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Metabolism of Cyclohexane Carboxylic Acid by *Alcaligenes* Strain W1

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Thirty-three microorganisms capable of growth with cyclohexane carboxylate as the sole source of carbon were isolated from mud, water, and soil samples from the Aberystwyth area. Preliminary screening and whole-cell oxidation studies suggested that, with one exception, all of the strains metabolized the growth substrate by beta-oxidation of the coenzyme A ester. This single distinctive strain, able to oxidize rapidly *trans*-4-hydroxycyclohexane carboxylate, 4-ketocyclohexane carboxylate, *p*-hydroxybenzoate, and protocatechuate when grown with cyclohexane carboxylate, was classified as a strain of *Alcaligenes* and given the number W1. Enzymes capable of converting cyclohexane carboxylate to *p*-hydroxybenzoate were induced by growth with the alicyclic acid and included the first unambiguous specimen of a cyclohexane carboxylate hydroxylase. Because it is a very fragile protein, attempts to stabilize the cyclohexane carboxylate hydroxylase so that a purification procedure could be developed have consistently failed. In limited studies with crude cell extracts, we found that hydroxylation occurred at the 4 position, probably yielding the *trans* isomer of 4-hydroxycyclohexane carboxylate. Simultaneous measurement of oxygen consumption and reduced nicotinamide adenine dinucleotide oxidation, coupled with an assessment of reactant stoichiometry, showed the enzyme to be a mixed-function oxygenase. Mass spectral analysis enabled the conversion of cyclohexane carboxylate to *p*-hydroxybenzoate by cell extracts to be established unequivocally, and all of our data were consistent with the pathway: cyclohexane carboxylate → *trans*-4-hydroxycyclohexane carboxylate → 4-ketocyclohexane carboxylate → *p*-hydroxybenzoate. The further metabolism of *p*-hydroxybenzoate proceeded by *meta* fission and by the oxidative branch of the 2-hydroxy-4-carboxymuconic semialdehyde-cleaving pathway.

The microbial metabolism of alicyclic hydrocarbons and alcohols is predominantly mediated by their conversion to alicyclic ketones, and these, in conjunction with natural alicyclic ketones, are cleaved by the introduction of a ring oxygen by a biological Baeyer-Villiger reaction (3) yielding lactones that are either inherently unstable or enzymatically hydrolyzed (12, 14, 18, 26, 29). Alicyclic ketone 1,2-monooxygeneases have been purified from a number of sources (13, 19) and characterized as flavoproteins requiring either reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide dinucleotide phosphate (NADPH) as the electron donor (13, 19).

There is now a considerably body of evidence to support the view that the introduction of a carboxyl group blocks cleavage by the above mechanism with the mandatory involvement of alternative pathways. Guilbert and Chung (20) have implicated the carnitine derivative of cyclopropane carboxylate in its degradation by *Fusarium oxysporum*, although the precise ring cleavage steps are still unclear (35).

Work by Dutton and Evans (15) and Whittle et al. (40) with *Rhodopseudomonas palustris* has shown that it can dissimilate benzoate anaerobically in the presence of light by a reductive pathway and that cyclohexane carboxylate (CHC) can be integrated into this sequence. Attack at the 1,2 position of the coenzyme A ester of the compound yields pimelate by a beta-oxidation sequence. Preliminary evidence also indicates that this pathway and modifications of it may be broadly distributed and are not confined to photosynthetic organisms (16, 30).

In contrast, Kaneda (21), Blakley (6), and Smith and Callely (37) have provided evidence to support an interesting alternative pathway in which hydroxylation of CHC at the 4 position is followed by dehydrogenation to yield the 4-keto compound, which, by removal of four electrons, yields *p*-hydroxybenzoate. Further metabolism of the *p*-hydroxybenzoate occurs either via the

β-ketoadipate pathway (27) or by the *meta*-fission sequence (10).

So far, this aromatization pathway has only been reported for strains of the gram-positive genera *Achromobacter* and *Corynebacterium*. The initial hydroxylation reaction has not been effectively demonstrated, and only limited studies of the aromatization step have been undertaken (22).

In this paper, we report the isolation of a number of microorganisms capable of growth with CHC as the sole source of carbon and the results of detailed studies with a strain of *Alcaligenes* that makes use of the aromatization pathway and yields cell extracts in which all of the enzymes of the sequence can be assayed.

MATERIALS AND METHODS

Bacterial strains. Bacteria were isolated by elective culture from a wide range of pasteurized and untreated soil samples gathered in the Aberystwyth area in Wales. Typically, CHC was used as the elective carbon source, although microorganisms obtained by election with *trans*-cyclohexane-1,2-diol and cyclopentane carboxylate were frequently also shown to be capable of growth with CHC.

Culture medium and growth of bacteria. Stock cultures were maintained on nutrient agar. After growth at 30°C, they were stored at 2°C and subcultured at monthly intervals. Induced cells were obtained by growth at 30°C in mineral salts medium containing (grams per liter): KH_2PO_4, 2.0; Na_2HPO_4, 4.0; $(NH_4)_2SO_4$, 2.0; and CHC, 1.0. The medium was adjusted to pH 7.2 with 5 M NaOH. A complex mixture of inorganic salts in aqueous solution (33) was added at 0.4% (vol/vol) before sterilization of the medium.

Large crops of cells were grown by the following regimen. A 50-ml amount of culture medium in a 250-ml conical flask was inoculated from a slant. After 24 h of growth at 30°C in an orbital shaker (Gallenkamp & Co. Ltd., Widnes, U.K.) at 150 cycles/min, the culture was used to inoculate 500 ml of medium in a 2-liter conical flask. After 8 h, the resulting culture was used to inoculate a 10-liter batch of medium in a Microferm laboratory fermentor (New Brunswick Scientific Co.) supplied with sterile air at 5 liters/min and stirred at 400 rpm. Cells were harvested in the late logarithmic phase of growth (typically at an absorbance of 200 Klett units in a Klett-Summerson photoelectric colorimeter with a 545- to 620-nm filter) in a Sharples centrifuge, washed by suspension in phosphate buffer (KH_2PO_4, 2 g/liter; Na_2HPO_4, 4 g/liter; pH 7.1), and centrifuged at 23,000 × *g* (maximum) for 15 min.

