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The Aerobic Metabolism of Cyclohexanecarboxylic Acid by *Acinetobacter anitratum*

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1. The aerobic metabolism of cyclohexanecarboxylic acid by a bacterium isolated from garden soil (*Acinetobacter anitratum*) was investigated. 2. Evidence for the formation of cyclohex-1-ene-1-carboxylate, 2-hydroxycyclohexanecarboxylate and pimelate when either cell suspensions or cell-free extracts were incubated in the presence of cyclohexanecarboxylic acid is presented. 3. Crude cell-free extracts required ATP, CoA, FAD and Mg^{2+} as cofactors for the production of pimelate from cyclohexanecarboxylic acid, suggesting the existence of an activating reaction with formation of CoA esters, in this system.

That certain aromatic compounds disappear from anaerobic environments has been known for some time (Tarvin & Buswell, 1934) but, until recently, the biochemistry of this phenomenon was unclear. Dutton & Evans (1969) demonstrated that the anaerobic photometabolism of benzoate by *Rhodopseudomonas palustris* proceeded by a reductive pathway. Williams & Evans (1973) isolated from soil a *Moraxella* sp. which grew anaerobically by nitrate respiration on benzoate; here also, the aromatic ring was reduced. Cyclohexanecarboxylate was a common intermediate in both of these anaerobic degradative pathways, as revealed by tracer studies using *ring*-^{14}C- and *carboxyl*-^{14}C-labelled benzoate. These observations raised the question of how micro-organisms utilize hydro-aromatic substrates aerobically. The present paper describes some of the steps involved in the oxidative metabolism of cyclohexanecarboxylate by a soil bacterium, *Acinetobacter anitratum*.

Materials and Methods

Organism

A bacterium capable of growth on cyclohexanecarboxylic acid as a sole source of organic carbon and energy was isolated from garden soil by enrichment culture. The organism was identified as *Acinetobacter anitratum* (Brisou, 1957; Rho, 1970), and has been deposited at the N.C.I.B., Torry Research Station, Aberdeen, U.K., under accession number N.C.I.B. 10487.

Culture conditions

The isolate was normally grown aerobically at 25°C in the following medium (%, w/v): $(NH_4)_2SO_4$ (0.1); K_2HPO_4 (0.05); $MgSO_4,7H_2O$ (0.01); $FeSO_4,7H_2O$ (0.025). The carbon source was added to a final concentration of 0.06% (w/v). When succinate was used as substrate, a final concentration of 0.15% (w/v) was used. The medium was prepared with tap water, the pH adjusted to 7.0–7.5 with 20% (w/v) NaOH and autoclaved for 15min at 13.8N/cm^2. The organism was grown in 10-litre batches at 30°C with forced aeration, and harvested early in the exponential phase.

Preparation of cell-free extracts

These were prepared as described in the preceding paper (Williams & Evans, 1975). Protein determinations in extracts were performed by the biuret reaction as described by Gornall *et al.* (1949), with crystalline bovine plasma albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) as standard.

Oxygen consumption

This was either measured in the constant-volume Warburg apparatus, or a Clark-type oxygen electrode was used. All measurements were done at 25°C.

Thin-layer chromatography

Analytical and preparative t.l.c. was performed on glass plates (20cm × 20cm) coated to a thickness of 0.25 or 0.5mm, as required, with silica gel G (E. Merck A.-G., Darmstadt, Germany). The following solvent systems were used: (*a*) benzene–dioxan–acetic acid (45:5:2, by vol.); (*b*) toluene–ethyl acetate–formic acid (5:4:1, by vol.); (*c*) benzene–methanol–acetic acid (80:1:1, by vol.); (*d*) butan-1-ol–ethanol–water (7:1:2, by vol.); (*e*) ethanol–aq. NH_3 (sp.gr. 0.88)–water (100:16:12, by vol.); (*f*) benzene–methanol–acetic acid (10:2:1, by vol.). Carboxylic acids were detected by spraying

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with 0.1% (w/v) Bromocresol Green in methanol (Lugg & Overall, 1948). Oxo acids were chromatographed as their 2,4-dinitrophenylhydrazones prepared by the method of Smith (1960).

