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Oxidation of 1-Alkenes to 1,2-Epoxyalkanes by *Pseudomonas oleovorans*

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Resting cells of *Pseudomonas oleovorans* PO-1R that had been grown on octane oxidized 1-alkenes containing 6 to 12 carbon atoms and 1,7-octadiene to their corresponding 1,2-epoxides. The microorganism was capable of growing on 1-octene but not on 1,7-octadiene as a sole carbon source. The optimal temperature, pH, and 1-octene concentration for 1,2-epoxyoctane production by the resting cells were 34 to 40 C, pH 7 to 8, and 1.5 mg of 1-octene per ml, respectively. Epoxide concentration reached a maximum after 150 min of incubation and subsequently declined. In the absence of 1-octene, the epoxide was metabolized readily by the resting cells. The amount of 1,2-epoxyoctane produced was dependent on the initial cell concentration. With larger cell populations, the amount of epoxide present after 60 min of incubation was less than the amount observed at lower population densities after the same time period. This relationship was attributed to the rapid depletion of 1-octene at high biomass concentrations and the resultant early initiation of epoxide degradation by the resting cells.

The initial oxidation step of 1-alkene metabolism by microorganisms may occur at either the methyl group or the double bond (5). Bruyn (2) found that large quantities of 1,2-hexadecanediol were produced by *Candia lipolytica* growing on 1-hexadecene. About 5% of the hydrocarbon consumed was accounted for as the diol. Subsequent studies confirmed diol production and implicated molecular oxygen incorporation into the product (5, 6). In addition to diols, other products of 1-hexadecene oxidation have been identified. These were the ω-unsaturated acid, ω-unsaturated primary and secondary alcohols, 1,2-epoxide, and 2-hydroxy acid (5). Each product had the same chain length as the alkene substrate.

Thijsse and Van der Linden (15) studied 1-heptene oxidation by resting cells of *Pseudomonas aeruginosa* grown on heptane. In the presence of chloramphenicol, 6-heptenoic, 4-pentenoic, and 2,4-pentadienoic acids accumulated. Van der Linden (17) demonstrated the production of 1,2-epoxyoctane from 1-octene by heptane-grown resting cells of the same microorganism. Chloramphenicol, which was necessary for acid accumulation, had no effect on epoxide formation. Epoxides were not produced from alkenes by peptone-grown cells. In addition, epoxides were not detected as products of alkane metabolism and were not oxidized by *P. aeruginosa* cultures. Similarly, the 1,2-epoxide of hexadecane was not oxidized by *C. lipolytica* and did not support the growth of this organism. Thus, the role of epoxides in alkene metabolism is uncertain. Van der Linden (17) postulated that the enzyme system that forms epoxides may be the same as the system that catalyzes the initial oxidation of alkanes. This possibility also is suggested by the data of other workers. Cardini (Ph.D. thesis, University of Texas Austin, 1969) found that a cell-free extract of a *Corynebacterium* oxidized 1-octene to 1,2-epoxyoctane in addition to hydroxylating octane. Others have observed that certain microorganisms capable of hydroxylating steroids also epoxidated similar steroids (1, 14). For example, microorganisms that converted 11-deoxycortisol to cortisol also converted $\Delta^{9(11)}$ deoxycortisol to the 9β,11β-epoxide (1).

Coon and his co-workers (7, 11-13, 16) isolated an enzyme system from *P. oleovorans* that catalyzed the hydroxylation of alkanes and fatty acids. Recently, May and Abbott (9, 10) reported that this same enzyme system catalyzed the epoxidation of 1-alkenes. The present study was initiated to determine whether

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whole cells of this microorganism also catalyzed out the epoxidation of alkenes. Subsequent studies were then performed to identify environmental factors that influence epoxide production by *P. oleovorans* and to establish optimal conditions for in vivo enzyme assay.

MATERIALS AND METHODS

Organism. The organism used in these studies was *P. oleovorans* PO-1R which was derived from a culture kindly provided by M. J. Coon. Stock cultures were maintained on nutrient agar slants in screw-cap test tubes. A small quantity of octane was added to each tube.