For specific purposes, crops of cells were also grown with *p*-hydroxybenzoate (1 g/liter) and sodium succinate (3 g/liter) as the sole sources of carbon, and the growth regimen was modified to account for various doubling times.

Preparation of cell extract (160,000 × *g* supernatant). Washed cells, suspended in the phosphate buffer (pH 7.1, 1.5 cell volume), were disrupted by passage through a French press as previously described (14), and unbroken cells and insoluble components were removed by centrifugation at 160,000 × *g* (average) and 3°C for 60 min.

Preparation of a washed membrane fraction. The pink upper layer of cell debris remaining from preparation of the 160,000 × *g* supernatant was carefully removed, suspended in phosphate buffer (pH 7.1), and again centrifuged at 160,000 × *g* for 30 min. A second suspension and centrifugation step completed the washing of the membrane fraction, which was finally suspended in a small volume of buffer and used within a short time.

Estimation of protein. The biuret assay previously described (15) was used routinely in estimating protein levels.

Gas exchange reactions. Gas exchange catalyzed by whole-cell suspensions or by cell extracts was measured in a Warburg apparatus or with a Clark-type O_2 electrode (oxygen monitor; model 53, Yellow Springs Instrument Co.) in an agitated vessel maintained at 30°C.

Enzyme units. All enzyme activities were expressed as micromoles of substrate consumed or product formed per minute.

Enzymes of *p*-hydroxybenzoate metabolism. *p*-Hydroxybenzoate hydroxylase was assayed by the procedure of Gibson (17). Protocatechuate 3,4-oxygenase and protocatechuate 4,5-oxygenase were assayed as described by Stanier and Ingraham (38) and Dagley et al. (11), respectively. 2-Hydroxy-4-carboxymuconic semialdehyde hydrolase activity was determined spectrophotometrically at 410 nm, and 2-hydroxy-4-carboxymuconic semialdehyde dehydrogenase was determined from the rate of disappearance of 2-hydroxy-4-carboxymuconic semialdehyde catalyzed by cell extract in the presence of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) (34).

Enzymes of CHC aromatization. CHC hydroxylase was assayed either by measuring the stimulation of NADH oxidation at 340 nm when 2.5 μmol of CHC was added to a 1-cm-light-path cuvette that contained (in a final volume of 1 ml) 90 μmol of phosphate buffer (pH 7.6), 0.3 μmol of NADH, and the test extract (0.2 to 2 mg of protein), or by measuring the rate of oxygen consumption when 5 μmol of CHC was injected into the vessel that contained (in 3 ml) 290 μmol of phosphate buffer (pH 7.6), 1 μmol of NADH, and the test extract (0.5 to 5 mg of protein). For stoichiometric studies, amounts of CHC and NADH were varied as required, and the simultaneous measurement of NADH oxidation and O_2 consumption was performed in an apparatus constructed as described by Ribbons et al. (32). *trans*-4-Hydroxycyclohexane carboxylate (*trans*-4-hydroxyCHC) dehydrogenase was assayed either by measuring the rate of NADH formation when 2 μmol of *trans*-4-hydroxyCHC was added to a 1-cm-light-path cuvette that contained (in a volume of 1 ml) 80 μmol of glycine-NaOH buffer (pH 9.0), 2 μmol of NAD, and the test extract (0.1 to 4 mg of protein), or in the reverse direction by measuring the rate of NADH oxidation when 1 μmol of 4-ketocyclohexane carboxylate (4-ketoCHC) was added to a cuvette containing (in a volume of 1 ml) 90 μmol of phosphate buffer (pH 7.0), 0.2 μmol of NADH, and the test extract (0.1 to 1 mg of protein).

Catalase activity of the 160,000 × *g* supernatant from *Alcaligenes* strain W1 was assayed in the oxygen monitor by following the rate of oxygen production when 2 μmol of H_2O_2 was injected into the vessel that contained (in 3 ml) 290 μmol of anaerobic phosphate buffer (pH 7.8) and 0.02 to 0.2 mg of protein. Observed oxygen evolution rates were corrected for boiled protein controls.

For specific spectrophotometric assays under anaerobic conditions, Thunberg cuvettes were evacuated three times with a Speedivac two-stage high-vacuum pump (Edwards High Vacuum Ltd., Manor Royal, U.K.), each evacuation being followed by an argon flush.

Chromatographic procedures. Thin-layer chromatography of CHC *p*-hydroxybenzoic acid and related compounds was performed on 0.25-mm-thick layers of Kieselgel GF_{254} (type 60, E. Merck, Darmstadt, W. Germany) developed with solvent A (toluene-dioxane-acetic acid, 67:19:4 [by volume]), solvent B (ethyl formate-petroleum ether [bp 60 to 80°C]-propionic acid, 14:6:3 [by volume]), or solvent C (propan-2-ol-toluene-ammonia, 3:1:1 [by volume]).

Detection of compounds on chromatograms. Carboxylic acids were detected by spraying thoroughly dried plates with 0.1% (wt/vol) bromocresol green in aqueous 95% ethanol adjusted to pH 6 with NaOH. Ketones were detected by spraying with 0.1% (wt/vol) 2,4-dinitrophenylhydrazine in 5 M HCl and observing the yellow derivatives thus formed. *p*-Hydroxybenzoate, protocatechuate, and related compounds were located (i) by direct visual observation under 254-nm UV light or (ii) after plates were sprayed with the Folin-Ciocalteau phenol reagent and exposed to ammonia, or (iii) after plates were sprayed with diazotized *p*-nitroaniline.

Gas-liquid chromatography. Gas-liquid chromatography was performed in a Pye series 104 chromatograph (Pye-Unicam, Cambridge, U.K.) on glass columns (4 mm by 1.5 m) packed with 1.25% (wt/wt) diglycerol, 3% (wt/wt) OV 17, or 10% (wt/wt) diethylene-glycol succinate on acid-washed Chromosorb W (60 to 80 mesh). A judicious use of these three column systems in combination with a variety of temperature-programmed and isothermal operating conditions allowed the methyl esters of all investigated intermediates of CHC metabolism and those of a range of related compounds, prepared by the procedure of Metcalfe and Schmitz (24), to be separated from each other.

