Biochem. J. (1971) **121**, 363–370
Printed in Great Britain

The Metabolism of Cyclohexanol by *Nocardia globerula* CL1

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(*Received* 14 *August* 1970)

1. *Nocardia globerula* CL1, isolated by enrichment on cyclohexanol and grown with it as carbon source, oxidized it with a Q_{O_2} of 39 μl/h per mg dry wt. and the overall consumption of 2.2 μmol of oxygen/mol of substrate. Cyclohexanone, 2-hydroxycyclohexan-1-one dimer and cyclohexane-1,2-dione were oxidized with Q_{O_2} values similar to that for cyclohexanol whereas ε-caprolactone and 6-hydroxycaproate were oxidized very slowly and adipate not all. 2. Disrupted cell suspensions could not be shown to catalyse the conversion of cyclohexanol into cyclohexanone. 3. A cyclohexanol-induced cyclohexanone oxygenase (specific activity 0.55 μmol of NADPH oxidized/min per mg of protein) catalysed the consumption of 1 mol of NADPH and 1 mol of O_2 in the presence of 1 mol of cyclohexanone. NADPH oxidation did not occur under anaerobic conditions. The only detected reaction product with 25000 *g* supernatant was 6-hydroxycaproate. 4. Extracts of cyclohexanol-grown cells contained a lactone hydrolase (specific activity 15.6 μmol hydrolysed/min per mg of protein), which converted ε-caprolactone into 6-hydroxycaproate. 5. Incubation of 6-hydroxycaproate with 25000 *g* supernatant in the presence of NAD^+ resulted in NAD^+ reduction under anaerobic conditions, oxygen consumption under aerobic conditions and the conversion of 6-hydroxycaproate into adipate. 6. Cyclohexanone oxygenase fractions devoid of ε-caprolactone hydrolase catalysed the stoicheiometric formation of ε-caprolactone from cyclohexanone in the presence of excess of NADPH. 7. The reaction sequence for the oxidation of cyclohexanone by *N. globerula* CL1 is: cyclohexanol → cyclohexanone → ε-caprolactone → 6-hydroxycaproate → adipate. 8. It is suggested that the adipate may be further dissimilated by β-oxidation.

Though the metabolism of substituted aromatic compounds has been the subject of intensive research (Dagley, Chapman, Gibson & Wood, 1964; Gibson, 1968; Evans, 1970) the substituted cyclohexanes have received comparatively little attention. The seven isomeric forms of methylcyclohexanol are excreted by rabbits as their glucuronides, whereas about 1% of methylcyclohexane fed is aromatized to benzoic acid (Elliott, Tao & Williams, 1965*a*,*b*). Posternak, Reymond & Friedli (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*-cyclohexane-1,2-diol to cyclohexane-1,2-dione, which was then cleaved, by the action of a hydrolase (EC 3.7.1.–), to form 6-oxocaproate. Ooyama & Foster (1965) reported the isolation of two microorganisms, one of which was tentatively identified as a species of *Nocardia*, capable of growth with a wide range of straight- and branched-chain hydrocarbons. Though neither of these organisms was capable of growth with cyclohexane or cyclohexanol, 2-methylbutane-grown cells formed cyclohexanone and an unidentified neutral compound when incubated with either cyclohexane or cyclohexanol. The unidentified neutral compound was neither cyclohexane-1,2-dione nor *trans*-cyclohexane-1,2-diol. Dr P. J. Chapman (personal communication) has demonstrated the induced oxidation of cyclohexanol to cyclohexanone by a species of *Acinetobacter* (N.C.I.B. 9871). Further oxidation of the cyclohexanone he indicated as being mediated by an oxygenase of the mixed-function type (Mason, 1957) and suggested that it formed 2-oxo-oxacycloheptane (ε-caprolactone), which was then cleaved hydrolytically to form 6-hydroxycaproate.

In the present paper we report some studies of the oxidation of cyclohexanol by a strain of *Nocardia* that extend the observations of Ooyama & Foster (1965) and Dr P. J. Chapman.

MATERIALS AND METHODS

Maintenance and growth of organisms. *Nocardia globerula* CL1 was isolated from woodland soil near Aberystwyth by elective culture in medium with cyclohexanol as sole source of carbon. Stock cultures were maintained on nutrient–agar slopes at 2°C.