Cell growth. *P. oleovorans* was cultivated in shake flasks in a mineral salts medium consisting of: $(NH_4)_2HPO_4$, 10 g; K_2HPO_4, 5 g; Na_2SO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $FeSO_4 \cdot 7H_2O$, 0.02 g; $MnSO_4 \cdot 4H_2O$, 0.02 g; NaCl, 0.02 g; H_3BO_3, 0.5 mg; $CuSO_4 \cdot 5H_2O$, 0.04 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg; $FeSO_4 \cdot 7H_2O$, 8.0 mg; $CaCl_2$, 50.0 mg; $CoCl_2 \cdot 6H_2O$, 0.2 mg, distilled H_2O, to 1 liter. The medium was dispensed in 100-ml volumes in 300-ml baffled shake flasks fitted with foam plugs. The flasks were autoclaved for 15 min at 121 C. At time of use, 1.0 ml of filter-sterilized octane was added as the substrate. The flasks were inoculated with cells washed from a nutrient agar slant and incubated at 30 C for 16 h at 350 rpm on a rotary shaker. Other experiments (not reported here) have shown that these cultures are in the late exponential growth phase.

P. oleovorans also was cultivated in 2 liters of the above medium in a 5-liter magnetically driven fermentor (Fermentation Design, Allentown, Pa.). Dissolved oxygen was monitored by a galvanic electrode, and the CO_2 content of the exit gas was measured with a CO_2 analyzer (Beckman Instrument Co., Fullerton, Calif.). A small quantity of polypropylene glycol (1.0 ml) was added to control foaming.

Biomass concentration was determined by optical density (OD) and dry weight measurements. A fraction of the cell suspension was diluted 1:15 in 0.1 N HCl, and the absorbance was determined at 660 nm (Spectronic 20, Bausch and Lomb, Rochester, N.Y.). For dry weight measurements, 5.0 ml of cell suspension was transferred to a tared centrifuge tube, sedimented by centrifugation, and washed with 0.1 N HCl to remove precipitated salts. The pellet remaining after the HCl wash was dried at 105 C overnight, cooled in a desiccator, and weighed. Dry weight measurements were performed in triplicate, and the averages were reported.

Chemicals. Epoxides with even carbon numbers from C_6 to C_{12} were purchased from Chemical Samples Co. (Columbus, Ohio). Epoxides with odd carbon numbers were synthesized from the appropriate 1-alkene using *m*-chloroperbenzoic acid (3). A similar procedure was used to synthesize 7,8-epoxy-1-octene from 1,7-octadiene. The latter epoxide was purified by distillation and its structure was confirmed by nuclear magnetic resonance.

Epoxide production. The cells harvested from shake-flask cultures were washed three times in 0.1 M phosphate buffer, pH 7.0, and the final pellet was resuspended in fresh buffer to obtain an OD of 0.22 when diluted 1:80. A 2-ml amount of this washed cell suspension (containing 20 mg dry cell mass) was placed in a 50-ml baffled shake flask containing 20 ml of 0.1 M phosphate buffer, pH 7, and 0.05 ml of Triton X. The 1-alkene substrate (0.3 ml) was added, and the flask was closed tightly to minimize evaporation. The reaction mixture was incubated at 30 C on a water bath rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 400 rpm.

Assay of 1,2-epoxides. A 1.0-ml sample of the resting cell suspension was removed and extracted with an equal volume of *n*-hexane containing 0.25 mg of 2-octanol per ml. The 2-octanol served as an internal standard to compensate for sampling errors and solvent losses during sample preparation. The emulsion formed during the hexane extraction was broken by centrifugation. The upper hexane layer was recovered and used for quantitative gas chromatographic analysis of the 1,2-epoxides.