Mass spectra. Mass spectra of authentic methyl *p*-hydroxybenzoate and of methylated reaction products were determined at an ionization potential of 24 eV on an A.E.I. MS 30 instrument coupled to a Pye series 104 gas-liquid chromatograph fitted with a 3% (wt/wt) OV 17 column.

Preparation of compounds. Ethyl *p*-hydroxybenzoate was prepared as described by Cavill and Vincent (9). *cis,trans*-4-HydroxyCHC (ethyl ester) was prepared by catalytic hydrogenation of ethyl *p*-hydroxybenzoate at 150°C and a pressure of 100 to 150 atm for 24 h (38) in the presence of Raney nickel (grade 4). The product (distillation range, 138 to 148°C at 8 to 9 mm of Hg) was saponified by refluxing with 10% (wt/wt) aqueous KOH, ether extracted from the acidified reaction mixture, and recrystallized from boiling ethyl acetate as a white solid that softened at 119°C and melted at 125 to 127°C. These data are compatible with the product consisting of a mixture of *cis* and *trans* isomers of 4-hydroxyCHC (8).

4-KetoCHC was prepared, as described by Artico et al. (1), by condensation of diethylmalonate and ethylacrylate in the presence of sodium ethoxide to yield the triethyl ester of 2,4,4-tricarboxycyclohexanone (bp 174 to 180°C at 5 mm of Hg). The ester was hydrolyzed by refluxing with aqueous ethanolic NaOH for 8 h, and the mixture was acidified and refluxed for a further 2 h to decarboxylate the product. The 4-ketoCHC was extracted with diethyl ether and distilled (bp 185 to 190°C at 50 mm of Hg) to give a clear viscous oil that crystallized on cooling.

Analysis of the *cis,trans*-4-hydroxyCHC and the 4-ketoCHC by thin-layer chromatography demonstrated the former compound to be a pure mixture of the geometric isomers and the latter to contain only very small traces of impurities.

2-Hydroxy-4-carboxymuconic semialdehyde was prepared biologically. Warburg flasks contained (in a volume of 2 ml) 47 μmol of phosphate buffer (pH 7.2), 2.25 μmol of L-cysteine, 3.3 μmol of $FeSO_4$, and 2 mg of dialyzed 160,000 × *g* supernatant from *p*-hydroxybenzoate-grown cells. Reactions at 30°C were initiated by the addition of 4 μmol of protocatechuate. When oxygen uptake ceased, flask contents were transferred to 0.5 ml of 2.5 M HCl, and the yellow reaction product was extracted with 10 ml of ethyl acetate. The aqueous layer was discarded, and the product was extracted from the ethyl acetate with 3 ml of 0.1 M NaOH (adjusted to pH 8), maintained at 3°C, and used the same day.

Chemicals. NAD, NADH, NADP, NADPH, and catalase were obtained from the Boehringer Corporation; CHC, diethylmalonate, 3,4-dihydroxybenzoic acid (protocatechuic acid), ethylacrylate, *p*-hydroxybenzoic acid, and phenazine methosulfate were obtained from B.D.H., Ltd.; Chromosorb W and diglycerol were obtained from Field Instruments Ltd.; OV 17 was supplied by the Pierce Chemical Co.; dithiothreitol was obtained from Sigma Chemical Co., and diethylene-glycol succinate was supplied by Wilkens Instruments. *cis*-4-HydroxyCHC, *trans*-4-hydroxy CHC, and 3-ketocyclohexane carboxylic acid were very generously donated by W. C. Evans.

Compounds used in growth-screening experiments were obtained from sources previously described (13), and the purest grades of organic solvents commercially available were dried and redistilled before use.

RESULTS

Isolation of bacteria. Microorganisms capable of growth with CHC are easily isolated from the biosphere. In addition to our strains isolated by elective culture from soil and mud samples obtained in the Aberystwyth area, all of the gram-positive organisms and four of the gram-negative strains that we obtained by elective culture with *trans*-cyclohexane-1,2-diol (12) were capable of growth with CHC.

The CHC-utilizing bacteria were generally also capable of growth with cyclopentane car-

boxylate and alicyclic alcohols and ketones. Preliminary studies with a number of them revealed that growth on CHC yielded cells that were capable of the oxidation of cyclopentane carboxylate, pimelate, and adipate in addition to the growth substrate, but incapable of oxidizing *p*-hydroxybenzoate or protocatechuate. We have tentatively assumed, without further investigation, that these organisms use the fatty acid beta-oxidation pathway (15, 30, 40) to cleave the cyclohexane ring.

One gram-negative organism isolated from garden soil was distinguished by its inability to grow with simple alicyclic alcohols and ketones and by the ability of CHC-grown cells to oxidize *p*-hydroxybenzoate and protocatechuate (3,4-dihydroxybenzoate) while being unable to oxidize pimelate.

Because these preliminary observations indicated that this gram-negative organism, designated strain W1, might possibly make use of the proposed aromatization pathway (6, 21, 37) for the dissimilation of CHC, it was subjected to a detailed study in the hope that it would add to our currently incomplete understanding of the enzymology of microbial aromatization of the alicyclic ring.

Identity of strain W1. Strain W1 is an obligately aerobic, gram-negative, coccal rod (0.5 by 1.5 μm) that is actively motile by polar or degenerate peritrichous flagella (35). It is catalase and oxidase positive, has simple growth requirements, is alkaline in litmus milk, and reduces nitrate to nitrite. It fails to grow at 40°C, is gelatinase and indole negative, does not accumulate poly-β-hydroxybutyrate, and is not proteolytic. It is unable to utilize arginine and fails to produce fluorescent pigments on King B medium. It is thus preferably classified as a strain of *Alcaligenes* rather than *Pseudomonas alcaligenes*, and we have designated it *Alcaligenes* strain W1.