Cells were grown at 30°C in media containing (per l): KH_2PO_4, 2g; Na_2HPO_4, 4g; $(NH_4)_2SO_4$, 2g; yeast extract, 0.1g; $CaCl_2,H_2O$, 0.1g; $FeSO_4,7H_2O$, 0.01g; $MgCl_2,7H_2O$, 0.4g. Cyclohexanol, sterilized by membrane filtration, was supplied to Erlenmeyer flasks from diffusion tubes as described by Claus & Walker (1964). For larger quantities of cells a 10-litre batch of medium in a New Brunswick Microferm vessel (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) was inoculated with 500ml of a freshly grown Erlenmeyer-flask culture, supplied with air sequentially passed through Dreschel bottles containing cyclohexanol and water at 10litres/min, and stirred at 300rev./min. After 48h growth, which yielded about 1mg dry wt. of cells/ml, the crop was harvested in a Sharples Super centrifuge and washed once by resuspension in phosphate buffer (KH_2PO_4, 2g/l; Na_2HPO_4, 4g/l), pH7.1, followed by centrifugation at 3°C and 13000*g* for 15min.

Disruption of cells. Washed cells, resuspended in phosphate buffer, pH7.1 (1.5 × cell vol.), were disintegrated by two passages through a French pressure cell (Milner, Lawrence & French, 1950) with a pressure difference at the orifice of 18000lb/in^2 or by exposure at 0°C to the output of an MSE 500W ultrasonic disintegrator at an average frequency of 20000Hz, 1.6 coulomb of output energy being used/20ml of cell suspension. The method of disruption employed was not critical to the reported observations.

Preparation of extracts. Broken-cell suspensions were clarified by centrifuging at 25000*g* and 0°C for 30min in a Sorvall RC2.B refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) and, where stated, further fractionated by centrifuging 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, Bardawill & David (1949).

Gas-exchange reactions. Gas exchange catalysed by whole-cell suspensions or cell extracts was measured in Warburg manometers at 30°C; for reactions involving uptake of O_2 the centre wells contained strips of Whatman no. 542 filter paper and 0.1ml of 20% (w/v) KOH.

Buffers. Phosphate buffer, pH7.1, contained 2g of anhydrous KH_2PO_4 and 4g of anhydrous Na_2HPO_4 in deionized water to a final volume of 1 litre. All concentrations are expressed as concentrations of phosphate.

Chromatography. T.l.c. of carboxylic acids and lactones was carried out on 0.25mm-thick layers of a mixture of Kieselguhr G and Kieselgel G (3:1, w/w) (E. Merck A.-G., Darmstadt, Germany) pretreated with acetic acid vapour and developed with solvent *A*, the upper phase of a benzene–acetic acid (40:7, v/v) mixture (Miyazaki, Suhara & Kobayashi, 1969), or on Kieselgel G developed with solvent *B*, benzene–dioxan–acetic acid (90:25:4, by vol.), or solvent *C*, benzene–methanol–acetic acid (45:8:4, by vol.) (Petrowitz & Pastuska, 1962). Carboxylic acids were detected by spraying thoroughly dried plates with 0.1% (w/v) Bromocresol Green in aq. 95% (v/v) ethanol, adjusted to pH6 with NaOH.

Detection and measurement of ε-caprolactone. ε-Caprolactone 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 ε-caprolactone. Detection on t.l.c. utilized a modification of the alkaline hydroxamate procedure, also described by Cain (1961).

Spectrophotometric assays. Cyclohexanone oxygenase was assayed as a routine by measuring the decrease in E_{340} that occurred when the test extract (0.05–0.5mg of protein) was added to a 1cm-light-path cell that contained, in 3ml, 130μmol of phosphate buffer, pH7.1, 0.5μmol of $NADP^+$ and 2μmol of cyclohexanone. Where required, anaerobic conditions were obtained by 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.

Determination of acid production by pH-*stat.* The formation of 6-hydroxycaproate from 1–5μmol of ε-caprolactone or cyclohexanone by 25000*g* supernatant was monitored by the controlled addition of 1mM-NaOH to an unbuffered reaction system, initial volume 5ml, maintained at pH7.0 by use of an Autotitration Controller (Pye Unicam Ltd., Cambridge, U.K.). Alkali consumption was monitored manually at suitable time-intervals.

Preparation of 6-hydroxycaproate. A solution of 1mmol (102mg) of ε-caprolactone in 16ml of 1.2M-NaOH was incubated on a boiling-water bath for 20min, cooled on ice and 20ml of phosphate buffer was added. The pH was adjusted to 7.1 by addition of 2M-HCl and the volume made up to 50ml by further addition of phosphate buffer. This solution of 6-hydroxycaproate (20μmol/ml) contained no detectable residual lactone.