The 1,2-epoxyalkanes were assayed by flame ionization gas chromatography by using a stainless-steel column (20 ft by ⅛ in. [ca. 6.09 m by 3.18 mm]) packed with 10% Carbowax 20 M on 80/100 Chromosorb W (Allied Sciences Laboratories, State College, Pa.). The column temperature was maintained isothermally at 190 C, and the carrier gas flow was 35 ml of helium per min. The various epoxide products were identified by retention time comparisons and co-chromatography with authentic standards. This identification was supplemented by observing the presence or absence of product peaks before and after bromination and acid hydrolysis as described by May and Abbott (9, 10). The amount of epoxide accumulated was determined by measuring the ratio of the epoxide peak area to the peak area of 2-octanol. A standard curve was constructed to convert the peak area ratio of 1,2-epoxyoctane to 2-octanol into epoxide concentration in the resting cell suspensions. Response factors were not obtained for the other 1,2-epoxides, therefore, the concentrations of these products were reported only as the peak area ratio.

The retention time of 1,2-epoxynonane was almost identical to that of 2-octanol. Therefore, quantitative estimation of 1,2-epoxynonane was performed by using 1-octanol as an internal standard. Similar response factors were obtained for 1-octanol and 2-octanol. Thus, peak area ratios measured for 1,2-epoxynonane were comparable to those measured for the other epoxides.

RESULTS

Resting cell suspensions of octane-grown cells of *P. oleovorans* oxidized 1-octene to 1,2-epoxyoctane which accumulated.

Time course of epoxide formation. Control experiments with heat-killed cells indicated that the epoxide was produced enzymatically. The epoxide concentration reached a maximum after 150 min of incubation and subsequently

declined (Fig. 1). The rate of epoxide production was linear for the first 60 min. Therefore, epoxide production was measured within this interval whenever the effect of variables was tested.

P. oleovorans was cultivated in a fermentor to determine whether the amount of 1,2-epoxyoctane subsequently accumulated by the resting cells was dependent on the growth stage at which the cells were harvested. The fermentation time course was divided into three stages: (i) exponential growth, (ii) linear growth due to zero dissolved oxygen tension, and (iii) no growth due to substrate depletion (Fig. 2). Cells harvested during the exponential or linear growth phases produced similar amounts of 1,2-epoxyoctane. Within an hour after substrate depletion, the amount of epoxide produced decreased by about 50%. After prolonged incubation in the absence of substrate, the cells were no longer capable of accumulating 1,2-epoxyoctane (Fig. 2). Thus, harvesting cells prior to substrate depletion insured epoxide production by the resting cells. Cells cultivated in shake flasks were capable of accumulating epoxide if harvested 16 h after inoculation.

Effect of pH, temperature, and 1-octene concentration. The effect of pH on 1,2-epoxyoctane production by the resting cells was examined. Tris(hydroxymethyl)aminomethane buffer (0.1 M) was used for pH values of 8.0 and 9.0, and 0.1 M phosphate buffer was used for values from 5.5 to 8.0. A pH between 7.0 and 8.0 appeared to be optimum for epoxide production (Fig. 3). The initial and final pH readings in these experiments differed by less than 0.5 pH unit, and similar amounts of epoxide were

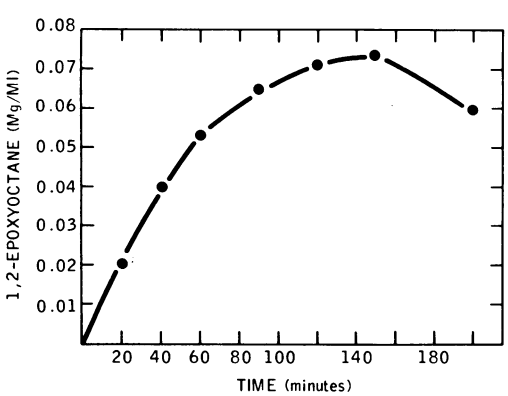

FIG. 1. *Time course of 1,2-epoxyoctane production by resting cells of P. oleovorans.*