Growth experiments. Batches of growth medium were inoculated with 1 ml of a freshly grown nutrient broth culture of *Alcaligenes* strain W1 and incubated in a gyratory shaker at 30°C. Cultures were visually examined at 24-h intervals. Good growth was observed with CHC, *cis,trans*-4-hydroxyCHC, 4-ketoCHC, *p*-hydroxybenzoate, protocatechuate, 1-oxa-2-oxocycloheptane, adipate, and pimelate. No growth occurred, even after an extended period of incubation, with *cis*-4-hydroxyCHC, cyclohexanol, *trans*-cyclohexane-1,2-diol, *cis,trans*-cyclohexane-1,3-diol, *cis,trans*-cyclohexane-1,4-diol, 2-hydroxycyclohexanone, cyclopentanone, cyclohexanone, cyclooctanone, 2-methylcyclohexanol, 3-methylcyclohexanol, 4-methylcyclohexanol, cyclopentane carboxylate, cyclohexane acetate, cyclohexane propionate, or cyclohexane butyrate.

Growth of this organism on simple alicyclic structures appears to be restricted to CHC and its *trans*-4-hydroxy and 4-keto derivatives, intermediates of the proposed pathway of aromatization by *C. cyclohexanicum* (21) and *Arthrobacter* strain PRL W15 (6).

Oxidation of substrates by whole cells. Freshly harvested CHC-grown cells of *Alcaligenes* strain W1 were capable of the rapid oxidation of the growth substrate after a short lag that was sometimes, but not always, observed, *trans*-4-hydroxyCHC, 4-ketoCHC, *p*-hydroxybenzoate, and protocatechuate (Fig. 1). Limited oxygen uptake occurred with *cis,trans*-4-hydroxyCHC. The rate of oxidation of pimelate

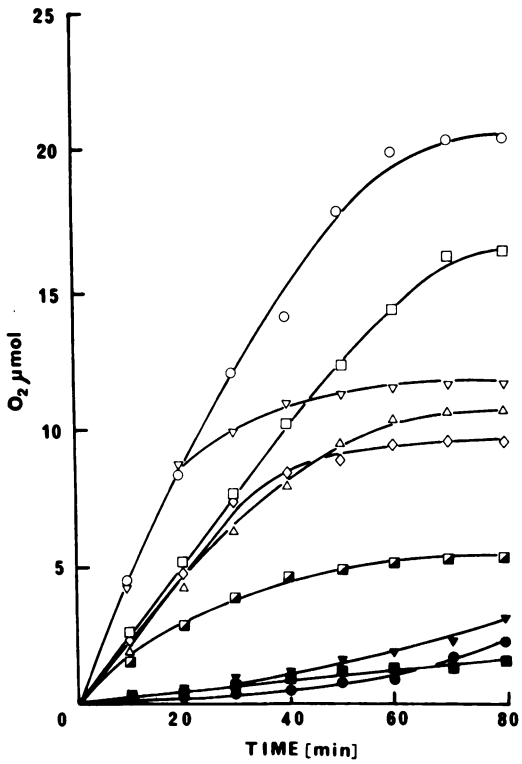

FIG. 1. *Substrate oxidation by whole cells of CHC-grown Alcaligenes strain W1. Warburg flasks contained (in a total volume of 2 ml) 1.5 mg (dry weight) of Alicaligenes strain W1 and 74 μmol of phosphate buffer (pH 7.1). Reactions at 30°C were started by the addition of substrates (3 μmol) from side arms. Center wells of flasks contained 0.1 ml of 20% KOH and a strip of filter paper to facilitate absorption of CO_2. Symbols: ○, CHC; ◪, cis,trans-4-hydroxyCHC; ■, cis-4-hydroxyCHC; □, trans-4-hydroxyCHC; △, 4-ketoCHC; ▽, p-hydroxybenzoate; ◇, protocatechuate; ●, adipate; ▼, pimelate. Cyclopentane carboxylate and benzoate were not oxidized.*

was very low, and no significant oxidation of *cis*-4-hydroxyCHC, cyclopentane carboxylate or benzoate occurred.

Cells grown with *cis,trans*-4-hydroxyCHC or 4-ketoCHC displayed similar substrate oxidation profiles, although with the former a lag period of about 30 min preceded the initiation of CHC oxidation, and cells grown with 4-ketoCHC did not show any stimulation of oxygen uptake by CHC during an experimental period of 2 h.

In all cases, Warburg flasks with protocatechuate in the side arm rapidly assumed a bright yellow color within a few minutes after the addition of substrate. The color faded in the later stages of oxygen consumption, strong visual evidence that protocatechuate was metabolized by a *meta*-fission sequence (10) in this organism.

Metabolism of CHC by extracts of *Alcaligenes* strain W1. The ability of CHC-grown *Alcaligenes* strain W1 to oxidize *p*-hydroxybenzoate and protocatechuate, concomitant with the induction of enzymes of the *meta*-fission pathway, was indicative that, as reported for *C. cyclohexanicum* and *Arthrobacter* strain PRL W15, it too makes use of a sequence of electron-withdrawing steps to generate *p*-hydroxybenzoate from the growth substrate.

The initial step in aromatization, presumed to be the hydroxylation of the growth substrate to yield *trans*-4-hydroxyCHC (6, 21), has not been effectively demonstrated in the gram-positive strains studied, although Kaneda et al. (22) have given a preliminary report of a highly unstable enzyme of low activity in crude extracts of *C. cyclohexanicum* that can only be assayed with [1-^{14}C]CHC and that, in the presence of NADH and O_2, generates *trans*-4-hydroxy[^{14}C]CHC.

Alcaligenes strain W1 differs from these gram-positive bacteria in that it possesses a CHC hydroxylase that can be assayed by conventional means, and the overall conversion of CHC to *p*-hydroxybenzoate by extracts can thus be demonstrated.

Cyclohexane carboxylate hydroxylase. The 160,000 × *g* supernatant from CHC-grown *Alcaligenes* strain W1 catalyzed the rapid oxidation of NADH and the consumption of O_2 in the presence of CHC. Figure 2 shows a simultaneous assay of O_2 consumption and NADH oxidation. The enzyme had a pH optimum of 7.6 and was inactive with NADPH as the electron donor, and NADH oxidation was not observed under anaerobic conditions. Specificity toward the alicyclic substrate was also strict. Limited activity toward cyclopentane carboxylate and cyclohexyl acetic acid could be detected (Table 1), but other substrate analogs were ineffective either as substrates or as competitive inhibitors.