Other materials. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49), NAD^+, $NADP^+$, NADH and NADPH were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, W. Germany. Adipic acid, αα′-bipyridyl, cyclohexanol and cyclohexanone were from British Drug Houses Ltd., Poole, Dorset, U.K. EDTA, hydroxylamine hydrochloride and Tiron (disodium 1,2-dihydroxybenzene-3,5-disulphonate) were from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. ε-Caprolactone, *cis*- and *trans*-cyclohexane-1,2-diol, *cis*- and *trans*-cyclohexane-1,4-diol, cyclohexane-1,2-dione, cyclohexane-1,3-dione and 2-hydroxycyclohexan-1-one (dimer) were from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Calcium phosphate gel was supplied by Sigma Chemical Co., St Louis, Mo., U.S.A. Whatman DEAE-cellulose (grade DE23) was from W. and R. Balston Ltd., Maidstone, Kent, U.K. Alumina C_γ gel prepared according to the method of Colowick (1955) was a gift from Dr L. J. Rogers of this department.

Enzyme units. All enzyme activities are expressed as μmol of substrate consumed or μmol of product formed/min.

RESULTS

Oxidation of substrates by whole cells. Freshly harvested cyclohexanol-grown cells of *Nocardia globerula* CL1 oxidized the growth substrate with

the consumption of 2.2 μmol of oxygen/μmol of cyclohexanol added and a Q_{O_2} (μl of oxygen/h per mg dry wt.) of 39.

Cyclohexanone was oxidized with the consumption of 1.65 μmol of oxygen/μmol of substrate and a Q_{O_2} of 41. 2-Hydroxycyclohexan-1-one dimer and cyclohexane-1,2-dione were also oxidized at similar rates whereas ε-caprolactone and 6-hydroxycaproate were oxidized with relatively low Q_{O_2} values, 11 and 9 respectively, and cyclohexane-1,3-dione, *cis*- and *trans*-cyclohexane-1,4-diol and adipate were not oxidized. None of these substrates stimulated oxygen consumption by *p*-hydroxybenzoate-grown cells.

Oxidation of cyclohexanol by cell extracts. Attempts to demonstrate cyclohexanol-stimulated oxygen uptake or, where relevant, nicotinamide nucleotide reduction by extracts obtained either by French-pressure-cell treatment or sonication have not been successful. No activity was observed in the presence of NAD^+, $NADP^+$, 2,6-dichlorophenol-indophenol, Methylene Blue or phenazine methosulphate over a pH range from 7 to 10. This contrasts with the NAD^+-linked cyclohexanol dehydrogenase of *Acinetobacter* (N.C.I.B. 9871), which was observed by Dr P. J. Chapman (personal communication) to have an alkaline pH optimum. Reconstituted broken-cell systems in which the light fraction of the pellet obtained by centrifuging broken-cell preparations at 25000 *g* for 30 min was resuspended in buffer and used alone, or in conjunction with the supernatant, failed to catalyse a cyclohexanol-stimulated consumption of oxygen in the Warburg apparatus.

Oxidation of cyclohexanone by cell extracts. When 25000 *g* supernatant was incubated with cyclohexanone and phosphate buffer an NADPH-dependent consumption of oxygen was observed. NADH failed to initiate cyclohexanone-dependent oxygen consumption. An investigation of the stoicheiometry of the reaction with high-speed supernatant, obtained by centrifuging the initial cell extract at 130000 *g* for 1 h, demonstrated that the oxidation of 1 μmol of cyclohexanone was accompanied by the consumption of 1 μmol of oxygen and the oxidation of 1 μmol of NADPH (Table 1). This stoicheiometry is that which is theoretically required for a mixed-function oxygenase, a postulate reinforced by the obligatory requirement for oxygen before substrate-stimulated NADPH oxidation can proceed.

A possible product of such a mixed-function oxygenase reaction would be a hydroxycyclohexanone, and the high Q_{O_2} observed with whole cells and 2-hydroxycyclohexan-1-one dimer would make this a likely candidate. However, studies on analogous reactions involving (+)-camphor (Conrad, DuBus & Gunsalus, 1965; Trudgill, DuBus & Gunsalus, 1966) and quercitin (J. M. Wood, personal communication) have revealed lactones to be first identifiable products of similar mixed-function oxygenase-mediated reactions.

