The Metabolism of Cyclopentanol by *Pseudomonas* N.C.I.B. 9872

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1. *Pseudomonas* N.C.I.B. 9872 grown on cyclopentanol as carbon source oxidized it at a rate of 228 μl of O_2/h per mg dry wt. and the overall consumption of 5.9 μmol of $\text{O}_2/\mu\text{mol}$ of substrate. Cyclopentanone was oxidized at a similar rate with the overall consumption of 5.2 μmol of $\text{O}_2/\mu\text{mol}$ of substrate. Cells grown with sodium acetate as sole source of carbon were incapable of significant immediate oxidation of these two substrates. 2. Disrupted cells catalysed the oxidation of cyclopentanol to cyclopentanone by the action of an NAD^+ -linked dehydrogenase with an alkaline pH optimum. 3. A cyclopentanol-induced cyclopentanone oxygenase (specific activity 0.11 μmol of NADPH oxidized/min per mg of protein) catalysed the consumption of 1 μmol of NADPH and 0.9 μmol of O_2 in the presence of 1 μmol of cyclopentanone. NADPH oxidation did not occur under anaerobic conditions. The only detectable reaction product with 100000g supernatant was 5-hydroxyvalerate. 4. Extracts of cyclopentanol-grown cells contained a lactone hydrolase (specific activity 7.0 μmol hydrolysed/min per mg of protein) that converted 5-valerolactone into 5-hydroxyvalerate. 5. Cyclopentanone oxygenase fractions obtained from a DEAE-cellulose column were almost devoid of 5-valerolactone hydrolase and catalysed the formation of 5-valerolactone in high yield from cyclopentanone in the presence of NADPH. 6. Incubation of 5-hydroxyvalerate with the 100000g supernatant, NAD^+ and NADP^+ under aerobic conditions resulted in the consumption of O_2 and the conversion of 5-hydroxyvalerate into glutarate. 7. The high activity of isocitrate lyase in cyclopentanol-grown cells suggests that the further oxidation of glutarate proceeds through as yet uncharacterized reactions to acetyl-CoA. 8. The reaction sequence for the oxidation of cyclopentanol by *Pseudomonas* N.C.I.B. 9872 is: cyclopentanol $\rightarrow$ cyclopentanone $\rightarrow$ 5-valerolactone $\rightarrow$ 5-hydroxyvalerate $\rightarrow$ glutarate $\rightarrow$ $\rightarrow$ acetyl-CoA.

Though the metabolism of substituted aromatic compounds has been the subject of intensive research (Gibson, 1968; Evans, 1970; Dagley, 1971) the substituted cycloalkanes have received comparatively little attention. Posternak *et al.* (1955) reported that *Acetobacter suboxydans* growing with either isomer of cyclohexane-1,2-diol released 2-hydroxycyclohexan-1-one into the culture medium, and Yugari (1961) showed that a species of *Pseudomonas* adaptively oxidized *trans*-cyclohexan-1,2-diol to cyclohexan-1,2-dione, which was then cleaved by the action of a hydrolase to form 6-oxohexanoate. Ooyama & Foster (1965) reported the isolation of two micro-organisms capable of growth with a wide range of straight- and branched-chain hydrocarbons. Though neither of these organisms was capable of growth with cycloalkanes, 2-methylbutane-grown cells transformed cyclohexane and cyclohexanol into cyclohexanone, and cyclopentane and cyclopentene into cyclopentanone. Further metabolism of the cyclic ketones was not observed.

Merritt & Tomkins (1959) reported the oxidation of cyclohexanol to cyclohexanone by purified alcohol

dehydrogenase (EC 1.1.1.1) from horse liver, but the enzyme was not active towards cyclopentanol.

Norris & Trudgill (1971) have described the reaction sequence for the oxidation of cyclohexanol by *Nocardia globerula* as cyclohexanol $\rightarrow$ cyclohexanone $\rightarrow$ 1-oxa-2-oxocycloheptane $\rightarrow$ 6-hydroxyhexanoate $\rightarrow$ adipate and suggested that the adipate is further metabolized by β -oxidation.

Shaw (1966) has reported the oxidation of cyclopentanone to glutarate by two species of *Pseudomonas* and an unidentified Gram-negative organism.

In the present paper we report some detailed studies of the oxidation of cyclopentanol by a strain of *Pseudomonas*.