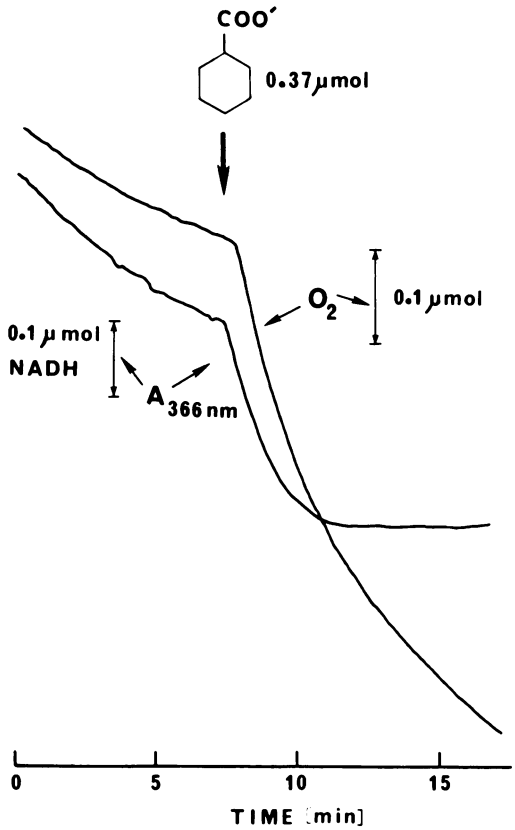

FIG. 2. *Simultaneous assay of oxygen consumption and NADH oxidation during CHC oxidation by cell extracts of Alcaligenes strain W1. The reaction mixture contained 335 μmol of KH_2PO_4-NaOH buffer (pH 7.6), 160,000 × g supernatant protein (5.5 mg), 0.93 μmol of NADH, and 0.248 μmol of CHC carboxylate, added as indicated. Temperature, 30°C. $A_{366\ nm}$, Absorbance at 366 nm.*

TABLE 1. *Monooxygenase activity of the 160,000 × g supernatant toward analogs of CHC*

Compound	mM	Relative rates of oxidation[a]
CHC	2	1.0
Cyclopentane carboxylate	0.4	0.05
	2	0.11
	18	0.21
Cyclohexyl acetate	0.4	0.14
	2	0.16
	10	0.16
Cyclopropane carboxylate	2	<0.01
trans-Cyclohexane 1,2-dicarboxylate	2	<0.01
1-Methylcyclohexane carboxylate	2	<0.01
Cyclohexylamine	2	<0.01

[a] Oxidation of NADH assayed spectrophotometrically. The concomitant consumption of oxygen was verified with an O_2 electrode.

Attempts to establish the stoichiometric relationships among the three substrates were not very satisfactory. The consumption of approximately 1 μmol of O_2 and the oxidation of approximately 1 μmol of NADH for each micromole of cyclohexane carboxylate added to the reaction system was evident (Fig. 2), but a precise assessment was not possible because the initial rapid phase of activity was followed by a phase of slower oxygen consumption and a suppression of endogenous NADH oxidation presumably associated with the further metabolism of the product of hydroxylation.

Attempts to separate the cyclohexane carboxylate monooxygenase from other enzymes of the sequence, thus enabling it to be studied in isolation, have been totally frustrated by the unstable character of the enzyme.

Enzyme activity was stable for extended periods in cell pastes and extracts stored at −20°C but was rapidly lost from extracts stored at 3°C. Attempts to stabilize the enzyme by addition of CHC, mercaptoethanol, riboflavine 5′-phosphate, flavine adenine dinucleotide, ferrous sulfate, or organic solvents, either singly or in combination, were without success. Maintenance of extract under an atmosphere of N_2 at 3°C did not retard inactivation. Limited success was obtained upon the inclusion of 1 mM dithiothreitol in the phosphate buffer in which the cells were suspended before disruption, and a small stimulation of activity was also observed when it was included in the assay buffer.

These procedures were employed routinely with the result that the 160,000 × *g* supernatant maintained at 3°C overnight still retained 30 to 40% of its original activity.

***trans*-4-HydroxyCHC dehydrogenase.** The 160,000 × *g* supernatant catalyzed a reduction of added NAD in the presence of *trans*-4-hydroxyCHC. No activity was observed with the *cis* isomer. Maximum activity was attained at pH 10, and the rapid cessation of reaction at lower pH values, which could be restored by addition of further quantities of substrates but not by an additional aliquot of protein, was indicative of an adverse reaction equilibrium as reported by Blakley (6). Extraction of a bulk reaction with diethyl ether, after acidification and removal of precipitated protein, yielded two compounds. One of these was chromatographically identical to *p*-hydroxybenzoate, and the other co-chromatographed with authentic 4-ketoCHC.

Formation of the 2,4-dinitrophenylhydrazone of the latter compound yielded a derivative that was spectroscopically and chromatographically identical with the derivative of authentic 4-ketoCHC.

The dehydrogenase was of a very restricted specificity, and the presence of a *trans*-carboxyl group in the 4 position relative to the secondary alcohol group was mandatory because neither cyclohexanol, nor *cis,trans*-cyclohexan-1,4-diol, nor 4-methylcyclohexanol was capable of acting as a substrate. This strict specificity for a single geometric isomer of 4-hydroxyCHC was reflected in the limited oxygen consumption by whole cells provided with the *cis,trans* mixture (Fig. 1).

The enzyme was more conveniently assayed in the reverse direction by following the consumption of NADH concomitant with the reduction of 4-ketoCHC. In this direction, the enzyme had a pH optimum of 7.2, and the addition of 1 μmol of 4-ketoCHC (1 mM) stimulated the oxidation of 0.8 μmol of NADH, confirming that, at a physiological pH, the reaction equilibrium lay in the direction of alcohol formation. Measurements of the equilibrium constant could not, of course, be made in a crude system of this nature because of the influence of NADH oxidase activity. Oxidized NADP ($NADP^+$) and NADPH are not capable of accepting or donating electrons in this reaction.

