The Metabolism of Benzoate by *Moraxella* Species Through Anaerobic Nitrate Respiration

EVIDENCE FOR A REDUCTIVE PATHWAY

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1. *Moraxella* sp. isolated from soil grows anaerobically on benzoate by nitrate respiration; nitrate or nitrite are obligatory electron acceptors, being reduced to molecular N₂ during the catabolism of the substrate. This bacterium also grows aerobically on benzoate. 2. Aerobically, benzoate is metabolized by *ortho* cleavage of catechol followed by the β -oxoadipate pathway. 3. Cells of *Moraxella* grown anaerobically on benzoate are devoid of *ortho* and *meta* cleavage enzymes; cyclohexanecarboxylate and 2-hydroxycyclohexanecarboxylate were detected in the anaerobic culture fluid. 4. [ring-U-¹⁴C]Benzoate, incubated anaerobically with cells in nitrate-phosphate buffer, gave rise to labelled 2-hydroxycyclohexanecarboxylate and adipate. When [carboxy-¹⁴C]benzoate was used, 2-hydroxycyclohexanecarboxylate was radioactive but the adipate was not labelled. A decarboxylation reaction intervenes at some stage between these two metabolites. 5. The anaerobic metabolism of benzoate by *Moraxella* sp. through nitrate respiration takes place by the reductive pathway (Dutton & Evans, 1969). Hydrogenation of the aromatic ring probably occurs *via* cyclohexa-2,5-dienecarboxylate and cyclohex-1-enecarboxylate to give cyclohexanecarboxylate. The biochemistry of this reductive process remains unclear. 6. CoA thiol esterification of cyclohexanecarboxylate followed by β -oxidation via the unsaturated and hydroxy esters, would afford 2-oxocyclohexanecarboxylate. Subsequent events in the *Moraxella* culture differ from those occurring with *Rhodopseudomonas palustris*; decarboxylation precedes hydrolytic cleavage of the alicyclic ring to produce adipate in the former, whereas in the latter the keto ester undergoes direct hydrolytic fission to pimelate.

The microbial metabolism of aromatic compounds in anaerobic environments, e.g. soils and sediments, stagnant waters, sewage sludge and the rumen, represent a part of the carbon cycle of Nature which, biochemically, is not well understood. Bacterial cultures have been isolated which dissimilate the benzene nucleus of many naturally occurring substrates in the absence of oxygen, fermentatively, photosynthetically, or by nitrate (anaerobic) respiration.

Oshima (1965) described a mixed culture of two bacteria which in combination, but not separately, grew on aromatic substrates by nitrate respiration. These denitrifying pseudomonads, when grown anaerobically with ¹⁸O-enriched nitrate, incorporated ¹⁸O to a greater extent with *p*-hydroxybenzoate or protocatechuate than with succinate as their carbon substrate. He concluded that in the anaerobic cleavage of the aromatic ring, the incorporation of oxygen atoms of nitrate into the reaction products occurred, in a manner similar to that of molecular oxygen. This definitive inference appears to us unwarranted, bearing in mind the nature of his experiments.

Taylor *et al.* (1970) have isolated, from soil, *Pseudomonas* PN-1 which grew in pure culture

anaerobically by denitrification on aromatic compounds. Aerobically it metabolized benzoate and *p*-hydroxybenzoate *via* protocatechuate using the '*meta*' cleavage pathway (Dagley *et al.*, 1960) but anaerobically in the presence of nitrate, this organism degraded benzoate by a pathway quite different from that induced aerobically. A scheme was proposed, which involved the addition of three molecules of water to the aromatic nucleus before cleavage. Subsequent radioactive-isotope and mutant studies confirmed the breakdown of the benzene ring under denitrifying conditions without, however, producing any direct evidence for this hypothetical pathway (Taylor & Heeb, 1972).

Chemical considerations indicate that for additions to an aromatic nucleus to occur, the system of bonding characterized by very extensive delocalization of the π electrons must be converted into one in which little of the delocalization energy remains. Accordingly, only a few reagents, hydrogen being among them, saturate the benzene ring under easily attainable conditions. Direct chemical hydration of the aromatic nucleus is not observed, although covalent hydration of many heterocyclic compounds, such as pteridines for example, is well known. Photochemical processes resulting in

hydration might perhaps occur under suitable circumstances, but they are not present in the phenomenon discussed here. Biochemically, then, it is difficult to visualize a mechanism involving the addition of a water molecule to the aromatic ring, whereas the activation and transfer of hydrogen is a reasonable possibility.

Since the ideas of Oshima (1965) and those of Taylor and his collaborators (Taylor *et al.*, 1970) on the anaerobic dissimilation of aromatic compounds by nitrate respiration appeared improbable to us, we have investigated this particular fermentation. The present paper presents evidence for the operation of a reductive pathway, a preliminary account of which has appeared (Williams & Evans, 1973).