A bulk reaction in which 20 μmol of cyclohexanone was incubated with an excess of NADPH and 3.2 mg of 25000 *g* supernatant yielded a product that was acidic and co-chromatographed with 6-hydroxycaproic acid in all three t.l.c. systems (see the Materials and Methods section). Though no lactone was detected in the products of this reaction this is readily explained, even if it is a reaction intermediate, since the same acid was also produced on incubation of 25000 *g* supernatant with ε-caprolactone.

The lactone hydrolase (EC 3.1.1.–) thus demonstrated has a specific activity 30-fold greater than

Table 1. *Stoicheiometry of oxygen and NADPH requirements for the oxidation of cyclohexanone by 130000 g supernatant*

(*a*) The main compartments of Warburg flasks contained, in a volume of 2.0 ml, 80 μmol of phosphate buffer, pH 7.1, 2.2 mg of 130000 *g* supernatant, 2 μmol of $NADP^+$, 20 μmol of glucose 6-phosphate and 7 units of glucose 6-phosphate dehydrogenase. The centre wells contained 0.1 ml of 20% (w/v) KOH. Manometers were equilibrated at 30°C and reactions started by additions of cyclohexanone from side arms as indicated.

Cyclohexanone added (μmol) (*A*)	O_2 consumed (μmol) (*B*)		Ratio *B*/*A*
0	0.44	—	—
2.5	2.49	2.04	0.82
5.0	5.00	4.56	0.93
7.5	6.94	6.50	0.87

(*b*) Reaction mixtures in 1 cm-light-path cuvettes contained, in a volume of 3.0 ml, 120 μmol of phosphate buffer, pH 7.1, 0.29 mg of 130000 *g* supernatant and 0.5 μmol of NADPH. Reactions, at 30°C, were started by addition of cyclohexanone as indicated and the decrease in E_{340} was followed until it ceased.

Cyclohexanone added (μmol) (*A*)	$-\Delta E_{340}$	NADPH† oxidized (μmol) (*B*)	Ratio *B*/*A*
0	0	0	—
0.05	0.10	0.048	0.96
0.10	0.20	0.097	0.97
0.20	0.42	0.203	1.01
0.40	0.74	0.358	0.90
0.40*	0	0	—

* Anaerobic Thunberg cuvette.

† Specific activity 0.55 μmol of NADPH oxidized/min per mg of protein.

Table 2. *Hydrolysis of ε-caprolactone by 25000g supernatant from cyclohexanol-grown* N. globerula *CL*1

Reaction mixtures in 125mm×15mm test tubes, incubated at 30°C in a Gallenkamp Metabolic Shaking Incubator, contained in a volume of 2.0ml, 92μmol of phosphate buffer, pH 7.1, and 45μg of 25000g supernatant, and reactions were started by addition of 12μmol of ε-caprolactone. Samples (1ml) were removed for assay of residual lactone by the method of Cain (1961) at the time-intervals indicated.

Reaction time (min)	ε-Caprolactone† remaining (μmol)	
0	12.0	12.0*
2	10.0	12.0
4	9.2	12.0
8	5.7	11.0
12	3.5	10.5
16	0.5	10.5

* Boiled extract control.

† Specific activity of lactone hydrolase 15.7μmol/min per mg.

Table 3. *Measurement of acid production from ε-caprolactone by 25000g supernatant with a* pH-*stat*

The reaction vessel (25ml beaker) contained 5ml of water and 0.06mg of 25000g supernatant, adjusted to pH 7.0. Reactions were started by addition of substrate, agitated with a magnetic stirrer and maintained at pH 7.0 by controlled addition of mM-NaOH with a pH-stat. Alkali addition was monitored manually until reactions ceased.

ε-Caprolactone added (μmol) (*A*)	NaOH consumed (μmol) Observed	Corrected (*B*)	Ratio *B*/*A*
None	0.37	—	—
1.08	1.53	1.16	1.07
2.70	3.21	2.84	1.05
5.40	5.07	4.70	0.87
5.40*	0.41	0.04	—

* The 25000g supernatant was boiled before incubation.

that of the mixed-function oxygenase acting on cyclohexanone (Table 2), revealing a situation in which even a transient accumulation of any intermediate lactone is unlikely.

A quantitative assessment of acid production by 25000g supernatant with cyclohexanone or ε-caprolactone as substrate was made by using a pH-stat (Table 3). A unitary stoicheiometry of acid production was directly demonstrated with ε-caprolactone as substrate. A more complex situation exists with cyclohexanone as substrate because of the stoicheiometric NADPH requirement. When 1mg of 25000g supernatant was incubated with 5μmol of cyclohexanone and a slight excess of NADPH no net pH change was recorded at the completion of reaction (determined by measuring E_{340} of a sample of the reaction mixture). This is to be expected in a reaction system that consumes 1mol of NADPH for the production of 1mol of acid and is thus in overall protein balance. Prolonged incubation of 25000g supernatant with 5μmol of 2-hydroxycyclohexan-1-one dimer or cyclohexane-1,2-dione did not result in the liberation of any titratable acid.