Experimental

Methods

Maintenance and growth of organisms. *Pseudomonas* N.C.I.B. 9872, obtained by elective culture on cyclopentanol, was a gift from Dr. P. J. Chapman. Stock cultures were grown on nutrient agar at 30°C, stored at 2°C and subcultured at monthly intervals.

Cells were grown at 30°C in media containing (per litre): KH_2PO_4 , 2g; Na_2HPO_4 , 4g; $(\text{NH}_4)_2\text{SO}_4$, 2g; yeast extract, 0.1g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01g; $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0.1g; NaCl , 0.1g. $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$ (0.4g/l) was added aseptically as a sterile aqueous 10% (w/v) solution to sterilized growth media. Cyclopentanol, sterilized by membrane filtration, was supplied to 25-ml cultures in 100ml Erlenmeyer flasks from diffusion tubes as described by Claus & Walker (1964); a direct addition of 2g of cyclopentanol/l was made to larger volumes of medium in 2-litre Erlenmeyer flasks. For large quantities of cells a 15-litre batch of medium in an L.H. type 1/1000 fermenter vessel (L.H. Engineering Co. Ltd., Bells Hill, Stoke Poges, Bucks., U.K.) was inoculated with 600ml of a freshly grown Erlenmeyer-flask culture and supplied with a total of 5g of cyclopentanol/l either by manual addition of portions or by continuous introduction as an aqueous 4% (w/v) solution by an electric pump during the course of growth. The culture was supplied with sterile air at 4 litres/min and stirred at 150 rev./min. After 15h of growth, which yielded about 1.2mg dry wt. of cells/ml, the crop was harvested in a Sharples Super centrifuge at 14300g and a flow rate of 0.3 litre/min and washed twice by resuspension in phosphate buffer (KH_2PO_4 , 2g/l; Na_2HPO_4 , 4g/l), pH 7.1, followed by centrifugation at 3°C and 13000g for 15 min.

When sodium acetate, sodium glutarate or sodium succinate was used as carbon source it was included in the basal medium at 2g/l.

Disruption of cells. Washed cells, resuspended in the phosphate buffer, pH 7.1 (1.5 × cell vol.), were disintegrated by passage through a French pressure cell (Milner *et al.*, 1950) with a pressure difference at the orifice of 20000 lb/in² (138 MN/m²).

Preparation of extracts. Broken cell suspensions were clarified by centrifuging at 100000g and 3°C for 1h in a Martin Christ Omega II preparative ultracentrifuge (Martin Christ, 3360 Osterode/Harz, W. Germany). The protein concentrations of extracts were determined by the method of Gornall *et al.* (1949).

Gas-exchange reactions. Gas exchange catalysed by whole-cell suspensions or cell extracts was measured either in Warburg manometers or with a Clark-type O_2 electrode in an agitated vessel maintained at 30°C (Oxygen Monitor, model 53; Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.).

Buffers. The phosphate buffer used in most instances contained 1g of anhydrous KH_2PO_4 and 2g of anhydrous Na_2HPO_4 in deionized water to a final volume of 1 litre and had a pH of 7.1. Other phosphate buffers were prepared by adjusting solutions of KH_2PO_4 of appropriate concentration to the required pH with NaOH. The composition and molarity of other buffers used are described at the appropriate points in the text.

Enzyme units. All enzyme activities are expressed

as μmol of substrate consumed or μmol of product formed/min.

Spectrophotometric assays. Cyclopentanol dehydrogenase was assayed by measuring the increase in E_{340} when the test extract (1–3mg of protein) was added to a 1cm-light-path cell that contained, in 3ml, 500 μmol of glycine–NaOH buffer, pH 10.0, 1.0 μmol of NAD^+ and 0.5 μmol of cyclopentanol. Cyclopentanone oxygenase was assayed as a routine by measuring the decrease in E_{340} that occurred when the test extract (0.5–2.0mg of protein) was added to a 1cm-light-path cell that contained, in 3ml, 60 μmol of phosphate buffer, pH 7.1, 0.5 μmol of NADPH and 2 μmol of cyclopentanone. Where required, anaerobic conditions were obtained by the use of Thunberg cells that were first evacuated with a Speedivac two-stage high-vacuum pump (Edwards High Vacuum Ltd., Manor Royal, Sussex, U.K.), followed by threefold repeated flushing with N_2 and re-evacuation. 5-Hydroxyvalerate dehydrogenase was assayed by measuring the increase in E_{340} when 1 μmol of 5-hydroxyvalerate was added to an anaerobic Thunberg cuvette that contained, in 3ml, 60 μmol of phosphate buffer, pH 7.1, 4 μmol of NAD^+ and 5–10mg of protein (100000g supernatant).