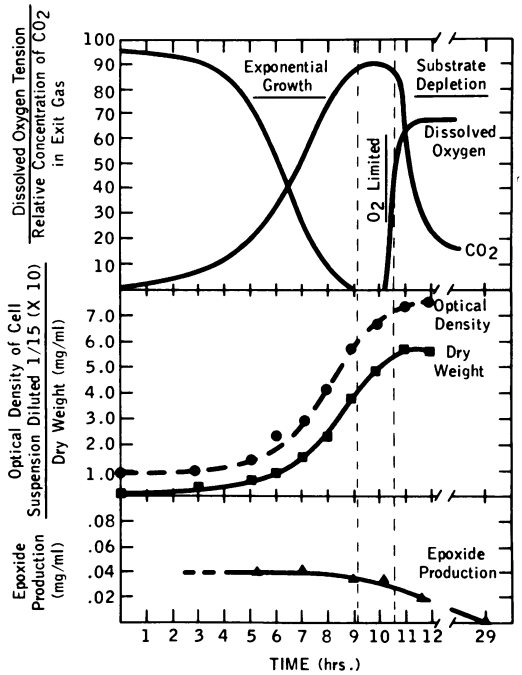

FIG. 2. *Production of 1,2-epoxyoctane by resting cells of P. oleovorans harvested at different stages of growth from a 5-liter fermentor.*

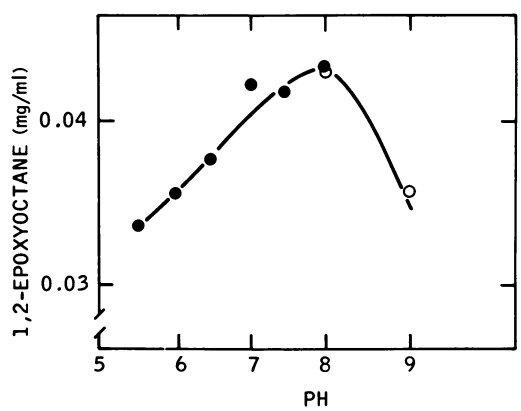

FIG. 3. *Effect of pH on the production of 1,2-epoxyoctane by resting cells of P. oleovorans. Symbols: ●, data obtained with 0.1 M phosphate buffer; ○, data obtained with 0.1 M tris(hydroxymethyl)aminomethane buffer.*

produced in either buffer at pH 8.0. Authentic samples of 1,2-epoxyoctane, final concentration 0.1 mg/ml, were added to heat-killed cell suspensions at pH 4.5, 7.0, and 10.0 to test for nonenzymatic degradation of 1,2-epoxyoctane. The epoxide concentration in these suspensions did not decrease significantly after 1 h of incubation indicating that nonenzymatic oxida-

tion or hydrolysis of epoxide did not occur under our assay conditions.

The temperature optimum for epoxidation by resting cell suspensions was about 40 C (Fig. 4). At higher temperatures there was a large decrease in the amount of epoxide accumulated, but below the optimum the temperature dependence of epoxidation was less.

Various concentrations of 1-octene were added to the resting cell suspensions. The amount of epoxide produced was dependent on the amount of substrate initially added (Fig. 5). A 1-octene concentration of about 1.0% (vol/vol) supported the greatest amount of epoxide production.

Effect of biomass concentration. The cell concentration also influenced the rate of epoxide production. The amount of epoxide accumulated after 1 h of incubation increased as the biomass concentration was increased up to about 1.0 mg/ml. At higher biomass concentrations, less product was observed to accumulate (Fig. 6).

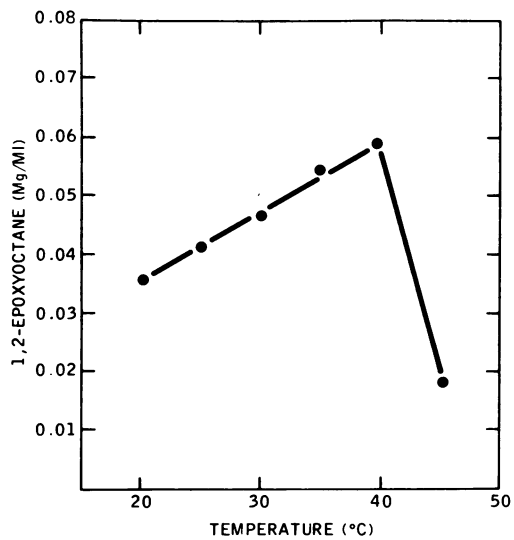

FIG. 4. *Effect of temperature on the production of 1,2-epoxyoctane by resting cells of P. oleovorans.*