Gas–liquid chromatography

This was performed in an Aerograph model 1520B gas chromatograph equipped with a flame-ionization detector and a Honeywell Electronic 15 recorder. A stainless-steel column [1.5 m × 0.32 cm (5 ft × 0.125 in)] and three different stationary phases were used; (A) 10% Carbowax 20M on AW-OMCS Chromosorb W (100–120 mesh) (Varian Aerograph); (B) 10% Apiezon on HMPS Chromosorb W (80–100 mesh) (Perkin–Elmer); (C) 10% diethyleneglycol succinate on AW-OMCS Chromosorb W (100–120 mesh) (Wilkens Instruments and Research Ltd.). The gas flow rates were N_2, 25 ml/min, H_2, 25 ml/min, air, 500 ml/min. Operation was isothermal at 120°C.

Preparation of samples for g.l.c.

Cell suspensions or cell-free systems in buffer were equilibrated for 15 min at 25°C; after addition of substrate (5–10 μmol/ml for cell suspensions; 0.1–1.0 μmol/ml for cell-free systems) samples were collected at different intervals of time, up to 30 min. The reaction was stopped by addition of 50% (v/v) H_2SO_4 until the mixture was acid to Congo-Red (i.e. below pH 3), and the ether-soluble acids were extracted as described by Dutton & Evans (1969). The final ethereal extract was evaporated to a small volume under reduced pressure and the methyl esters of carboxylic acids were prepared by addition of ethereal diazomethane until a permanent yellow colour was obtained. The mixture was left for a few minutes, and then the solvent was removed under reduced pressure. The residue was resuspended in a small volume of light petroleum (b.p. 40–60°C) and stored at −20°C. Qualitative standards were prepared by dissolving the authentic acids in a small volume of ether and the solution was methylated as above. The samples were evaporated and resuspended in 1–2 ml of light petroleum (b.p. 40–60°C) for injection.

Isotopic experiments

Washed cells (1.5–3.0 g wet wt.) were suspended in 200 ml of 0.05 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0, and incubated for 15 min in the presence of 200–500 mg of cyclohexanecarboxylate. [*carboxyl*-C^{14}]Cyclohexanecarboxylic acid (100 μCi), purified as described by Dutton & Evans (1969), was added and 10–30 ml samples were taken at intervals, up to 30 min. The reaction was stopped by acidification with H_2SO_4 (50%, v/v to pH lower than 3), and the suspension was sonicated for 10 min. Ether-soluble acids of the supernatant obtained after 30 min of centrifugation at 18000*g* were extracted in ether and converted into their ammonium salts, as described by Dutton & Evans (1969), and the final ethereal extract was applied to silica gel plates. After the plates had been developed two-dimensionally, radioactive areas were located by radioautography on Ilfex non-screen X-ray films (Ilford Ltd., Mobberley, Knutsford, Cheshire, U.K.), after development with Phenisol X-ray developer (Ilford Ltd.).

Mass-spectral analysis

This was performed by the Physico-chemical Measurement Unit, Harwell, Didcot, Berks., U.K.

Chemicals

2-Oxocyclohexanecarboxylic acid, *trans*-2-hydroxycyclohexanecarboxylic acid, cyclohex-1-enecarboxylic acid, cyclohex-2-enecarboxylic acid, cyclohex-3-enecarboxylic acid, *trans*- and *cis*-4-hydroxycyclohexanecarboxylic acid were obtained as described by Dutton & Evans (1969); *trans*- and *cis*-3-hydroxycyclohexanecarboxylic acid were obtained by combining the methods described by Perkin & Tattersal (1907) and Boorman & Linstead (1935). Cyclohexa-2,5-dienecarboxylic acid was prepared as described by Kuehne & Lambert (1963).

2-Oxopimelic acid was purchased from K & K Laboratories Inc., Plainview, N.Y., U.S.A., and [*carboxyl*-^{14}C]cyclohexanecarboxylic acid (specific radioactivity 21.8 μCi/mg) was obtained from C.E.A., Department des Radioelements, Gif-sur-Yvette, France. CoA was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K.; other cofactors were from Sigma (London) Chemical Co., London S.W.6, U.K. Unless otherwise stated, all other chemicals were from British Drug Houses Ltd., Poole, Dorset, U.K.

Experiments using cell-free extracts

The activity of crude cell-free extracts of *A. anitratum* grown on cyclohexanecarboxylate, towards this substrate and other likely intermediates, was examined by following O_2 consumption, and exploration of acid–ether extracts, after methylation, by g.l.c. The reaction mixture contained in 3 ml of buffer (0.05 M-Tris–HCl, pH 7.0, and in a parallel experiment 0.05 M-potassium phosphate buffer, pH 7.0): substrate, 0.5 μmol; ATP, 0.4 μmol; CoA, 0.4 μmol; $MgCl_2$, 10 μmol; FAD, 0.4 μmol; and cell-free extract, 0.3 ml (3 mg of protein).