Determination of acid production by pH-stat. The formation of 5-hydroxyvalerate from 10–100 μmol of 5-valerolactone by the 100000g supernatant was monitored by the controlled addition of 10mM-NaOH to an unbuffered reaction mixture, initial volume 10ml, maintained at pH 7.0 by the use of an Autotitration Controller (Pye Unicam Ltd., Cambridge, U.K.). NaOH consumption was monitored manually at suitable time-intervals.

Detection and measurement of 5-valerolactone. 5-Valerolactone was determined by the procedure of alkaline hydroxamate formation followed by acidification, conversion into the ferric hydroxamate and measurement of E_{510} as described by Cain (1961). A standard curve was prepared with authentic commercial 5-valerolactone.

Preparation of hydroxamic acid esters for chromatography. Hydroxamates derived from authentic 5-valerolactone and from the reaction products were prepared by the alkaline procedure of Cain (1961) and treated for chromatographic purposes as follows. Where necessary, protein was precipitated by addition of boiling ethanol and the protein was removed by centrifugation. Ethanol was then removed under a flow of N_2 at 50°C, the aqueous residue adjusted to pH 6.5 with 2M-HCl and the hydroxamic acid esters were extracted with ethyl acetate. The ethyl acetate extracts were dried over anhydrous Na_2SO_4 , decreased in volume under a flow of N_2 and chromatographed in appropriate solvents.

Chromatography. T.l.c. of 2,4-dinitrophenylhydrazine derivatives of neutral ketones was carried out on 0.25mm-thick layers of Kieselgel G developed with

solvent A, benzene–tetrahydrofuran (19:1, v/v) or with solvent B, light petroleum (b.p. 80°–100°C)–diethyl ether (7:3, v/v) (Byrne, 1965), or on 0.25 mm-thick layers of neutral aluminium oxide (Brockmann grade I activity) developed with solvent C, light petroleum (b.p. 80°–100°C)–ethyl formate (7:3, v/v). T.l.c. of carboxylic acids was carried out on 0.25 mm-thick layers of a mixture of Kieselguhr G and Kieselgel G (3:1, w/w) pretreated with acetic acid vapour and developed in solvent D, the upper phase of a benzene–acetic acid (40:7, v/v) mixture (Miyazaki *et al.*, 1969) or on Kieselgel G developed with solvent E, benzene–methanol–acetic acid (45:10:4, by vol.) (Petrowitz & Pastuska, 1962) or with solvent F, benzene–ethyl acetate–formic acid (25:25:2, by vol.). T.l.c. of carboxylic acids was also carried out on 0.25 mm-thick layers of microgranular cellulose developed with solvent G, butan-1-ol–propionic acid–water (178:125:90, by vol.) (Calvin & Benson, 1949), or with solvent H, propan-1-ol–formic acid–water (6:3:1, by vol.) (Weimberg, 1959), or with solvent J, propan-1-ol–eucalyptol–formic acid–water (15:15:6:4, by vol.), a minor modification of the system of Cheftel *et al.* (1951). T.l.c. of hydroxamate esters formed from lactones was carried out on 0.25 mm-thick layers of Kieselgel G, developed with solvent K, dioxan–acetic acid (5:2, v/v) or with solvent L, ethyl acetate–acetic acid (5:3, v/v), or on 0.25 mm-thick layers of microgranular cellulose developed with solvent M, pyridine–acetic acid–water (10:7:3, by vol.) (Cain, 1961).

Detection of compounds on chromatograms. Hydroxamic acid esters of lactones were detected by conversion into their ferric derivatives as described by Cain (1961). Carboxylic acids were detected by spraying the thoroughly dried plates with 0.1% (w/v) Bromocresol Green in aq. 95% (v/v) ethanol, adjusted to pH 6 with NaOH.

Materials

5-Hydroxyvalerate. A solution of 1 mmol (100 mg) of 5-valerolactone in 16 ml of 1.2 M-NaOH was incubated on a boiling-water bath for 20 min, cooled in ice, and 20 ml of phosphate buffer, pH 7.1, was added. The pH was adjusted to 7.1 by addition of 2 M-HCl and the volume was made up to 50 ml by further addition of phosphate buffer. This solution of 5-hydroxyvalerate (20 μ mol/ml) contained no detectable lactone residue.