Effect of inhibitors on cyclohexanone oxygenase activity of 25000g supernatant. The cyclohexanone-linked oxidation of NADPH was not inhibited by the metal-ion chelating agents αα′-bipyridyl, EDTA and Tiron, nor was any effect observed with sodium azide or sodium arsenite. A 40% inhibition of activity was, however, observed in the presence of 1mM-iodoacetamide.

Separation of cyclohexanone oxygenase from ε-caprolactone hydrolase and investigation of the products of the oxygenase reaction. Attempts to obtain the oxygenase free from contamination by lactone hydrolase by use of ammonium sulphate fractionation, selective adsorption on alumina C_γ gel or calcium phosphate gel and attempted selective inactivation by heat treatment were not successful.

Chromatography of 130000g supernatant that had been treated with protamine sulphate (0.1mg/mg of soluble protein) on a column (35cm×2.5cm) of Sephadex G-200 that had been calibrated with lactate dehydrogenase (EC 1.1.1.27), glucose 6-phosphate dehydrogenase and horse heart cytochrome *c* effected no significant separation of cyclohexanone oxygenase and ε-caprolactone hydrolase, but indicated them to have molecular weights of 95000 and 98000 respectively as determined by the method of Leach & O'Shea (1965). However, chromatography of 130000g supernatant on DEAE-cellulose yielded oxygenase fractions that were totally devoid of lactone hydrolase activity (Fig. 1). These fractions were pooled and protein precipitated by addition of ammonium sulphate to 70% saturation was separated by centrifuging at 2°C and 25000g for 15min and redissolved in 2ml of phosphate buffer.

Product of cyclohexanone oxygenation. Partially purified ε-caprolactone hydrolase-free cyclohexanone oxygenase catalysed the consumption of 1mol of oxygen and the formation of approx. 1mol of ε-caprolactone/mol of cyclohexanone added in the presence of excess of NADPH (Table 4). This establishes that, like the mixed-function oxygenases that utilize the cyclic ketones (+)-camphor (Conrad *et al.* 1965; Trudgill *et al.* 1966) and quercitin (Dr J. M. Wood, personal communication),

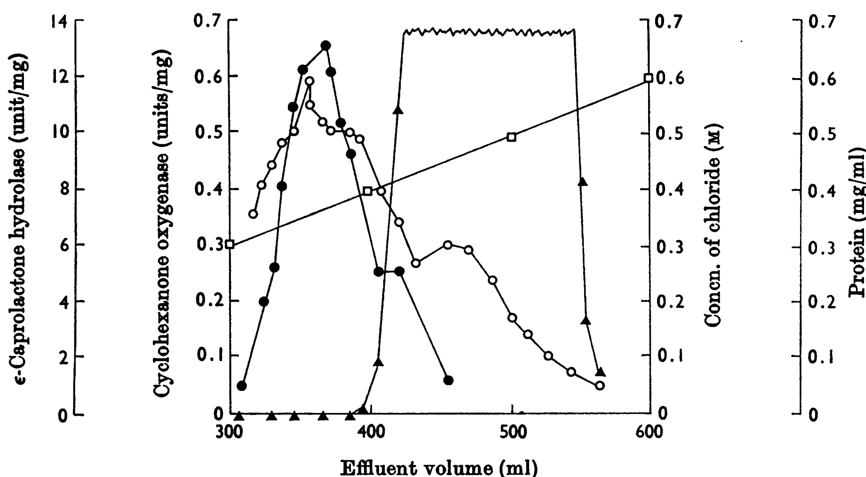


Fig. 1. Chromatography of 192mg of 130000g supernatant on a column (35cm × 2.5cm) of DEAE-cellulose (DE23) equilibrated with 0.023M-phosphate buffer, pH7.1. Cyclohexanone oxygenase and ε-caprolactone hydrolase were eluted with an increasing concentration of KCl in the same buffer and 7ml fractions collected. ○, Protein measured by $E^{1\,cm}_{260/280}$; ●, cyclohexanol oxygenase activity; ▲, ε-caprolactone hydrolase activity measured by the procedure described in Table 2; □, calculated molarity of KCl gradient.