Results

A. anitratum grew abundantly in mineral salts liquid medium supplemented with 0.06% (w/v) cyclohexanecarboxylate under aerobic conditions (cell yield, 2 g wet wt./litre). Cells were usually harvested during the first half of the exponential phase (16–20 h).

Oxidation of cyclohexanecarboxylic acid

O_2 uptake by *A. anitratum* cells grown on cyclohexanecarboxylate in the presence of various substrates is shown in Table 1. Cells immediately oxidized cyclohexanecarboxylate, cyclohex-1-enecarboxylate and 2-oxocyclohexanecarboxylate; after a lag period, pimelate was also oxidized. Cells grown aerobically on succinate showed no significant increase above the endogenous rate in the presence of the substrates listed above.

Crude cell-free extracts of cyclohexanecarboxylate-grown cells consumed O_2 at a very low rate in the presence of the following substrates: cyclohexanecarboxylate, cyclohex-1-enecarboxylate, 2-hydroxycyclohexanecarboxylate, 2-oxocyclohexanecarboxylate and pimelate. When ATP, Mg^{2+} ions and CoA were also added, cyclohexanecarboxylate, cyclohex-1-enecarboxylate and 2-hydroxycyclohexanecarboxylate showed an increased rate of O_2 uptake, which reached about double the endogenous control; the 2-oxocyclohexanecarboxylate and pimelate reaction mixtures, however, showed no increased response.

Identification of intermediates

Ether-soluble acidic compounds from *A. anitratum* cell suspensions (4.0 g wet wt./200 ml of 0.05 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0), metabolizing cyclohexanecarboxylate (200 mg), were analysed after 30 min of incubation by two-dimensional t.l.c. in solvent (*a*). Two major acidic spots were detected: one co-chromatographed with cyclohexanecarboxylate (R_F 0.88), and the other with authentic pimelic acid (R_F 0.38).

A radioactive spot, co-chromatographic with pimelic acid, was also found when the acidic ether-soluble fraction from cell suspensions, metabolizing [*carboxyl*-^{14}C]cyclohexanecarboxylate, was chromatographed two-dimensionally in solvent mixture (*a*) followed by solvent (*b*). Further chromatographic runs with solvent mixtures (*c*) and (*d*) failed to separate the radioactivity from authentic pimelic acid. Finally, more carrier was added to the material eluted from the radioactive area, and the mixture recrystallized to constant specific radioactivity, by the procedure described by Dutton & Evans (1969).

Ether extracts of acidified incubation mixtures of cells and cyclohexanecarboxylate in Tris–HCl buffer were analysed after methylation in a gas–liquid chromatograph coupled to a mass spectrometer. The column used was similar to B (see the Materials and Methods section); the methyl ester fraction was resolved into four sharp peaks, with retention times of 10, 18, 26 and 40 min and *m/e* of 142, 140, 158 and 188 respectively. These correspond to authentic methyl cyclohexanecarboxylate, methyl cyclohex-1-enecarboxylate, methyl 2-hydroxycyclohexanecarboxylate and dimethyl pimelate. A control experiment in which the substrate (cyclohexanecarboxylate) was omitted, gave no such peaks.

Evidence for the presence of a CoA-activating system in crude cell-free extracts

Since g.l.c. of acid–ether extracts of cell incubates, after their methylation, had proved a sensitive method of resolving components, a similar technique was used in experiments with cell-free extracts.

Crude cell-free extract was incubated with cyclohexanecarboxylate in the presence of various cofactors; after 30 min at 25°C, the acid–ether extract after methylation gave the following results on g.l.c. (*a*) With no additions, or in the presence of ATP or FAD, added singly, no acid other than the substrate was detected. (*b*) When both FAD and ATP

Table 1. *Oxidation of various substrates by A. anitratum cells grown aerobically on cyclohexanecarboxylate*

Each Warburg flask contained, in 3 ml of 0.05 M-Tris–HCl buffer, pH 7.0: cells, 5 mg dry wt./ml; substrate (in the side arm) 3 μmol. The centre well contained 0.2 ml of 20% KOH. Temperature, 25°C; atmosphere, air.