Other materials. NAD⁺, NADP⁺, NADH and NADPH were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, W. Germany. Cyclopentanol and 2,4-dinitrophenylhydrazine were from British Drug Houses Ltd., Poole, Dorset, U.K. Yeast extract was from Difco Laboratories, Detroit, Mich., U.S.A. Cycloheptanone and 5-valerolactone were from Ralph N. Emanuel Ltd., Wembley, Middx.,

U.K. Hydroxylamine hydrochloride was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Cyclohexanone, cyclopentanone and glutaric acid were from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Sodium DL-isocitrate was from Sigma Chemical Co., St. Louis, Mo., U.S.A. DEAE-cellulose (grade DE52) was from W. and R. Balston Ltd., Maidstone, Kent, U.K. Kieselgel G and Kieselguhr G were from E. Merck A.-G., Darmstadt, Germany. Microgranular cellulose/CT was from Reeve Angel Scientific Ltd., London E.C.4, U.K. Neutral aluminium oxide (Brockmann grade I activity) was from H. Woelm, Eschwege, Germany.

Results

Oxidation of substrates by whole cells

Freshly harvested cyclopentanol-grown cells of *Pseudomonas* N.C.I.B. 9872 oxidized the growth substrate at a rate of 228 μ l of O₂/h per mg dry wt. with the consumption of 5.9 μ mol of O₂/ μ mol of cyclopentanol added.

Cyclopentanone was oxidized with the consumption of 5.2 μ mol of O₂/ μ mol added and a rate of 194 μ l of O₂/h per mg dry wt. Glutarate was oxidized at a rate of 68 μ l of O₂/h per mg dry wt., and 5-valerolactone and 5-hydroxyvalerate at the same slow rate of only 36 μ l of O₂/h per mg dry wt. None of these substrates stimulated significant O₂ consumption by acetate-grown cells.

Oxidation of cyclopentanol by cell extracts

The 100000g supernatant from cyclopentanol-grown cells catalysed the stoicheiometric reduction of NAD⁺ in the presence of cyclopentanol at alkaline pH values (Fig. 1). The product of a similar reaction carried out under anaerobic conditions formed a 2,4-dinitrophenylhydrazone of low solubility in aqueous reagents that co-chromatographed with authentic cyclopentanone 2,4-dinitrophenylhydrazone in three solvent systems (Table 1).

Oxidation of cyclopentanone by cell extracts

When 100000g supernatant was incubated with cyclopentanone and the phosphate buffer, an NADPH-dependent consumption of O₂ was observed. NADH failed to initiate cyclopentanone-dependent O₂ consumption. An investigation of the stoicheiometry of the reaction demonstrated that the oxidation of 1 μ mol of cyclopentanone was accompanied by the consumption of approx. 1 μ mol of O₂ and 1 μ mol of NADPH (Table 2). This stoicheiometry is close to that which is theoretically required for a mixed-function oxygenase, a postulate reinforced by the obligatory requirement for O₂ before

Fig. 1. Effect of pH on the activity of cyclopentanol dehydrogenase

Reaction mixtures in 1cm-light-path cuvettes contained, in a volume of 3.0ml, 100000g supernatant (3.1 mg of protein), 1.0 μ mol of NAD⁺ and 500 μ mol of the following buffers: ○, phosphate; ●, tris-HCl; △, glycine-NaOH. Reactions were started by additions of 0.5 μ mol of cyclopentanol and the increase in extinction was followed at 340nm under anaerobic conditions.

substrate-stimulated NADPH oxidation can proceed.

A reaction mixture in which 20 μ mol of cyclopentanone was incubated with an excess of NADPH and 14.8mg of 100000g supernatant yielded a product that was acidic and co-chromatographed with 5-hydroxyvaleric acid in five t.l.c. systems (Table 3, reaction a).

The formation of 5-hydroxyvalerate from cyclopentanone by high-speed supernatant from *Pseudomonas* N.C.I.B. 9872 in the presence of NADPH is a parallel to the observed formation of 6-hydroxyhexanoate from cyclohexanone by a cell extract of cyclohexanone-grown *N. globerula* CL1. In this latter organism 1-oxa-2-oxocycloheptane was demonstrated to be the initial detectable oxygenation product, which was then hydrolysed to the hydroxy acid by a very active lactone hydrolase present in the extract.