Materials and Methods

Isolation of the organism

The organism was isolated from soil by an enrichment technique with the following medium (g/l): $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (7.9); KH_2PO_4 (1.5); NH_4Cl (0.3); KNO_3 (2); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1); trace element solution (5 ml/l). The trace element solution was similar to that used by Vishniac & Santer (1957) and had the following composition (g/l): EDTA (50); $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (22); CaCl_2 (5.54); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (5.06); $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (4.99); $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (1.1); $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.57); $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (1.61). It was adjusted to pH 6 with KOH solution (10%, w/v). The trace element solution and the MgSO_4 were sterilized separately; complete medium was reconstituted as above, with the addition of sodium benzoate (0.72 g/l; 5 mm); the pH was adjusted to 8.5 with NaOH and the medium autoclaved for 20 min at 13.8 N/cm^2 . This medium (20 ml) was dispensed into sterile universal bottles, fresh garden soil (0.1 g) was added, and the screw caps were loosely fitted. Several such cultures, with a variety of samples from different parts of a soil profile, were incubated at 30°C in an anaerobic jar containing alkaline pyrogallol and filled with N_2 (O_2 -free). Growth was evident in some cultures within 2–4 days: these were subcultured into new sterile media and incubated in an identical manner under anaerobic conditions. The cultures that showed growth were subcultured four times by this procedure, and then streaked on to the same medium solidified with Ionagar (1%); after 2–3 days of anaerobic incubation at 30°C , discrete colonies appeared. Single colonies were now subcultured into the sterile benzoate–nitrate–mineral salts medium and incubated anaerobically; growth appeared within 2–3 days. Replating the culture on to nutrient agar (Oxoid, code CM3; Oxoid Ltd., London SE1 9HF, U.K.) gave rounded pale-white non-fluorescent colonies when grown anaerobically, and the culture appeared

to be pure. On benzoate–mineral salts–agar medium the organism grew aerobically as rounded almost colourless colonies; these plates noticeably darkened around the colonies within a few days [cf. Sala-Trepas & Evans (1971) where a similar phenomenon was observed with *Azotobacter* strains growing on this medium]. The present isolate was a Gram-negative stumpy rod; staff at the N.C.I.B., Torrey Research Institute, Ministry of Agriculture, Fisheries and Food, Aberdeen, U.K., have classified it as a *Moraxella* sp. and given it the accession number N.C.I.B. 11086. In a preliminary communication, Williams & Evans (1973) incorrectly called it *Pseudomonas stutzeri*; the error arose by the inadvertent mixing of labels during the process of isolating pure strains of competent bacteria from our primary mixed cultures.

Anaerobic growth of the organism

Cultures were started under aerobic or anaerobic conditions by seeding from a nutrient agar slope into benzoate–nitrate–mineral salts medium and incubating on a rotary shaker at 30°C ; a profuse growth occurred within 24–48 h. A 10% inoculum from this culture was transferred into similar medium (100 ml) in 250 ml conical flasks fitted with rubber stoppers through which passed gas inlet and outlet tubes; filtered N_2 was bubbled through the sterile medium for 15 min, the ports were closed by screw clips and the flasks placed in the rotary incubator at 30°C . The organism took longer to multiply during this first transfer into anaerobic conditions; growth was usually appreciable within 3–5 days. However, a 10% inoculum of this culture into fresh medium, proliferates rapidly under anaerobic conditions at 30°C , e.g. a 10-litre culture only takes 12–24 h to grow.

The 2-litre fermentation vessels (Pyrex, Jobling Quickfit Catalogue, FV 2L, p. 3/11, 1973) and the 10-litre Pyrex bottles were fitted with magnetic stirrers activated through the Perspex bottom of the constant-temperature water bath (30°C) in which they were immersed. These vessels were also fitted with an inlet–outlet system through which was passed a slow stream of N_2 gas during the whole period of growth. The fermentation vessel had an additional sampling tube through which a portion of the liquor could be blown out without disturbing anaerobiosis.

The N_2 supply was of high purity grade ($<1.0 \text{ p.p.m}$ of O_2 ; Air Products, Sharp St., Walkden, Worsley, Manchester, U.K.) and was passed through pyrogallol solution [10% (w/v) in 10% (w/v) NaHCO_3 solution] followed by chromous chloride solution [$\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ (100 g) dissolved in 260 ml of water, and 40 ml of conc. HCl added; after some mercury and granulated zinc had been added, the suspension

was shaken vigorously to reduce the chromic salt to the chromous salt giving a blue solution; Vogel (1961)].

Aerobic growth of the organism

The *Moraxella* sp. strain was grown aerobically in the same medium as that used for anaerobic growth except that the nitrate was omitted and the NH_4Cl content increased to 1 g/litre. For cell production, 10-litre cultures were grown at 30°C in Pyrex bottles under forced aeration with sterile air.

Harvesting of cells

Aerobic cultures were harvested towards the end of the exponential-growth phase in a Sharples Super centrifuge, fitted with a hard cellophan lining to the rotor. They were washed once with 0.05 M-potassium phosphate buffer, pH 7.2, and either used immediately or stored at -20°C.