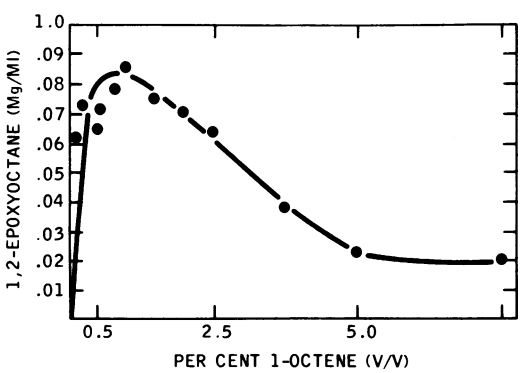

FIG. 5. *Effect of 1-octene concentration on the production of 1,2-epoxy-octane by resting cells of P. oleovorans.*

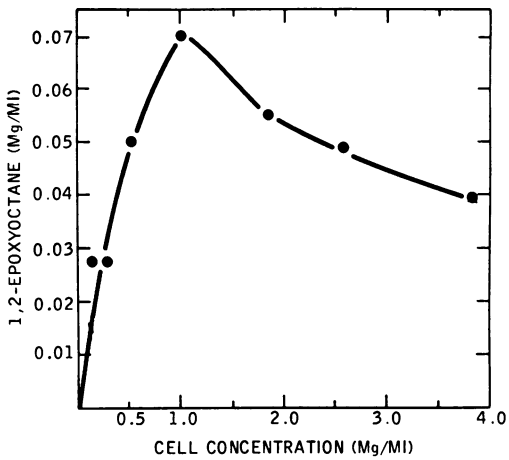

FIG. 6. *Effect of biomass concentration on the production of 1,2-epoxyoctane by resting cells of P. oleovorans.*

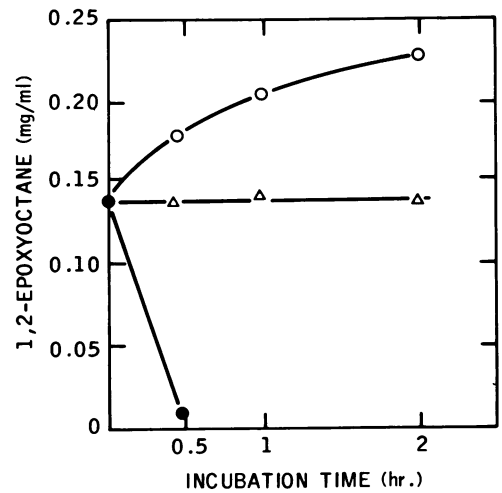

FIG. 7. *Degradation of 1,2-epoxyoctane by resting cell suspensions of P. oleovorans. Viable cell preparation plus 1,2-epoxyoctane (●); heat-killed cell preparation plus 1,2-epoxyoctane (Δ); viable cell preparation plus 1,2-epoxyoctane and 1-octene (○).*

Epoxide degradation. Examination of the time course of epoxide formation revealed that the epoxide concentration decreased during prolonged incubation, suggesting that 1,2-epoxyoctane might be further metabolized by *P. oleovorans*. To test this possibility, 2.8 mg of

1,2-epoxyoctane was added to viable or heat-killed cell preparations and incubated under standard conditions. No decline was observed in epoxide concentration in the heat-killed cell suspension, but epoxide rapidly disappeared in the presence of viable cells (Fig. 7). When 1-octene, final concentration 15 mg/ml, was added to a cell suspension along with the exogeneously supplied epoxide, a net increase in epoxide production was detected. From these data it appears that the rapid decline in epoxide concentration was due to the absence of 1-octene.