Incubation of 100000g supernatant from cyclopentanol-grown cells with 5-valerolactone in the pH-stat resulted in the liberation of about 1 μ mol of acid for each μ mol of lactone introduced (Table 4). This acidic reaction product, possibly contaminated with a small amount of glutaric acid, co-chromatographed with authentic 5-hydroxyvaleric acid in five t.l.c. systems (Table 3, reaction b).

The lactone hydrolase thus demonstrated has a specific activity 60-fold greater than the mixed-

function oxygenase acting on cyclopentanone (Table 2). In this situation, if the product of oxygenation is the lactone, as occurs in the metabolism of cyclohexanone by *N. globerula* CL1 (Norris & Trudgill, 1971), even a transient accumulation of the lactone is unlikely.

Separation of cyclopentanone oxygenase from 5-valerolactone hydrolase and identification of the product of the oxygenase reaction

Chromatography of the 100000g supernatant on DEAE-cellulose yielded oxygenase fractions that were almost devoid of lactone hydrolase activity (Fig. 2). Fractions collected between 1068 and 1116ml of column eluate were pooled and concentrated by addition of dry Sephadex G-25 followed by centrifugation in filtering centrifuge tubes (Millipore Ltd., London N.W.10, U.K.).

Incubation of this oxygenase preparation with cyclopentanone and NADPH under aerobic conditions yielded a product that, when converted into its hydroxamic acid ester, co-chromatographed with the derivative of 5-valerolactone in three t.l.c. systems (Table 5). Assay of the 5-valerolactone formed in the reaction gave a yield of 13 μ mol. This must be considered as a high yield in view of the small residue of 5-valerolactone hydrolase in the oxygenase preparation.

Oxidation of 5-hydroxyvalerate to glutarate by 100000g supernatant

When 100000g supernatant was incubated with 5-hydroxyvalerate and NAD⁺ under anaerobic conditions a limited and non-stoichiometric reduction of the NAD⁺ occurred. Addition of NADP⁺ caused a greater, but still limited, increase in E_{340} . The 5-hydroxyvalerate-stimulated O₂ consumption in the Warburg flask was NAD⁺-dependent and accelerated by the addition of NADP⁺. Stoichiometrically 1 μ mol of O₂ was consumed for each μ mol of 5-hydroxyvalerate added, presumably being mediated by NADH and NADPH oxidase activities, which are present in the extract.

The product of 5-hydroxyvalerate oxidation co-chromatographed with glutaric acid in solvent systems D, E, F, G and J (see the Experimental section).

Concentrations of isocitrate lyase in the 100000g supernatant from cyclopentanol-, acetate-, succinate- and glutarate-grown cells

The high activity of *threo*-D₂-isocitrate glyoxylate-lyase (EC 4.1.3.1) present in extracts of cyclopentanol- and glutarate-grown cells, is even higher than that obtained from cells grown with acetate and in marked contrast to that found when the organism is grown on succinate (Table 6).

Table 1. *Identification of the product of the cyclopentanol dehydrogenase reaction*

An anaerobic Thunberg cuvette contained, in a volume of 3.0ml, 600 μ mol of glycine-NaOH buffer, pH 10.0, 6.25mg of 100000g supernatant and 10 μ mol of NAD⁺. The reaction, at 30°C, was started by the addition of 10 μ mol of cyclopentanol from the side arm. After 15 min the cuvette was opened, the reaction was stopped by the addition of 1 ml of aq. 10% (w/v) trichloroacetic acid and protein was removed by centrifugation. The 2,4-dinitrophenylhydrazones of the reaction product was formed by incubating the reaction supernatant with 2 ml of 0.25% (w/v) 2,4-dinitrophenylhydrazine in 2M-HCl. The yellow precipitate that appeared at 4°C after 24 h incubation was harvested by centrifugation, washed with 2M-HCl to remove excess of reagent, then with water to remove excess of HCl, and finally freeze-dried. Solutions of the reaction product (1 mg/ml in ethanol) and of the derivatives of standard cyclic ketones were then chromatographed. For a description of the solvents see the Experimental section.