Anaerobic cultures were initially centrifuged under N_2 when relatively small in volume; this became impracticable with 10-litre cultures. It was found that bubbling N_2 gas through the culture fluid as it was being sucked into the rotor of the Sharples Super centrifuge gave cell sediments which rapidly regained their ability to utilize benzoate under anaerobic conditions by nitrate respiration. When stored in the frozen state, however, the cells lost this property.

Chemicals

Sodium benzoate, pimelic acid, cyclohexanone and cyclohexanecarboxylic acid were from British Drug Houses Ltd., Poole, Dorset, U.K. *cis,trans*-Cyclohexane-1,2-diol, 2-hydroxycyclohexanone, cyclohexane-1,2-dione, ϵ -hexanolactone and adipic acid came from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Adipic acid semialdehyde was freshly prepared, as required, by the method of Baer (1942). Cyclohex-1-enecarboxylic acid and cyclohex-2-enecarboxylic acid were prepared as described by Dutton & Evans (1969); cyclohex-3-enecarboxylic acid, α -oxopimelic acid and ethyl 2-cyclohexanonecarboxylate were supplied by Ralph Emanuel Ltd., London S.E.1, U.K. *trans*-2-Hydroxycyclohexanecarboxylic acid (m.p. 111°C) was prepared by the reduction of ethyl cyclohexan-2-onecarboxylate with sodium amalgam (Dieckman, 1894) and *cis*-2-hydroxycyclohexanecarboxylic acid (m.p. 80–81°C) by the catalytic reduction of the keto ester, by the method of Pascual *et al.* (1949). Cyclohexa-2,5-dienecarboxylic acid (m.p. 15–17°C) was prepared by the method of Kuehne & Lambert (1963); cyclohexa-1,4-dienecarboxylic acid (m.p. 120–121°C) was supplied by Nipa Laboratories, Treforest Industrial Estate, Pontypridd, Mid-Glamorgan, U.K. Benzoyl-CoA was synthesized by the method of Schachter & Taggart (1953). [*ring*-U- ^{14}C]Benzoic

acid (specific radioactivity, 369 $\mu\text{Ci}/\text{mg}$) and [*carboxy*- ^{14}C]benzoic acid (specific radioactivity, 470 $\mu\text{Ci}/\text{mg}$) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. They were purified by two-dimensional t.l.c. on silica gel G, with benzene-dioxan acetic acid (90:25:4, by vol.) as solvent, located by radioautography, and eluted with potassium phosphate buffer (0.05 M, pH 7). From these purified stock solutions, about 50 μCi was used for each tracer experiment.

Analytical techniques

Spectrophotometric determinations were carried out in the Unicam SP.800B recording spectrophotometer. Growth of organisms in culture was followed by measuring the E_{610} . The supernatant after centrifugation was scanned in the u.v. region 250–300 nm, and benzoate utilization was estimated from a standard curve of E_{273} against known benzoate concentrations dissolved in the same medium. Nitrite determinations were carried out by the Greiss-Ilosvay colorimetric method (Vogel, 1961). A standard plot of nitrite concentration (0.01–0.001 g/litre) against E_{520} was used, and the unknown values were adjusted to fall within this range.

Enzyme assays

Cells of *Moraxella* sp. grown aerobically and anaerobically on benzoate, in the media indicated above, and harvested towards the end of the exponential phase of growth, were sonicated (2 g wet weight in 10 ml of potassium phosphate buffer, pH 7.5, 0.1 M) for 10–15 min at 0–4°C in an MSE 100 W ultrasonic disintegrator. They were then centrifuged (30000 g for 30 min) to give the cell-free extract.

Catechol 1,2-dioxygenase (catechol-oxygen 1,2-oxidoreductase; EC 1.13.11.1) was assayed by the method of Hayaishi *et al.* (1957).

Catechol 2,3-dioxygenase (catechol-oxygen 2,3-oxidoreductase; EC 1.13.11.2) was measured by the method of Sala-Trepat & Evans (1971).

Chromatography

T.l.c. was carried out on glass plates (20 cm $\times$ 20 cm) spread to a thickness of 0.25 mm with silica gel (Kieselgel G; E. Merck A.-G., Darmstadt, Germany) and with the following solvent systems: (A) benzene-dioxan-acetic acid (90:25:4, by vol.) and (B) light petroleum (b.p. 40–60°C)-diethyl ether-acetic acid (50:50:1, by vol.).

Organic acids were detected by 0.1% (w/v) Bromocresol Green in ethanol (Lugg & Overall, 1948).

Radioactive areas on t.l.c. plates were located by radioautography on an X-ray film (Ilflex No-Screen X-ray film; Ilford Ltd., Mobberley, Knutsford, Cheshire, U.K.) in a light-proof holder, and subsequent development in Phenisol X-ray developer (Ilford Ltd.).