Aromatization of 4-ketoCHC. Cell extract from CHC-grown *Alcaligenes* strain W1 catalyzed the consumption of oxygen in the presence of 4-ketoCHC. The maximum rate of oxygen consumption was obtained at pH 7.8. Attempts to demonstrate that the pink membrane fraction from CHC-grown cells constitutes a natural electron acceptor system have, as yet, met with failure even though spectrophotometric assays demonstrated the limited ability of potassium ferricyanide and 2,6-dichlorophenolindophenol to accept electrons from the soluble protein system. The in vivo donation of electrons from an aromatization enzyme directly to oxygen, followed by a catalase-mediated decomposition of the hydrogen peroxide thus formed, appears to be a particularly wasteful oxidative route. It is possible that effective electron transfer to the cytochrome chain requires a degree of physical organization that is lost during the preparation of subcellular fractions.

Addition of phenazine methosulfate to assays in the oxygen monitor increased the rate of substrate-stimulated oxygen consumption about fivefold, and the enzyme was routinely assayed in this manner in the presence of an optimum (0.8 mM) level of the artificial electron acceptor.

The introduction of 4-ketoCHC into the oxygen monitor vessel stimulated the consumption of a stoichiometric equimolar amount of oxygen (Table 2). The autooxidation of reduced phenazine methosulfate generated hydrogen peroxide. However, it was experimentally determined

TABLE 2. *Stoichiometry of 4-ketoCHC aromatization*[a]

4-KetoCHC added (nmol)	Oxygen consumed (nmol)	Oxygen consumed/ substrate (mol/mol)
50	43	0.86
100	105	1.05
150	149	0.99
200	192	0.96
250	217	0.87
300	328	1.09

[a] Oxygen monitor vessels, at 30°C, contained (in a volume of 3 ml), 280 μmol of phosphate buffer (pH 7.8), 2.2 mg of the 160,000 × *g* supernatant protein, and 0.8 μmol of phenazine methosulfate. Reactions were initiated by addition of 4-ketoCHC.

that, under the assay conditions used, extracts of CHC-grown *Alcaligenes* strain W1 exhibited catalase activity (specific activity, 1.3 μmol of O_2 produced per min) that was not inhibited by the phenazine methosulfate in the reaction vessel. The observed oxygen consumption therefore corresponded to the removal of four electrons from the alicyclic ring. The product of the reaction, extracted from an acidified reaction mixture with diethyl ether, was subjected to thin-layer chromatography in solvents A, B, and C and shown to be chromatographically identical to *p*-hydroxybenzoate (R_f values, 0.29, 0.42, and 0.16, respectively). In addition, the quenching of fluorescence, the acidic reaction with bromocresol green, and the coloration when the product was sprayed with diazotized *p*-nitroaniline or the Folin-Ciocalteau reagent followed by exposure to ammonia were identical to the behavior of authentic *p*-hydroxybenzoic acid.

Kinetic studies with phenazine methosulfate as the electron acceptor at 0.8 mM established a K_m of 0.43 mM for 4-ketoCHC. This value is of the same order as that reported by Blakley (6) for the aromatizing enzyme of *Arthrobacter* strain PRL W15 assayed with 2,6-dichlorophenolindophenol as the electron acceptor.

Cell extracts of CHC-grown *Alcaligenes* thus contained a series of enzymes capable of converting CHC to *p*-hydroxybenzoate. All three enzymes were induced by growth with CHC and absent from extracts of cells grown with *p*-hydroxybenzoate or succinate (Table 3).

Identity of the product of CHC hydroxylation and conversion of CHC into *p*-hydroxybenzoate by the 160,000 × *g* supernatant. When relatively large amounts of the 160,000 × *g* supernatant were incubated with CHC and excess NADH in the Warburg apparatus, the initial rapid phase of oxygen consumption was followed by a slower phase that terminated with the consumption of approximately 2 μmol of O_2 for each micromole of CHC supplied. Two reaction flasks that each contained 140 μmol of KH_2PO_4-NaOH buffer (pH 7.6), 15 μmol of NADH, and 10 μmol of CHC were equilibrated at 30°C, and the reaction was initiated by the addition of the 160,000 × *g* supernatant (13 mg of protein) from the side arms. Reactions were stopped by the addition of 1 ml of 6 M HCl when substrate-stimulated O_2 consumption had reached 8 μmol. Denatured protein was removed by centrifugation, and the acidic reactants were extracted three times with diethyl ether (5 ml each). The ether layer was dried over anhydrous Na_2SO_4, evaporated to a small volume, and subjected to chromatographic analysis. In addition to a trace of residual CHC, two products chromatographically identical to 4-hydroxyCHC (the chromatographic systems were incapable of resolving the *cis* and *trans* isomers) and *p*-hydroxybenzoate were detected. The contents of a second pair of flasks, which were allowed to react until substrate-stimulated O_2 consumption had virtually ceased and were similarly processed, yielded a single product chromatographically identical to *p*-hydroxybenzoate.

Irrefutable proof of identity was provided by a mass spectral analysis of the methyl ester of the product and comparison of it with an authentic standard (Fig. 3).

Further metabolism of *p*-hydroxybenzoate by *Alcaligenes* strain W1. Preliminary studies rapidly established the presence of enzymes of *p*-hydroxybenzoate metabolism in extracts of CHC-grown cells. Manometric studies demonstrated the consumption of 2 μmol of O_2 for each micromole of *p*-hydroxybenzoate added only in the presence of NADPH; no substrate-stimulated O_2 uptake or pyridine nucleotide oxidation was observed with NADH. The metabolism of each added micromole of protocatechu-

TABLE 3. *Enzymes of CHC aromatization in extracts of Alcaligenes strain W1 grown with CHC, p-hydroxybenzoate, or succinate*

Enzyme activity	Sp act in extracts of cells grown on:		
	CHC	*p*-Hydroxybenzoate	Succinate
CHC hydroxylase	0.08[a]	<0.0005	<0.0005
trans-4-HydroxyCHC dehydrogenase	0.28[b]	0.001	<0.0005
	0.32[c]	NA[d]	NA
4-KetoCHC oxidase (aromatizing)	0.05[e]	<0.0006	<0.0006

[a] Because specific activity tends to increase with increasing amounts of protein in the assay, this is not a maximum value.