Sample chromatographed	Solvent system ...	<i>R_F</i> values		
		A	B	C
2,4-Dinitrophenylhydrazine reagent		0.54	0.51	0.59
Cycloheptanone 2,4-dinitrophenylhydrazone		0.65	0.64	0.71
Cyclohexanone 2,4-dinitrophenylhydrazone		0.62	0.63	0.68
Cyclopentanone 2,4-dinitrophenylhydrazone		0.57	0.61	0.65
Reaction product 2,4-dinitrophenylhydrazone		0.59	0.60	0.64

Table 2. *Stoichiometry of O₂ and NADPH requirements for the oxidation of cyclopentanone by 100000g supernatant*

For experiment (a) the oxygen monitor contained, in a volume of 3.0ml, 60 μ mol of phosphate buffer, pH 7.1, 1.5mg of 100000g supernatant and 1 μ mol of NADPH. Reaction mixtures were equilibrated at 30°C and reactions started by additions of cyclopentanone as indicated. For experiment (b) reaction mixtures in 1 cm-light-path cuvettes contained, in a volume of 3.0ml, 60 μ mol of phosphate buffer, pH 7.1, 1.5mg of 100000g supernatant and 0.5 μ mol of NADPH. Reactions at 30°C were started by additions of cyclopentanone as indicated and the decrease in *E*₃₄₀ was followed.

Experiment (a)

Cyclopentanone added (μ mol) (A)	O ₂ consumed (μ mol)		Ratio B/A
	Total	Blank value subtracted (B)	
0	0.13	—	—
0.125	0.24	0.11	0.88
0.250	0.35	0.22	0.88
0.375	0.48	0.35	0.93
0.500	0.56	0.43	0.86

Experiment (b)

	$-\Delta E_{340}$	NADPH oxidized (μ mol)†		
		Total	Blank value subtracted (B)	
0	0.04	0.02	—	—
0.125	0.31	0.15	0.13	1.04
0.250	0.56	0.27	0.25	1.00
0.325	0.70	0.34	0.32	0.98
0.500	1.01	0.49	0.47	0.94
0.500*	0	0	0	—

* Anaerobic Thunberg cuvette.

† Specific activity, 0.17 μ mol of NADPH oxidized/min per mg of protein.

Table 3. *Chromatographic characterization of 5-hydroxyvaleric acid formed from cyclopentanone and 5-valerolactone by 100000g supernatant*

Reaction (a): from cyclopentanone. A Warburg flask contained, in a volume of 2.0 ml, 400 μ mol of phosphate buffer, pH 7.1, 14.7 mg of 100000g supernatant, 20 μ mol of NADPH and 20 μ mol of cyclopentanone. The centre-well contained 0.1 ml of 20% (w/v) KOH. The manometer was equilibrated at 30°C and reaction started by addition of the cyclopentanone from the side arm. When cyclopentanone-stimulated O₂ uptake had ceased the reaction mixture was adjusted to pH 2 with 1 M-HCl, and then frozen and thawed to complete the precipitation of protein, which was removed by centrifugation. The aqueous supernatant was extracted with diethyl ether, the ether layer dried over anhydrous sodium sulphate, the ether removed under a stream of N₂ and the residue taken up in 0.2 ml of water for chromatography. Reaction (b): from 5-valerolactone. The product of incubation of 100 μ mol of 5-valerolactone with 100000g supernatant under the conditions described in Table 4 was acidified to pH 2 with 1 M-HCl and prepared for chromatography as described above. For a description of the solvents see the Experimental section.

Solvent system ...	<i>R_F</i> values				
	D	E	F	G	H
5-Hydroxyvaleric acid	0.21	0.36	0.32	0.50	0.47
Product from reaction (a)	0.20	0.36	0.32	0.51	0.47
Product from reaction (b)	0.20	0.36	0.32	0.51	0.47
Glutaric acid	0.32	0.43	0.48	0.80	0.90

Table 4. *Measurement of acid production from 5-valerolactone by 100000g supernatant with a pH-stat*

The reaction vessel (25 ml beaker) contained 10 ml of water and 0.78 mg of 100000g supernatant adjusted to pH 7.0. Reactions, at 23°C, were started by addition of substrate, and the mixtures were agitated with a magnetic stirrer and maintained at pH 7.0 by controlled addition of 10 mM-NaOH with a pH-stat. Alkali addition was monitored manually until reactions ceased.

5-Valerolactone added (μ mol) (A)	NaOH consumed (μ mol)		Ratio B/A
	Observed	Corrected (B)	
0	2	—	—
25	24	22	0.88
50	45	43	0.86
100	91	89	0.89

Induced and basal concentrations of the enzymes catalysing cyclopentanol oxidation

Sodium acetate was used as a growth substrate to obtain cells for measurement of basal enzyme activities. Cells were harvested from the late-exponential phase of growth, and the specific activities of relevant enzymes in 100000g supernatant prepared from these and cyclopentanol-grown cells are shown in Table 7. A greater than 250-fold increase in specific activity of

all assayed enzymes was stimulated by growth with cyclopentanol.

