the end of growth.

**Metabolism of 2-hydroxypenta-2,4-dienoate by extracts.** Extracts of cells grown on nutrient broth-styrene were able to transform both 2-hydroxypenta-2,4-dienoate and 4-hydroxy-2-oxopentanoate into acetaldehyde and pyruvate.

**Conclusions.** The results in this paper strongly suggest that *R. rhodochrous* NCIMB 13259 degrades styrene by the pathway illustrated in Fig. 3. This pathway is supported by, or consistent with, the following lines of evidence. (i) It is consistent with the pattern of substrate utilization as revealed by growth tests and by experiments on oxygen uptake induced and noninduced bacteria (Table 1). (ii) All requisite enzyme activities were demonstrated in extracts, the only exception being the putative styrene-toluene dioxygenase. Failure to demonstrate this activity is not particularly unexpected since such enzymes are notoriously unstable (19), and in any case the incorporation of both atoms of $^{18}$O and the presence of cis-glycol dehydrogenase activity make the case for a styrene dioxygenase virtually unassailable. (iii) Many of the substrates and postulated substrates (e.g., styrene and styrene cis-glycol) were shown to be converted by intact cells or by cell extracts into the appropriate products (e.g., 3-vinylcatechol, 2-vinylmuconic acid, 2-hydroxy-6-oxoocta-2,4,7-trienoic acid, and acrylic acid). As far as we can see from the literature, *R. rhodochrous* is the first organism proven to metabolize styrene through a cis-glycol route, although other workers (6, 20) have obtained evidence for initial ring attack. The types of reaction involved in the upper part of the pathway in Fig. 3 are well established in the breakdown of other noncarboxylated aromatic substrates such as benzene, toluene, ethylbenzene, and naphthalene and are known to occur in other rhodococci, notably in the metabolism of tetratin and o-xylene by *Rhodococcus* (Corynebacterium) sp. strain C125 (28, 29). The enzymes of this pathway seem to have very relaxed substrate specificities, and it is known that toluene dioxygenases from various organisms can convert styrene into styrene cis-glycol. It is therefore possible that some organisms that have already been shown to degrade toluene may also be able to degrade, and perhaps grow on, styrene, especially if tested at low concentrations.

The productive route used by *R. rhodochrous* NCIMB 13259 to cleave 3-vinylcatechol is meta cleavage by 3-vinylcatechol 2,3-dioxygenase, followed by further metabolism of the 2-hydroxy-6-oxoocta-2,4,7-trienoic acid to give acyclic acid and 2-hydroxy-2-oxopentanoate (Fig. 3). This general type of pathway is known to occur in a wide range of organisms, and there is no evidence that the pathway in strain NCIMB 13259 differs from similar pathways established in other organisms, for example, that encoded by the TOL plasmid (9). Catechol 2,3-dioxygenases appear to be nonspecific in all organisms so far tested, but there are significant variations in relative specificities towards different catechols and none appears to have been tested with vinylcatechol as the substrate. The relative specificity of the catechol 2,3-dioxygenase activity in extracts of *R. rhodochrous* NCIMB 13259 grown on styrene-nutrient broth (Table 2) is similar to that of o-xylene-grown *Rhodococcus* sp. strain C125, which has an activity ratio of 100:240:56 for catechol-3-methylcatechol-4-methylcatechol (29).

The meta-cleavage product of 3-vinylcatechol is metabolized by a 2-hydroxymuconic acid semialdehyde hydrolase to give acyclic acid and 2-hydroxypent-2,4-dienoate. This activity is very unstable at 55°C, as are equivalent hydrolases from other organisms (4). The final stage of the established meta-cleavage pathway for catechols is the transformation of 2-hydroxypenta-2,4-dienoate into acetaldehyde and pyruvate by the consecutive action of 2-hydroxypenta-2,4-dienoate hydratase and 2-hydroxy-2-oxopentanoate (2-hydroxy-2-oxovalerate) aldolase (9). Extracts of *R. rhodochrous* NCIMB 13259 grown on nutrient broth-styrene could indeed transform both 2-hydroxypenta-2,4-dienoate and 4-hydroxy-2-oxopentanoate into acetaldehyde and pyruvate, presumably because of the presence of the appropriate hydratase and aldolase.

Acrylic acid is produced by the hydrolysis of 2-hydroxy-6-oxoocta-2,4,7-trienoic acid by 2-hydroxymuconic acid semial-
dehydrate hydrolase (Fig. 3). Since there is no appreciable accumulation of acrylic acid in the growth medium, *R. rhodochrous* NCIMB 13259 must be able to metabolize this substrate. However, growth did not occur on acrylic acid at either 5 or 0.5 mM, though 5 mM vinlylactic acid, which has one more methylene group than acrylic acid, did support growth. Simultaneous induction experiments showed a low rate of oxygen consumption when acrylic acid was added to cells grown on styrene (Table 1; a similar rate was observed with cells grown on glucose). Although acrylic acid is known to be toxic to many microorganisms, several strains of bacteria that can grow on it have been isolated but low concentrations are often necessary (1, 24).

A catechol 1,2-dioxygenase is clearly present, but there is no sign of any further metabolism of cis,cis-muconic acid or of 2-vinlylmucolate. This raises several questions: Why does the muconic acid accumulate? Why is the catechol 1,2-dioxygenase expressed? Is it possible for vinlylmuconic acid to be metabolized by an ortho pathway?

The wide range of substrates oxidized by *R. rhodochrous* NCIMB 13259, together with the persistence in the environment of other rhodococci (32), and the apparent lack of catabolite repression suggest that this strain could be useful in bioremediation and waste treatment.

ACKNOWLEDGMENTS

A. M. W. acknowledges the award of a CASE studentship by Zeneca Bio Products and the U.K. Science and Engineering Research Council. C. A. F. acknowledges the award of a U.K. Natural Environment Research Council equipment grant and also thanks the Leverhulme Trust as well as T. Leisinger and A. M. Cook for support and hospitality during the isolation of this organism. We thank N. J. Blunt and A. J. Scott for assistance with some of the experiments.

REFERENCES