Isotope-dilution experiments

Cells (2g wet wt.) grown anaerobically on benzoate-nitrate-mineral salts medium, were suspended in potassium phosphate buffer (0.05M, pH 8.5, 100ml) containing benzoate (100mg) and KNO₃ (0.25g); anaerobic conditions were re-established and incubation was continued at 30°C. Spectrophotometric scanning of centrifuged samples (5ml) at appropriate intervals showed that 90% of the benzoate was utilized in 2–3h under these conditions. For isotope-dilution studies, several such fermentations were set up with [¹⁴C]benzoate (50μCi) supplied in addition to the unlabelled benzoate and likely intermediates (3.5mg) incorporated as carriers. After a further 90min each flask was acidified to a pH below 3 with dilute H₂SO₄, the cells were centrifuged at 5000g for 5min, and the supernatant was extracted with ether (4×100ml portions). The combined ether extracts were dried with anhydrous MgSO₄ and the solvent was removed under reduced pressure; the acid-ether extracts were taken up in ether (1ml) and subjected to two-dimensional t.l.c. on silica gel G and with solvent mixtures (A) and (B). A sample was also methylated with diazomethane and examined by g.l.c. Radioactive spots which appeared on t.l.c. to correspond with some of the added intermediates, were eluted, mixed with more authentic carrier and the whole recrystallized to constant specific radioactivity. Assays were done in a Philips liquid-scintillation analyser.

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, with a chart speed of 25cm (10in)/h. Operation was isothermal at 130°C in a stainless-steel column [1.5m×0.32cm (5ft×0.125in)], with 10% Carbowax 20M on AW-OMCS Chromosorb W (100–120 mesh) as the stationary phase. The gas flow rates were N₂, 30ml/min, H₂, 35ml/min, and air, 500ml/min.

A Pye Series 104 chromatograph coupled to a flame-ionization detector and Speedomax W recorder with a chart speed of 38cm (15in)/h was also used intermittently in this work. It was operated isothermally at 160°C and had a glass column (1.5m×4mm) filled with 15% Carbowax 20M on AW-DMCS chromosorb W (100–120 mesh). The gas flow rates were N₂, 40ml/min, H₂, 40ml/min, and air, 500ml/min.

Identification of compounds transiently appearing in growing cultures and cell-incubation experiments

Cultures (10 litres), grown anaerobically in the benzoate-nitrate-mineral salts medium, were centrifuged at various stages of substrate utilization. The cells were used for incubation experiments, and the supernatant was adjusted to pH 7 and con-

centrated to 1 litre in a cyclone evaporator under reduced pressure at 25–30°C. The neutral concentrate was extracted with ether (4×500ml), then acidified to pH 3 with dilute H₂SO₄ and re-extracted with ether in a similar manner, and finally subjected to constant ether extraction for 48h. The acid-ether extracts were combined, dried with anhydrous MgSO₄ and the solvent was removed. A sample of the residue was subjected to t.l.c. by using a variety of solvent mixtures. Another portion was esterified with diazomethane in ether, the solvent was removed and the methyl esters were dissolved in a small volume of light petroleum (b.p. 40–60°C) for injection into a gas chromatograph. The components of the residual acid-ether-soluble fraction were separated by running as a band on t.l.c. plates in the most suitable solvent mixture; elution of each band with methanol afforded almost pure samples of the acids present for identification by spectrophotometry, g.l.c. and mass spectrometry. Similar procedures were used to identify products formed in cell-incubation experiments under anaerobic conditions.

Results

Aerobic metabolism of benzoate by Moraxella sp.

When the organism was grown in the benzoate-mineral salts medium under aerobic conditions, cultures gave positive colour reactions for catechol and β-ketoadipate (Evans, 1947). Cells were harvested, washed and sonicated in phosphate buffer to produce a cell-free extract suitable for assay of ring-fission enzymes. Such extracts showed the presence of catechol 1,2-dioxygenase only; cell-free extracts prepared from acetate-grown cells showed no activity. The presence of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase in extracts of cells grown anaerobically in benzoate-nitrate-mineral salts medium was also tested but none was found.

Anaerobic metabolism of benzoate by Moraxella sp. through nitrate respiration

Utilization of benzoate in culture under anaerobic conditions. Cultures (1.5 litres) were set up in anaerobic fermentation vessels at 30°C fitted with a sampling device, magnetic stirrer and supplied with a continuous slow stream of N₂ (O₂-free) and a 10% (w/v) inoculum from a starter culture; these were assayed for growth and benzoate concentration. Benzoate (5mM) was utilized completely within about 9h by which time growth had also reached its maximum (cell yield = 1g wet wt./litre or 288mg dry wt./litre).

Preliminary trials showed that 10-litre cultures could be harvested in a Sharples Super centrifuge (as described under 'Harvesting of cells') and these concentrated cell suspensions when incubated

anaerobically in benzoate–nitrate–phosphate buffer, regained their ability to metabolize the substrate. This technique was used in subsequent experiments.