Discussion

The ability of cyclopentanol-grown cells of *Pseudomonas* N.C.I.B. 9872 to oxidize cyclopentanol and cyclopentanone at nearly equivalent rates is compatible with a role for cyclopentanone as an intermediate in cyclopentanol oxidation. The failure of such cells to oxidize 5-valerolactone, 5-hydroxyvalerate and glutarate at equivalent rates may reflect the inability of these more polar compounds to penetrate the bacteria rapidly, rather than indicating their non-participation in cyclopentanol oxidation.

Our demonstration of a soluble NAD⁺-linked cyclopentanol dehydrogenase in the 100000g supernatant from cyclopentanol-grown cells parallels the observation of an NAD⁺-linked cyclohexanol dehydrogenase in extracts of cyclohexanol-grown *Acinetobacter* N.C.I.B. 9871 by Dr. P. J. Chapman (personal communication); like the latter enzyme it has an alkaline pH optimum. Both of these observations are at variance with the inability of Norris & Trudgill (1971) to detect a cyclohexanol dehydrogenase in extracts of cyclohexanol-grown *Nocardia globulara* CL1.

The cyclopentanone oxygenase of *Pseudomonas* N.C.I.B. 9872 is tightly coupled, NADPH oxidation being relatively slow in the absence of cyclopentanone. The near 1:1 stoichiometry of O₂ and NADPH consumption, and the total dependence of substrate-stimulated NADPH oxidation on the presence of O₂,

Fig. 2. Chromatography of the 100000g supernatant on DEAE-cellulose

Cell extract (100000g supernatant) containing 1.08 g of protein was loaded on a column (15 cm × 5 cm) of DEAE-cellulose (DE52) equilibrated with 0.023 M-phosphate buffer, pH 7.1. Cyclopentanone oxygenase and 5-valerolactone hydrolase were eluted with an increasing concentration of KCl in the same buffer and 12 ml fractions were collected. ○, Protein measured by $E_{260}^{1cm}/E_{280}^{1cm}$; ●, cyclopentanone oxygenase activity; ▲ (scale 1) and △ (scale 2), 5-valerolactone hydrolase activity measured by the procedure of Cain (1961); - - - - -, calculated concentration of the KCl gradient.

Table 5. Identification of the product of the oxidation of cyclopentanone by partially purified cyclopentanone oxygenase

A 25 ml Erlenmeyer flask contained, in a volume of 3.0 ml, 240 μmol of phosphate buffer, pH 7.1, 2.4 mg of partially purified cyclopentanone oxygenase (0.67 unit) from DEAE-cellulose chromatography and 20 μmol of NADPH. Reaction, at 30°C on a Gallenkamp Metabolic Shaking Incubator, was started by the addition of 20 μmol of cyclopentanone and terminated after 30 min by cooling in an ice bath. A sample was withdrawn for determination of lactone by the hydroxamate method (Cain, 1961) and the remainder was used to prepare the hydroxamate derivative for chromatographic analysis. This derivative and the authentic compound, prepared from 5-valerolactone as described in the Experimental section, were subjected to t.l.c.

Solvent system	<i>R_F</i> values of hydroxamic acid esters	
	5-Valerolactone	Reaction product*
K	0.49	0.47
L	0.26	0.25
M	0.80	0.80

* A control from which cyclopentanone was omitted did not yield any detectable lactone.

Table 6. Isocitrate lyase activity in 100000g supernatant from acetate-, cyclopentanol-, glutarate- and succinate-grown *Pseudomonas* N.C.I.B. 9872

Reaction mixtures in 125 mm × 15 mm test tubes, incubated at 30°C in a Gallenkamp Metabolic Shaking Incubator, contained, in a volume of 6.0 ml, 138 μmol of phosphate buffer, pH 7.1, 18 μmol of cysteine, 18 μmol of MgCl₂ and 100000g supernatant in the range 0.5–10 mg of protein. Reactions were started by the addition of 20 μmol of DL-isocitrate and stopped after suitable time-intervals by withdrawing 1 ml samples into 1 ml of 20% (w/v) trichloroacetic acid. Protein was removed by centrifugation and the glyoxylate formed was determined by the procedure of Friedemann & Haugen (1943). The identity of the reaction product was checked by comparing the absorption spectrum of its 2,4-dinitrophenylhydrazine derivative in alkali with that of the derivative of authentic glyoxylate.