Substrate	O_2 consumed (μl) 10 min	20 min
Cyclohexanecarboxylate	127	232
Cyclohex-1-ene-1-carboxylate	104	210
2-Oxocyclohexanecarboxylate	83	105
Pimelate	27	85
Cyclohex-2-ene-1-carboxylate	38	76
Cyclohex-3-ene-1-carboxylate	21	64
Cyclohexa-2,5-diene-1-carboxylate	17	37
2-Hydroxycyclohexanecarboxylate	27	45
trans-3-Hydroxycyclohexanecarboxylate	30	61
cis-3-Hydroxycyclohexanecarboxylate	18	34
trans-4-Hydroxycyclohexanecarboxylate	21	41
cis-4-Hydroxycyclohexanecarboxylate	20	56
Benzoate	29	63
Adipate	20	50
Catechol	19	52
Salicylate	22	32
Cyclohexane-1,2-diol	27	47
Cyclohexanol	21	38
Cyclohexanone	23	50
2-Oxopimelate	34	71
None	30	65

were present, a peak with a retention time corresponding to methyl cyclohex-1-enecarboxylate (18min) appeared. (*c*) A peak corresponding to dimethyl pimelate (retention time 40min) in addition to that due to methyl cyclohex-1-enecarboxylate, was observed when CoA, FAD and ATP were all present. With the further addition of Mg^{2+} ions, the relative peak height of dimethyl pimelate increased.

Discussion

The fate of cyclohexanecarboxylate in both animals (Beer *et al.*, 1951; Mitoma *et al.*, 1958; Babior & Bloch, 1966) and micro-organisms (Emmerling & Abderhalden, 1903; Rogoff, 1958; Gross, 1958; Tresguerres *et al.*, 1970; Kaneda, 1974) frequently involves an aromatization step but, whereas the resulting aromatic acid is first conjugated with glycine and then excreted by animals (Adamson *et al.*, 1970), bacteria and fungi frequently utilize the aromatic intermediates as carbon and energy sources and degrade them completely (Evans, 1970). The hexahydrohippuric acid (cyclohexanoylglycine), detected in the urine of cattle (Suemitsu *et al.*, 1971), may conceivably have arisen from 11-cyclohexylundecanoic acid, shown by Hansen (1967) to be a component of the fatty acids of bovine rumen bacteria. However, in the light of the anaerobic metabolism of benzoate by rumen liquor, described by Clark & Fina (1952) and in the preceding paper (Williams & Evans, 1975), a direct reduction of benzoate (or of the benzoate moiety of hippurate) to cyclohexanecarboxylate is now a viable alternative origin for this compound.

Colla & Treccani (1960) isolated adipic acid and pimelic acid from the culture fluid of a *Flavobacterium* sp. grown aerobically on decalin. Their interpretation of this result is curious; adipate was believed to be a genuine intermediate, whereas pimelate was adjudged an 'artifact'. In the aerobic metabolism of limonene by a soil pseudomonad, Dhavalikar & Bhattacharyya (1966) detected β-isopropenylpimelate; the precursor of this was perillic acid (4-isopropenylcyclohex-1-enecarboxylate) formed by the oxidation of the methyl group of the monocyclic terpene to the carboxylic acid. This pathway is therefore analogous to that described in the present paper. However, Kaneda (1974) found that when *Corynebacterium cyclohexanicum* was grown aerobically on cyclohexanecarboxylate, 4-oxocyclohexanecarboxylate appeared in the culture medium at an early growth phase. A cell-free extract oxidatively aromatized this to *p*-hydroxybenzoate, subsequently to be metabolized by one of the aromatic pathways. Apparently no CoA ester was involved, and O_2 could be replaced by another electron acceptor [e.g. $K_3Fe(CN)_6$].

In the present study, isotopic labelling of cyclohexanecarboxylate showed that pimelate was produced by *A. anitratum* under aerobic conditions; cyclohex-1-enecarboxylate and 2-hydroxycyclohexanecarboxylate were also detected. Cell-free extracts were inactive towards the substrate without certain cofactor additions; when ATP and FAD were supplied some cyclohex-1-enecarboxylate was formed, but it required the further addition of CoA and Mg^{2+} ions for pimelate to be detected as a reaction product. Our cell-free system is not very active, and requires a more detailed study; the evidence suggests that this *Acinetobacter* sp. uses the β-oxidation pathway for the aerobic metabolism of cyclohexanecarboxylate. A thiolytic cleavage of the CoA ester of 2-oxocyclohexanecarboxylate would give rise to a pimelate moiety since the α-C atom (C-1) of the keto ester is also a part of the alicyclic ring. This pathway is analogous to Scheme 1 (part *a*) of the preceding paper (Williams & Evans, 1975) advanced for the reductive anaerobic photometabolism of benzoate by *R. palustris* [see also Dutton & Evans (1969)] and differs from the oxidative 'aromatization' pathway of cyclohexanecarboxylate metabolism used by *Corynebacterium* sp. (Kaneda, 1974).

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