Specificity of the terminal electron acceptor for the anaerobic metabolism of benzoate. Growth experiments were set up using a variety of possible electron acceptors instead of nitrate in the basal medium; NO_2^- , but not SO_4^{2-} or $\text{S}_2\text{O}_3^{2-}$, could act as a terminal electron acceptor and, if used without NH_4Cl , also as a source of N_2 . The NO_2^- , SO_4^{2-} and $\text{S}_2\text{O}_3^{2-}$ were incorporated at equivalent concentrations to the NO_3^- used in the basal medium. Experiments with Durham tubes in the medium showed the accumulation of N_2 gas during the fermentation of benzoate in the presence of NO_3^- or NO_2^- .

Concomitant disappearance of benzoate and NO_2^- in culture. A culture was set up in the 2-litre fermentation vessel, with a basal medium in which the NO_3^- was replaced by NO_2^- , and NH_4Cl was omitted; the previous experiment had shown that this medium allowed good growth of the organism. Fig. 1 shows the concomitant utilization of benzoate and NO_2^- during the growth of the culture.

When air was admitted into the fermentation vessel, benzoate utilization stopped almost immediately; flushing the culture with N_2 (O_2 -free), however, was successful in re-establishing the conditions for substrate disappearance.

Detection of cyclohexanecarboxylic acid and 2-hydroxycyclohexanecarboxylic acids in growing cultures. The 'acid-ether-soluble' fraction, prepared as described in the Materials and Methods section from 10-litre anaerobically-grown benzoate cultures stopped at different stages of substrate utilization, were examined by t.l.c., and the methyl esters by g.l.c. T.l.c. (solvent mixture A) gave a major acidic spot at R_F 0.77, corresponding to benzoic acid, and two other well-defined acidic spots at R_F 0.83 and 0.54; these are the R_F values of cyclohexane-

carboxylic acid and 2-hydroxycyclohexanecarboxylic acid respectively. The methyl ester fractions gave g.l.c. peaks with retention times of 3 min (similar to methyl cyclohexanecarboxylate or methyl cyclohex-3-enecarboxylate), 7 min (corresponding to methyl benzoate) and 24 min (identical with methyl 2-hydroxycyclohexanecarboxylate). The acidic components of the fraction were separated by preparative t.l.c. and the above retention times of their methyl esters confirmed. Table 1 shows R_F values and retention times on g.l.c. of authentic chemicals which could be involved as intermediates. Mass spectrometry (performed by the Physico-chemical Measurement Unit, Harwell, Didcot, Berks., U.K.) of the methyl esters has subsequently also established their identity as methyl cyclohexanecarboxylate (m/e , 142) and methyl 2-hydroxycyclohexanecarboxylate (m/e , 158).

Fig. 1. Growth, benzoate and nitrite utilization, in a culture of *Moraxella* sp. growing anaerobically in a benzoate–nitrite–salts medium at 30°C

■, Cell density (E_{610}); ▲, benzoate ($\mu\text{mol/ml}$); ●, nitrite ($\mu\text{mol/ml}$).

Table 1. Thin-layer and gas chromatography of authentic reference compounds

For further details see the text.

Substance	Solvent system (R_F values)		Retention time of methyl esters on g.l.c. (min)
	A	B	
Benzoic acid	0.77	0.88	7
Cyclohexa-1,4-dienecarboxylic acid	0.77	0.83	5.75
Cyclohexa-2,5-dienecarboxylic acid	0.77	0.82	5.0
Cyclohex-1-enecarboxylic acid	0.91	0.95	4.0
Cyclohex-3-enecarboxylic acid	0.91	0.95	3.0
Cyclohexanecarboxylic acid	0.83	0.92	3.0
2-Hydroxycyclohexanecarboxylic acid	0.54	0.21	24.0
Pimelic acid	0.60	0.39	26.0
Adipic acid	0.57	0.30	16.0
6-Hydroxyhexanoic acid	0.43	0.14	5.5

Fig. 2. Radioautogram of the separation by silica gel G of the organic acids produced by *Moraxella* sp. exposed to [ring- U - ^{14}C]benzoate in nitrate-phosphate buffer for 3 h under anaerobic conditions

When [carboxy- ^{14}C]benzoate was used in a parallel experiment, the radioautogram of the labelled acids produced was identical, except for the absence of band 4 corresponding to adipic acid. The solvent was benzene-dioxan-acetic acid. 1, Benzoate, cyclohexanecarboxylic acid, cyclohexadienecarboxylic acid and cyclohexanecarboxylic acid (R_F 0.7–0.95); 2,3, unidentified (R_F 0.69, 0.62); 4, adipic acid (R_F 0.57); 5, 2-hydroxycyclohexanecarboxylic acid (R_F 0.54); 6, 7, 8, unidentified (R_F 0.45, 0.40, 0.35).