Table 4. *Stoicheiometry of oxygen utilization and product formation for the oxidation of cyclohexanone by partially purified cyclohexanone oxygenase*

The main compartments of Warburg flasks contained, in a volume of 2.0ml, 50μmol of tris buffer, pH7.2, 0.34mg of purified cyclohexanone oxygenase (0.43 unit) from DEAE-cellulose chromatography and cyclohexanone and NADPH as indicated. The centre wells contained 0.1ml of 20% (w/v) KOH. Monometers were equilibrated at 30°C and reactions started by additions of protein from the side arms.

Cyclohexanone added (μmol)	NADPH added (μmol)	O_2 consumed (μmol)	ε-Caprolactone* formed (μmol)
0	0	0	0
10.3	0	0	0.1
0	12	0	0
10.3	12	11.0	8.5
5.15	6	5.5	4.7

* Assayed by the method of Cain (1961) and identified by co-chromatography with authentic ε-caprolactone as described in the Materials and Methods section.

cyclohexanone oxygenase also forms a lactone as the detected reaction product.

Oxidation of 6-hydroxycaproate to adipate by 25000g supernatant. When 25000g supernatant was incubated with 6-hydroxycaproate and NAD^+ under anaerobic conditions reduction of 2μmol of NAD^+ for each μmol of 6-hydroxycaproate was observed. 6-Hydroxycaproate-stimulated oxygen consumption in the Warburg apparatus was NAD^+-dependent, presumably being mediated by NADH oxidase present in the crude extract. Stoicheiometrically 1μmol of oxygen was consumed for each μmol of 6-hydroxycaproate added, and a compound that co-chromatographed with adipic acid in all three t.l.c. systems (see the Materials and Methods section) was the only detectable acid product (Table 5).

Induced and basal concentrations of the enzymes catalysing cyclohexanol (cyclohexanone) oxidation. *p*-Hydroxybenzoate (2g/l) was used as a growth substrate to obtain cells for measurement of basal enzyme activities, since *N. globerula* CL1 failed to grow with glucose or any one of the following more polar compounds, acetate, glutamate, malate or succinate, as a carbon source. Cells were harvested from the mid-exponential phase of growth, and the specific activities of relevant enzymes of 25000g supernatant prepared from these and cyclohexanol-

Table 5. *Chromatographic characterization of the reaction product formed upon oxidation of 6-hydroxycaproate by 25000g supernatant*

The reaction mixture contained, in a volume of 2.0ml, 80μmol of phosphate buffer, pH 7.1, 9.7mg of 25000*g* supernatant, 5μmol of NAD^+ and 20μmol of 6-hydroxycaproate. After incubation at 30°C for 30min the reaction was stopped by the addition of 1ml of 5M-HCl and protein denaturation was completed by a cycle of freezing and thawing. Denatured protein was removed by centrifuging at 25000*g* for 10min. The supernatant was extracted with an equal volume of redistilled diethyl ether. The ether layer was dried over anhydrous Na_2SO_4, ether was removed under a stream of N_2 and the residue was dissolved in 0.4ml of acetone and chromatographed.

	R_F values		
Solvent system	Adipic acid	6-Hydroxycaproic acid	Reaction product
A	0.59	0.41	0.59
B	0.59	0.43	0.60
C	0.73	0.63	0.74

Table 6. *Activities of enzymes of cyclohexanol oxidation in extracts of* N. globerula *CL*1

The rates of reactions in 25000*g* supernatants are expressed as specific activities with respect to the assayable component of the reaction. For conditions of assay, see the Materials and Methods section or appropriate table.

	Specific activity in extracts of *N. globerula* CL1 grown on	
	Cyclohexanol	*p*-Hydroxybenzoate
Cyclohexanol dehydrogenase	?	?
Cyclohexanone oxygenase	0.55	0.0003
ϵ-Caprolactone hydrolase	15.6	Not detectable
6-Hydroxycaproate → adipate	0.05	Not detectable

grown cells are shown in Table 6. A greater than 100-fold increase in specific activity of all three assayed activities was stimulated by growth with cyclohexanol.

DISCUSSION

The ability of cyclohexanol-grown *N. globerula* CL1 to oxidize cyclohexanone, 2-hydroxycyclohexan-1-one dimer and cyclohexan-1,2-dione at rates similar to that obtained with the growth substrate might be considered as indicating that these compounds are intermediates in cyclohexanol oxidation, whereas the inability of such cells to oxidize ϵ-caprolactone, 6-hydroxycaproate and adipate at significant rates might be judged as excluding them.