Substrate specificity. The ability of *P. oleovorans* to epoxidate alkenes other than 1-octene was determined. Epoxides were produced from 1-alkenes containing 6 to 12 carbon atoms (Table 1). *P. oleovorans* also oxidized 1,7-octadiene to 7,8-epoxy-1-octene. Although both 1,7-octadiene and 1-octene were epoxidated, only 1-octene supported growth. The microorganism was also unable to grow on 7,8-epoxy-1-octene or on 1,2-7,8-diepoxyoctane.

DISCUSSION

May and Abbott (9, 10) reported that the ω-hydroxylation enzyme system of *P. oleovorans* catalyzed both the hydroxylation of alkanes and the epoxidation of alkenes. When 1-octene was supplied as a substrate, both 8-hydroxy-1-octene and 1,2-epoxyoctane were formed. In addition, it was found that the methyl group of the latter compound was also susceptible to hydroxylation. The ability of the ω-hydroxylation system to catalyze these multiple reactions accounts for several observations made in the present study. For example, *P. oleovorans* epoxidated 1,7-octadiene, but 1,7-octadiene, 7,8-epoxy-1-octene, and 1,2-7,8-dieposyoctane were unable to sustain growth. These results indicate that the microorganism was unable to metabolize the epoxide functionality. Thus, the metabolism of 1,2-epoxyoctane by resting cells and the ability of the microorganism to grow on 1-octene must be accounted for by oxidation of the methyl groups of these molecules. This conclusion is also supported by the observation that 1-octene inhibited 1,2-epoxyoctane metabolism (cf. Fig. 7). Since the latter compounds are substrates of the same enzyme a large excess of one (1-octene) should competitively inhibit the oxidation of the other (1,2-epoxyoctane). Such a competition has been observed with the cell-free ω-hydroxylation enzyme system (10).

TABLE 1. *Epoxidation of 1-alkenes and 1,7-octadiene by resting cells of P. oleovorans grown on n-octane*

Substrate	Product	Peak area ratio (1,2-epoxyalkane/2-octanol)[a]
1-hexene	1,2-epoxyhexane	Trace
1-heptene	1,2-epoxyheptane	0.03
1-octene	1,2-epoxyoctane	0.17
1-nonene	1,2-epoxynonane	0.19
1-decene	1,2-epoxydecane	0.25
1-undecene	1,2-epoxyundecane	0.08
1-dodecene	1,2-epoxydodecane	Trace
1,7-octadiene	1,2-epoxy-7-octene	0.30

[a] Product analyses were performed after 0.5 h of incubation of the substrate with the resting cells.

Epoxide accumulation from 1-octene by *Pseudomonas aeruginosa* was accompanied by the metabolism of a large quantity of 1-octene via methyl group oxidation (4). These oxidations, which in *P. oleovorans* can be attributed to the ω-hydroxylation enzymes, may result in the depletion of 1-octene. As shown in Fig. 7, the absence of 1-octene permitted the metabolism of 1,2-epoxyoctane to occur. Since 1-octene is oxidized more rapidly by higher biomass concentrations it will be depleted sooner enabling epoxide metabolism to begin earlier. If epoxide oxidation is initiated before the assay for 1,2-epoxyoctane is performed, it would appear (as in Fig. 6) that a high biomass concentration resulted in less epoxide accumulation.

Huybregtse and Van der Linden (4) reported that 1,2-epoxyoctane was slowly oxidized by octane-grown cells of *P. aeruginosa*. They postulated that the slow oxidation was due to hydrolysis of the epoxide to a 1,2-diol and oxidation of the diol to α-hydroxyoctanoic acid. The latter compound was detected by gas chromatography. The data in the present study shows that *P. oleovorans* rapidly degrades 1,2-epoxyoctane in the absence of 1-octene. Although the degradation products of the epoxide were not isolated, our data suggest that they arise via oxidation of the methyl group, and not by hydrolysis and subsequent oxidation of the epoxide functionality.

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