Cell-incubation experiments. Cells of *Moraxella* sp. (2g wet wt.) grown anaerobically in benzoate-nitrate-mineral salts medium were resuspended in phosphate buffer (100ml, pH 7.5, 0.05M) containing KNO_3 (100mg) and one of a variety of substrates (50mg) in conical flasks (250ml), flushed with N_2 gas with the ports closed; the incubations were allowed to continue at 30°C. Substrates used were: benzoate, 2-hydroxycyclohexanecarboxylate, cyclohexanone, *cis,trans*-cyclohexane-1,2-diol, cyclohexane-1,2-dione, and 2-hydroxycyclohexanone. Samples were removed periodically, centrifuged, and the supernatants scanned spectrophotometrically between 200–540nm to follow the disappearance of the substrates. After 3h of incubation, the acid-ether-soluble fraction of each fermentation was examined by t.l.c. (solvent mixture A) and, after

methylation, by g.l.c. The results, interpreted with the assistance of the data given in Table 1, were as follows.

With benzoate as substrate, four acidic components were present, with R_F 0.77 (corresponding to residual benzoate and a cyclohexadienecarboxylic acid), 0.57 (adipic acid), 0.54 (2-hydroxycyclohexanecarboxylic acid) and 0.4 (possibly 6-hydroxyhexanoic acid). The methylated fraction gave four peaks, with retention times (min) of 5.8 (possibly a methyl cyclohexadienecarboxylate or 6-hydroxyhexanoate), 7 (methyl benzoate), 16 (dimethyl adipate) and 24 (methyl 2-hydroxycyclohexanecarboxylate).

Both the *cis*- and *trans*-2-hydroxycyclohexanecarboxylate incubations had two acidic components, with R_F 0.54 (residual substrate) and 0.57 (adipic acid). G.l.c. of the methylated fraction gave two peaks with retention times of 16 and 24 min, in agreement with the t.l.c. results.

All the cyclohexane derivatives were utilized to some extent, as indicated by a decrease in E_{255} (for cyclohexane-1,2-dione) or appreciable shifts in the end absorption for the others; only 2-hydroxycyclohexanone gave rise to an acidic component, with R_F 0.57 and a retention time of its methylated derivative of 16 min, corresponding to adipic acid.

Tracer experiments. Cell-incubation experiments with [ring- U - ^{14}C]benzoate and [carboxy- ^{14}C]benzoates as tracers, with the addition of 2-hydroxycyclohexanecarboxylate and adipate as carriers (see the Materials and Methods section), afforded acid-ether-soluble fractions which were subjected to t.l.c. in solvent mixture A. Fig. 2 is a photocopy of the developed plate from the ring-labelled benzoate experiment; eight separate radioactive areas were visible (designated 1–8). The carboxy-labelled benzoate incubate gave a similar pattern except that position 4 (R_F 0.57) was not radioactive; spraying this t.l.c. plate with Bromocresol Green, however, immediately revealed the presence of carrier adipic acid in this position.

With the aid of Table 1, some of the radioactive intermediates illustrated in Fig. 2 may be tentatively identified as follows.

The major labelled area, designated 1, with an R_F ranging from 0.7 to 0.95, could include (apart from residual benzoate) cyclohexanecarboxylic acid, and any of the cyclohexene- and cyclohexadienecarboxylic acids which might be formed. Radioactive components 2 and 3 (R_F 0.69 and 0.62) remain unidentified. The labelled area 4 is coincident with adipic acid (R_F 0.57); it carried no label when [carboxy- ^{14}C]benzoate was used as substrate. Area 5 corresponds to 2-hydroxycyclohexanecarboxylic acid (R_F 0.54) and was radioactive in both experiments. The remaining labelled areas 6, 7 and 8 are unidentified metabolites with R_F 0.45, 0.40 and 0.35 respectively.

These experiments have been repeated several times, and although the pattern of labelled intermediates formed is reproducible, the intensity of radioactivity carried by each area is variable. Elution of the labelled acids 4 and 5 (R_F 0.57 and 0.54) and admixture with authentic adipic acid and 2-hydroxycyclohexanecarboxylic acid respectively followed by t.l.c. in solvent mixture B, failed to separate carrier and radioactivity. Methods similar to those used by Dutton & Evans (1969) were used to prove the identity of the labelled intermediates with authentic chemicals which moved coincidentally on t.l.c. in the two solvent systems used (A and B). The radioactive material with R_F 0.57 was mixed with more adipic acid as carrier, and the whole recrystallized from concentrated HNO_3 ; the molar specific radioactivity remained constant after four recrystallizations from this solvent. Similarly, the labelled material with R_F 0.54 was mixed with more carrier 2-hydroxycyclohexanecarboxylic acid and crystallized from benzene to constant specific radioactivity (see Table 2).