[b] Measured in the forward direction with NAD^+ as the electron acceptor.

[c] Measured in the reverse direction with NADH as the electron donor.

[d] NA, Not assayed.

[e] With phenazine methosulfate as electron acceptor.

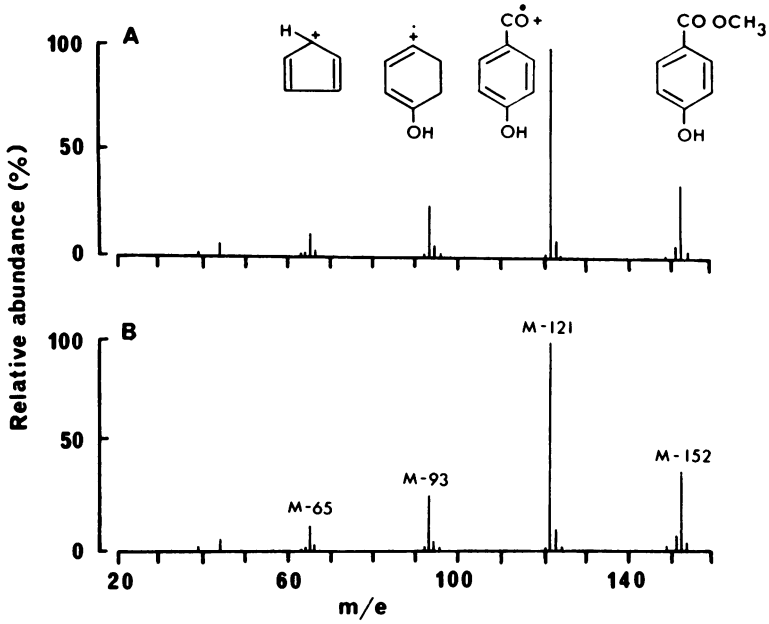

FIG. 3. *Mass spectra of methyl p-hydroxybenzoate. Authentic methyl p-hydroxybenzoate (A) and methylated reaction product (B) dissolved in diethyl ether were injected onto a 3% OV 17 column operating isothermally at 154°C with a carrier gas flow rate of 90 ml/min. Mass spectral analysis of the authentic methyl p-hydroxybenzoate and the peak with the same retention time in the reaction sample was performed at an ionizing voltage of 24 eV and an ion source temperature of 220°C. No peak corresponding to methyl p-hydroxybenzoate was detectable in a control flask (minus CHC) that was identically processed.*

ate was accompanied by the consumption of O_2 and the accumulation of a bright yellow reaction product. This product, extracted from acidified reaction systems with ethyl acetate and reextracted with 1 M NaOH, had an absorption maximum at 410 nm. In acid solution, this absorption and the yellow color were abolished, behavior typical of a substituted muconic semialdehyde (10).

Our failure to detect 2-hydroxy-4-carboxymuconic semialdehyde hydrolase in the extract and the apparently exclusive metabolism of the compound by the agency of a pyridine nucleotide-dependent dehydrogenase, as reported by Nishizuka et al. (25), was compatible with the very slow fading of the yellow color formed when protocatechuate was supplied as substrate in the absence of added NAD^+ or $NADP^+$. Identical observations were made with extracts of *p*-hydroxybenzoate-grown cells.

Key enzymes of the oxidative branch of the *meta*-fission pathway for *p*-hydroxybenzoate oxidation were present in extracts of cells grown with CHC or *p*-hydroxybenzoate, and their inducible nature was clearly demonstrated by reference to the extract of succinate-grown cells (Table 4).

TABLE 4. *Enzymes of p-hydroxybenzoate metabolism in extracts of Alcaligenes strain W1 grown with CHC, p-hydroxybenzoate, or succinate*

Enzyme activity	Sp act in extracts of cells grown on:		
	CHC	*p*-Hydroxybenzoate	Succinate
p-Hydroxybenzoate hydroxylase	0.20	0.69	0.0003
Protocatechuate 3,4-oxygenase	0	0	0
Protocatechuate 4,5-oxygenase	0.18	0.20	0.004
2-Hydroxy-4-carboxymuconic semialdehyde hydrolase	0.001	0.001	<0.0005
2-Hydroxy-4-carboxymuconic semialdehyde dehydrogenase	0.40[a]	0.73[a]	0.02[a]
	0.19[b]	0.29[b]	NA[c]

[a] Assayed with $NADP^+$ as the electron acceptor.
[b] Assayed with NAD^+ as the electron acceptor.
[c] NA, Not assayed.

Pathway of CHC metabolism. The demonstrated activities of a complete sequence of enzymes and the unequivocal identification of key intermediates allow a reaction sequence for the metabolism of cyclohexane carboxylate by *Alcaligenes* strain W1 to be formulated (Fig. 4).

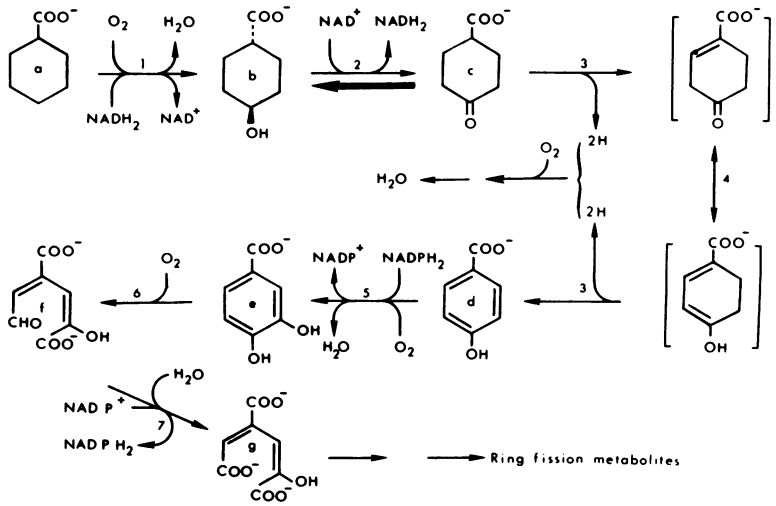

FIG. 4. *Pathway of CHC metabolism by Alcaligenes strain W1. Enzymes: (1) CHC 4-hydroxylase, (2) trans-4-hydroxyCHC dehydrogenase, (3) 4-ketoCHC oxidase(s), (4) spontaneous rearrangement, (5) p-hydroxybenzoate hydroxylase, (6) protocatechuate 4,5-dioxygenase, (7) 2-oxo-4-hydroxymuconicsemialdehyde dehydrogenase. Compounds: (a) CHC, (b) trans-4-hydroxyCHC, (c) 4-ketoCHC, (d) p-hydroxybenzoate, (e) 3,4-dihydroxybenzoate (protocatechaute), (f) 2-hydroxy-4-carboxymuconic semialdehyde, (g) 2-hydroxy-4-carboxymuconate.*