Evidence obtained with subcellular systems does not justify the inclusion of cyclohexane-1,2-dione and 2-hydroxycyclohexan-1-one dimer as intermediates, but firmly establishes ϵ-caprolactone, 6-hydroxycaproate and adipate as intermediates in cyclohexanone oxidation.

Possible explanations of these anomalous observations may reside in gratuitous induction phenomena (2-hydroxycyclohexan-1-one dimer-grown cells oxidize cyclohexanol and cyclohexanone), the equivocal status of commercial 2-hydroxycyclohexan-1-one because of its dimerized state and the inability of polar substrates to penetrate into this organism, reflected by its inability to grow with a variety of polar compounds.

Our inability to demonstrate what we believe to be the first step of the pathway, the oxidation of cyclohexanol to cyclohexanone, in a variety of broken-cell systems with both natural and artificial electron acceptors is unexplained and is in contrast with the NAD^+-linked dehydrogenase found in cyclohexanol-grown *Acinetobacter* N.C.I.B. 9871 by Dr P. J. Chapman (personal communication).

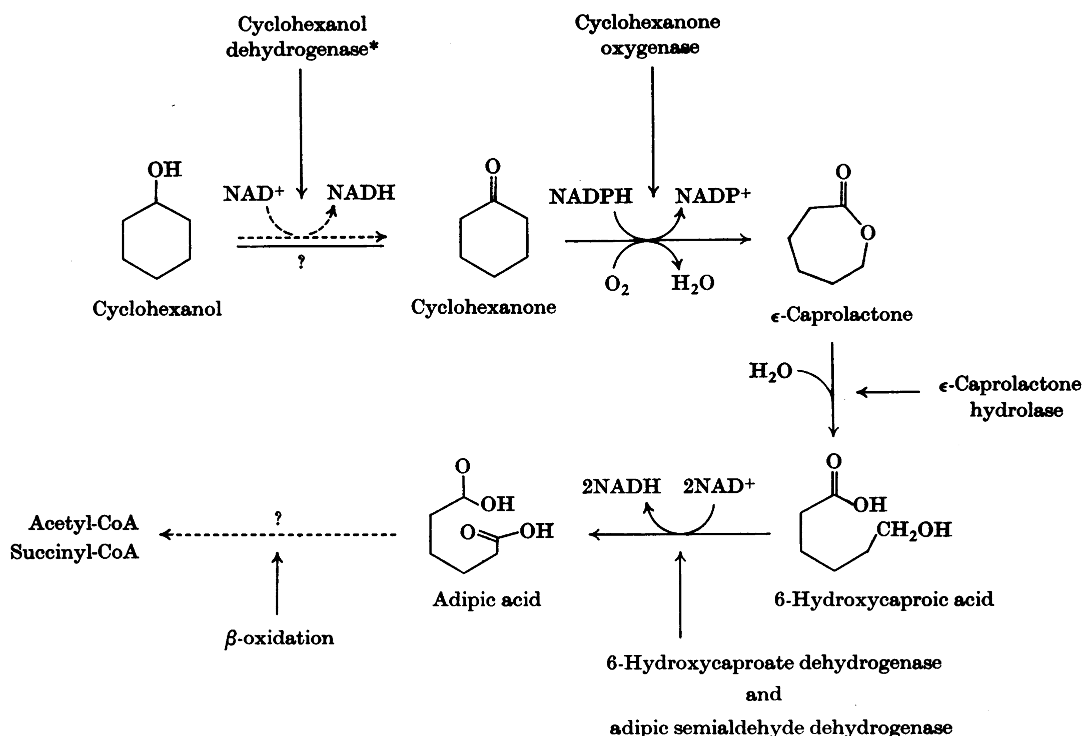
The cyclohexanone oxygenase of *N. globerula* CL1 is tightly coupled: NADPH oxidation does not occur in the absence of substrate. The near unitary stoicheiometry of cyclohexanone, oxygen and NADPH consumption and the total dependence of substrate-stimulated NADPH consumption in the presence of oxygen indicates this to be an example of a classical mixed-function oxygenase, as first described by Mason (1957). The lack of inhibitory effects displayed by metal-ion chelating agents

provisionally assigns this enzyme to the group of external mono-oxygenases that do not require metal ions for activity (Hayaishi, 1969).