Ability of Moraxella sp. to utilize a range of aromatic substrates under anaerobic conditions in nitrate-mineral salts medium. Although this organism was isolated because it metabolized benzoate anaerobically through nitrate respiration, it was of interest to ascertain the range of aromatic compounds which it could utilize under these conditions. Growth was estimated by measuring E_{610} of a 20% (v/v) inoculum from an anaerobic benzoate culture into substrate (0.07%, w/v)-nitrate-mineral salts medium after 24 and 48 h anaerobic incubations at 30°C. Measurement of utilization of substrates by benzoate-grown cells was carried out as described in the Materials and Methods section and the results are recorded in Table 3. It is noteworthy that the substrates capable of supporting growth or utilized by benzoate-grown cells, were the same as those found by Dutton & Evans (1969) for *Rhodopseudo-*

monas palustris growing photosynthetically under anaerobic conditions. None of the common phenols except phloroglucinol supported growth or were utilized; this behaviour contrasts markedly with aerobic aromatic metabolism where catechol is a key intermediate before ring-fission. Among the monohydroxybenzoates, salicylate was not metabolized; protocatechuate, the other central intermediate in aerobic aromatic pathways, was used anaerobically by both organisms. Of note is the ready dissimilation, except for phenylpropionate, of the $\text{C}_6\text{--C}_3$ group of aromatic acids; these, together with phloroglucinol, are the units mainly involved in the structure of plant phenolics.

Results obtained with the reduced benzoates and derivatives are consonant with the view that cyclohexa-2,5-dienecarboxylate, cyclohex-1-enecarboxylate, cyclohexanecarboxylate and 2-hydroxycyclohexanecarboxylate participate in the anaerobic metabolism of benzoate by *Moraxella* sp. together with some of the oxygenated cyclohexane derivatives before the formation of adipate.

Table 2. Crystallization of carrier compounds to constant specific radioactivity after re-isolation from suspensions of *Moraxella* sp. grown anaerobically on benzoate

Each carrier (3.5 mg) was incubated for 75 min in 0.05 M-potassium phosphate buffer, pH 7.0, containing: cells, 4–5 mg dry wt./ml; benzoic acid (unlabelled), 100 mg; KNO_3 , 300 mg; [*ring*- ^{14}C]benzoate, 20 μCi . Adipic acid was recrystallized from conc. HNO_3 . 2-Hydroxycyclohexanecarboxylic acid was recrystallized from benzene.

Carrier compound		Observed specific radioactivity (c.p.m./ μmol)			
No. of times crystallized	...	1	2	3	4
Adipic acid	37.20	—	—	—	35.16
2-Hydroxycyclohexanecarboxylic acid	48.9	48.3	—	—	48.0

Table 3. Ability of *Moraxella* sp. to utilize a range of aromatic substrates under anaerobic conditions in nitrate-mineral salts medium

- Substrates which support growth, and were utilized by benzoate-grown cells
Benzoic acid, benzaldehyde, benzyl alcohol, benzoylglycine, *m*- and *p*-hydroxybenzoic acids, protocatechuic acid, phenylacetic acid, cinnamic acid and *p*-hydroxycinnamic acid, caffeic acid, cyclohexa-2,5-dienecarboxylic acid and cyclohexanecarboxylic acid, phloroglucinol, adipic acid and pimelic acid.
- Substances which did not support growth, but were utilized by benzoate-grown cells
Phenylalanine, tyrosine, cyclohex-1-enecarboxylic acid, *cis*- and *trans*-2-hydroxycyclohexanecarboxylic acid, cyclohexanol, cyclohexanone, *cis,trans*-cyclohexane-1,2-diol, cyclohexane-1,2-dione, 2-hydroxycyclohexanone.
- Substrates not utilized
Phenol, catechol, resorcinol, quinol, *p*-cresol, salicylic acid, phthalic acid, gentisic acid, phloroglucinolcarboxylic acid, phenylpropionic acid, tryptophan, cyclohexa-1,4-dienecarboxylic acid and cyclohex-3-enecarboxylic acid.

Discussion

Under aerobic conditions, a variety of micro-organisms are able to degrade aromatic substrates by hydroxylating and ring-cleavage enzymes which convert the benzenoid structures into assimilable aliphatic acids (Dagley *et al.*, 1960; Evans, 1970).

Some denitrifying soil bacteria exhibit a facultative nature; they are able to grow aerobically on aromatic substrates, or anaerobically in the presence of nitrate as terminal electron acceptor. *Pseudomonas* PN1 (Taylor *et al.*, 1970; Taylor & Heeb, 1972) metabolized benzoate aerobically *via* protocatechuate followed by a *meta* cleavage to α -hydroxy- γ -carboxymuconic semialdehyde and subsequently the α -oxo acid pathway. When grown anaerobically on benzoate by nitrate respiration, a speculative pathway involving the addition of three molecules of water to the benzene ring followed by a hydrolytic cleavage was advanced.

Moraxella sp. metabolized benzoate aerobically *via* catechol and the β -oxoadipate pathway, in which it differs from *Pseudomonas* PN1. When this substrate is utilized anaerobically the organism uses a reductive pathway analogous to that already advanced by Dutton & Evans (1969) for the anaerobic photometabolism of benzoate by *Rhodopseudomonas palustris*. Scheme 1 is tentatively put forward as the most likely representation of the events (identified intermediates are numbered in **bold** type and the hypothetical ones are in normal type). Benzoate (I) is first reduced to cyclohexanecarboxylate (II), probably through cyclohexa-2,5-dienecarboxylate and cyclohex-1-enecarboxylate (III). A fatty acid-activation system then becomes operative, with the participation of the CoA esters of compounds (II) and (III), 2-hydroxycyclohexanecarboxylate (IV) and 2-oxocyclohexanecarboxylate (V).