Growth substrate	DL-Isocitrate lyase (specific activity)
Acetate	0.015
Cyclopentanol	0.028
Glutarate	0.028
Succinate	0.0004

Table 7. Activities of enzymes of cyclopentanol oxidation in extracts of *Pseudomonas N.C.I.B. 9872*

The rates of reactions in 100000g supernatant are expressed as specific activities with respect to the assayable component of the reactions. For conditions of assay see the Experimental section or the appropriate table.

	Specific activity in extracts of <i>Pseudomonas N.C.I.B. 9872</i> grown on	
	Cyclopentanol	Acetate
Cyclopentanol dehydrogenase	0.68	0.00085
Cyclopentanone oxygenase	0.11	Not detectable
5-Valerolactone hydrolase	7.0	0.003
5-Hydroxyvalerate dehydrogenase	0.11	0.0004

Scheme 1. Reaction sequence for the oxidation of cyclopentanol by *Pseudomonas N.C.I.B. 9872*

indicates this to be an example of a classical mixed-function oxygenase as described by Mason (1957).

The demonstration of an induced 5-valerolactone hydrolase in extracts of cyclopentanol-grown cells and of the formation of 5-valerolactone by a partially purified cyclopentanone oxygenase, essentially devoid of 5-valerolactone hydrolase activity, puts the cyclopentanone oxygenase in the same group as the (+)-camphor oxygenase of *Pseudomonas putida* C1B (Conrad *et al.*, 1965; Trudgill *et al.*, 1966a,b), the cyclohexanone oxygenase of *N. globerula* CL1 (Norris & Trudgill, 1971) and a bacterial quercetin oxygenase (J. M. Wood, personal communication), all of which produce lactones as the oxygenated products, in contrast with the majority of mixed-function oxygenases, which generate alcohols.

The chemical equivalent of this reaction is the oxygenation of cyclic ketones by per-acids, first described by Baeyer & Villiger (1899) and applied to cyclopentanone to yield 5-valerolactone by Friess (1949).

The use of oxygenases to generate lactones from cyclic structures, which are then amenable to ring cleavage by hydrolysis, has also been reported for furans where the ring O is already present and the ketonic O is introduced (Trudgill, 1969; Griffin *et al.*, 1971).

The ability of 100000g supernatant to oxidize 5-hydroxyvalerate, formed from cyclopentanone in the presence of NADPH, to glutaric acid is indicative of the presence of the necessary alcohol and aldehyde dehydrogenases. An induced 5-hydroxyvalerate dehydrogenase has been demonstrated (Table 7) and

shown to require NAD^+ . 5-Oxovalerate dehydrogenase has not been directly demonstrated, because we have been unable to obtain the substrate, though stimulation of the conversion of 5-hydroxyvalerate into glutarate by the additional presence of NADP^+ indicates that the enzyme may be specific for this cofactor. Ichihara & Ichihara (1961) have demonstrated the presence of an induced NAD^+ -linked 5-oxovalerate dehydrogenase in extracts of a strain of *Pseudomonas* grown on DL-lysine.

The identical high activities of *threo*-D₃-isocitrate glyoxylate-lyase (EC 4.1.3.1) present in extracts of cyclopentanol- and glutarate-grown cells, even greater than that obtained with acetate-grown cells, and in marked contrast to that found when the organism, is grown on succinate (Table 6), lead us to suggest that glutarate oxidation yields only acetyl-CoA as the utilizable carbon fragment. Though we have not investigated glutarate oxidation in this organism, a pathway yielding acetyl-CoA has been described by Numa *et al.* (1964) for a strain of *Pseudomonas fluorescens* that yields CO_2 and 2 mol of acetyl-CoA by the reaction sequence: glutarate $\rightarrow$ glutaryl-CoA $\rightarrow$ glutaconyl-CoA $\rightarrow$ crotonyl-CoA $\rightarrow$ β -hydroxybutyryl-CoA $\rightarrow$ acetoacetyl-CoA $\rightarrow$ 2 acetyl-CoA.

Scheme 1 depicts the pathway of cyclopentanol oxidation which is compatible with all the results presented.

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