DISCUSSION

Evidence accumulated by a number of groups of workers has clearly established that the microbial metabolism of alicyclic carboxylic acids proceeds by routes that are quite distinct from the monooxygenase-mediated formation of lactones involved in the cleavage of simple or substituted alicyclic ketones.

Earlier studies, both in vivo and in vitro with CHC and animal systems, indicate that CHC can be converted to benzoate (5). The direct dehydrogenation of cyclohexane carboxyl-coenzyme A to benzoyl-coenzyme A has been reported by Babior and Bloch (2) in a subcellular system of guinea pigs. The aromatization of *trans*-4-hydroxyCHC (6) and 4-ketoCHC (21) by extracts of CHC-grown *Arthrobacter* strain PRL W15 and *C. cyclohexanicum* is, in conjunction with other data, clearly indicative of an aromatization route for microbial CHC metabolism, although in neither case has the detailed enzymology of all implied reaction steps been demonstrated. The involvement of 4-hydroxyCHC and 4-ketoCHC as intermediates, with an implied hydroxylation of the ring at the 4 position, and the lack of requirement for coenzyme A both contrast markedly with the characteristics of the aromatization route reported for mammalian systems (2, 7). In contrast to the mammalian systems, it would appear that bacterial formation of cyclohexane carboxyl-coenzyme A, possibly derived naturally from *n*-alkyl-substituted cycloalkanes (4), is associated with beta-oxidative cleavage and, unlike the aromatization route, is of course capable of accepting compounds of varied ring size.

The further metabolism of *p*-hydroxybenzoate formed from CHC by *Arthrobacter* strain PRL W15 and *C. cyclohexanicum* has been reported to occur by *ortho* fission (6, 21) and by the well-documented β-ketoadipate pathway. More recently, Smith and Callely (37) have reported another strain of *Arthrobacter* that metabolizes *p*-hydroxybenzoate, formed from CHC, by *meta* fission.

Our preliminary search for CHC-utilizing microorganisms revealed the ease with which they can be isolated and the comparative rarity of strains that make use of the aromatization route because, on the basis of growth spectra and whole-cell oxidation studies, all of our strains except for *Alcaligenes* strain W1, seem to make use of the beta-oxidation sequence. Of additional interest was the observation that *Alcaligenes* strain W1 has a very restricted growth spectrum with alicyclic compounds, being capable of growth with CHC, trans-4-hydroxyCHC, and 4-ketoCHC but incapable of growth with all other substituted cyclohexane and cyclopentane rings that were tested. Data currently available also indicate that *C. cyclohexanicum* (39) is similarly restricted. This contrasts markedly with the

broad spectrum of alicyclic compounds utilized by organisms employing the beta-oxidation route for CHC oxidation. It suggests that, in the case of *Alcaligenes* strain W1 and *C. cyclohexanicum*, some factors associated with induction or control make growth with compounds such as 4-ketoCHC and cyclohexanone mutually exclusive phenomena.

One characteristic that made *Alcaligenes* strain W1 distinctive was the presence of an easily assayable CHC hydroxylase. This enzyme, like the majority of microbial methylene and methyl hydroxylases, was specific for NADH as electron donor. Chromatographic analysis of the reaction products established that the ring was hydroxylated in the 4 position. The systems used did not distinguish between the *cis* and *trans* isomers of 4-hydroxyCHC, but, on the basis of whole-cell oxidation studies and 4-hydroxyCHC dehydrogenase specificity, the *trans* isomer is the likely reaction product.

CHC 4-hydroxylase is an inherently unstable enzyme, and this has prevented us from achieving any degree of purification up to the present time. Its labile nature may have been responsible for the delayed oxidation of the growth substrate sometimes observed with whole cells harvested in the late log or early stationary phase, a phenomenon also observed by Blakley (6).

The conversion of CHC to *p*-hydroxybenzoate by the 160,000 × *g* supernatant was unequivocally demonstrated and required the presence of oxygen and NADH for the initial hydroxylation step and NAD^+ and oxygen for the subsequent dehydrogenation and aromatization reactions.

The aromatization step was markedly stimulated by the presence of artificial electron acceptors, but the nature of the in vivo acceptor is still obscure because all attempts to demonstrate a stimulation of electron transfer by washed membrane fractions have, as yet, met with failure.

For 4-ketoCHC in the aromatization step, the high K_m value of 0.43 mM was of the same order as the value of 0.15 mM reported by Blakley for the partially purified *Arthrobacter* enzyme (6). A 1 mM aqueous solution of cyclohexanone equilibrates to yield 0.2 μM enol form (36). On the assumption that the situation for 4-ketoCHC is only slightly different, a 0.43 mM solution of the compound would yield an approximately 86 nM solution of the enol. Although Kaneda (21) suggested the enol form of 4-ketoCHC as one of the alternative substrate states for electron withdrawal, this atypically low K_m would make it an unlikely candidate and lend support to an alternative sequence of withdrawal of two electrons from the ring, enolization, and removal of the second pair of electrons (Fig. 4).

The exact order of the sequence of aromatization steps remains to be elucidated. Alternative possibilities include the initial removal of all four electrons followed by the enolization step to yield the aromatic ring.

Current studies are aimed at purification of the more stable enzymes of the aromatization sequence followed by a detailed study of individual reaction steps.

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