The identification of 6-hydroxycaproate as the product of cyclohexanone oxygenation by crude 25000*g* supernatant confirms the observation by Dr P. J. Chapman without, however, divulging the nature of the initial product of the oxygenase reaction. The possibility that, as in the case of (+)-camphor (Conrad *et al.* 1965; Trudgill *et al.* 1966) and quercitin oxygenations, the reaction with cyclohexanone, likewise a ketone, might also yield a lactone is suggested by the presence of an induced ϵ-caprolactone hydrolase in 25000*g* supernatant from cyclohexanol-grown cells that hydrolyses this substrate with the stoicheiometric formation of 6-hydroxycaproate, the same product as is formed subsequent to the hydroxylation of cyclohexanone.

The stoicheiometric yield of 6-hydroxycaproate from cyclohexanone is indirectly demonstrated by the lack of titratable acid production when cyclohexanone and NADPH are incubated with 25000*g* supernatant in the pH-stat. Since the reaction was demonstrated, by monitoring of E_{340}, to have proceeded to completion, proton balance could only have been maintained by the production of an amount of acid equivalent to the NADPH oxidized.

Because of the very high specific activity of ϵ-caprolactone hydrolase, an investigation designed to establish whether or not ϵ-caprolactone was the primary product of cyclohexanone oxygenation necessitated the use of an oxygenase fraction devoid of ϵ-caprolactone hydrolase activity. Of the separation procedures utilized only chromatography on DEAE-cellulose was sufficiently discriminating to yield oxygenase fractions totally devoid of ϵ-caprolactone hydrolase activity. Though the use of such partially purified oxygenase fractions enabled the product of cyclohexanone oxygenation to be established as ϵ-caprolactone, it provides no indication as to the mechanism of this type of oxygenase reaction. The inability of 25000*g* supernatant (capable of oxidizing cyclohexanone to adipate in the presence of NADPH and NAD^+) to metabolize either 2-hydroxycyclohexan-1-one dimer or cyclohexane-1,2-dione eliminates these compounds as metabolic intermediates in cyclohexanone oxidation. The possibility that a cyclohexanone epoxide



* P. J. Chapman (personal communication).

Scheme 1. Reaction sequence for the oxidation of cyclohexanol by *Nocardia globerula* CL1.

or a hydroxycyclohexanone monomer is formed as a precursor of the ε-caprolactone cannot be excluded. Unfortunately our present lack of knowledge concerning oxygenated intermediates in mono-oxygenase systems hinders development of this consideration. The mono-oxygenase-catalysed epoxidation of a double bond in squalene that initiates cyclization (Corey, Russey & Ortiz de Montellano, 1966; Van Tamelen, Willett, Clayton & Lord, 1966) involves a substrate so dissimilar to cyclohexanone that it is difficult to draw comparisons.

There is no evidence for the oxidation by *N. globerula* CL1 of 2-hydroxycyclohexan-1-one to cyclohexane-1,2-dione and the further metabolism of the dione to adipic semialdehyde and adipate as described by Yugari (1961) for a species of *Pseudomonas*. Although we acknowledge that whole-cell oxidation studies clearly demonstrate the presence of enzyme able to dissimilate these compounds, we feel justified, for the reasons stated above, in excluding them from the proposed catabolic sequence outlined in Scheme 1.

An induced oxidation of 6-hydroxycaproate to adipate in which NAD^+ is reduced under anaerobic conditions, or coupled to oxygen consumption in air, has been demonstrated. It is noteworthy that homologous straight-chain dicarboxylic acids have been identified in cultures of bacteria utilizing *n*-paraffin hydrocarbons (Ali Khan, Hall & Robinson, 1964; Kester & Foster, 1963) and alicyclic hydrocarbons (Colla & Treccani, 1960). The further oxidation of adipate by *N. globerula* CL1 remains to be investigated, but might be expected to occur via β-oxidation to yield acetyl-CoA, since indirect evidence for the conversion of adipate into β-oxoadipyl-CoA by *Pseudomonas aeruginosa* has been obtained by Ornston (1966) and cleavage of β-oxoadipyl-CoA to acetyl-CoA and succinyl-CoA by bacterial enzymes has been demonstrated by Katagiri & Hayaishi (1957). Further support for this pathway where succinyl-CoA or succinate then enters the tricarboxylic acid cycle has been obtained with mutants of *P. aeruginosa* by Chapman & Duggleby (1967).

We thank Dr M. E. Rhodes, who performed the taxonomic classification of *N. globerula*, Dr P. J. Chapman, who engaged in frequent useful discussions of this work, Mr J. S. Rees, who provided expert technical assistance, and Miss J. T. Peel for typing the manuscript.

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