In the case of *Rhodopseudomonas palustris* a hydrolytic cleavage of 2-oxocyclohexanecarboxylate (V) occurs to give pimelate (VI), and adipate (XIII) was identified as a product of the alicyclic ring in the *Moraxella* fermentation; a decarboxylation must therefore take place at some stage probably after (V). Scheme I depicts two ways by which the bacterium could accomplish this. 2-Oxocyclohexanecarboxylate (V) might be decarboxylated directly to cyclohexanone (VII) or its enolic form hydrated to give cyclohexane-1,2-diol-1-carboxylate (XIV); decarboxylation of this or its dehydrogenated product (XV) would result in convergence at some stage between cyclohexanone (VII) and cyclohexane-1,2-dione (X). 2-Hydroxycyclohexanone (IX) gave rise to adipate (XIII) in cell-incubation experiments, which makes it likely that a hydrolytic cleavage occurs to give 6-hydroxyhexanoate (XI); dehydrogenases could then convert this into adipate (XIII) through adipic acid semialdehyde (XII).

We have been unable so far, to prepare subcellular fractions from *Moraxella* cells grown anaerobically on benzoate which are capable of reducing the aromatic ring, even with the addition of a variety of cofactors which might be required. However, certain steps postulated in Scheme 1 resemble reactions known to occur in the aerobic process, for example, as follows.

In the aerobic metabolism of phloroglucinol by *Pseudomonas* sp., the first step is a reductive one, producing dihydrophloroglucinol; this reaction was reconstituted with a cell-free extract and NADPH as source of reducing power (Hang, 1967; Jamieson *et al.*, 1970). Decarboxylation of 2-oxocyclohexanecarboxylate (V) has its counterpart in the non-oxidative decarboxylation of hydroxybenzoates (Ribbons & Evans, 1960; Grant & Patel, 1969) by *Pseudomonas* sp. and *Klebsiella aerogenes* (*Aerobacter aerogenes*). The alternative mechanism bears some analogy to the formation of catechol from benzoate aerobically by *Acinetobacter*, *Alkaligenes*, *Azobacter* and some *Pseudomonas* sp. through decarboxylation of the diol intermediate (cyclohexa-3,5-diene-1,2-diol-1-carboxylate) as demonstrated enzymically by Reiner (1971) and Reiner & Hegeman (1971). Both ring-fission mechanisms shown in Scheme 1 are well known to take place aerobically. Thus Yugari (1961) demonstrated the cleavage of cyclohexan-1,2-dione (X) by a hydrolase from *Pseudomonas* sp. to give adipic acid semialdehyde (XII) and its oxidation to adipate (XIII) by an NAD-dependent dehydrogenase. However, Norris & Trudgill (1971) found that *Nocardia globerula* contained a mixed-function oxygenase system requiring NADP and molecular oxygen, which gave 6-hydroxyhexanoate (XI) from cyclohexanone (VII); 2-hydroxycyclohexanone (IX), a presumed intermediate, was thought to undergo re-arrangement to ϵ -hexanolactone followed by hydrolysis to 6-hydroxyhexanoate (XI). NAD-dependent dehydrogenases then oxidized the hydroxy acid to adipate (XIII). Further consolidation of the reductive pathway suggested for the anaerobic metabolism of benzoate through nitrate respiration awaits a reconstitution of each step at an enzymic level.

It is difficult, at present, to gauge the extent to which the anaerobic pathway of aromatic ring catabolism operates in Nature, compared with the better known aerobic processes of dissimilation. The Athiorhodaceae (e.g. *Rhodopseudomonas* and *Rhodospirillum* sp.) require light and anaerobiosis for it to be possible; such habitats exist in oceans and lakes (Stewart, 1968). Rumen liquor and sewage sludge can produce methane and CO₂ from certain plant phenolics under anaerobic conditions; this overall reaction probably requires the co-operation of at least two bacterial species. About 6–8% of the gross energy of the feed of ruminants is lost as

Scheme 1. Reaction sequences in the anaerobic metabolism of benzoate

(a) Photometabolism (*Rhodospseudomonas palustris*); (b) nitrate respiration (*Moraxella* sp.). The intermediates denoted by **bold** Roman numerals have been chemically identified, the rest are hypothetical.

methane (Blaxter, 1961), but the contribution of the aromatic components of the diet is unknown. Considering the soil environment, anaerobic conditions are frequently generated by impeded drainage, with reduction of tervalent iron (producing the visual phenomenon called gleying of the profile); soil aggregates can also be anaerobic internally, with reduction of sulphate and nitrate by the relevant microbial species.

Note added in Proof (Received 13 February 1975)

We have since identified cyclohexanone as its 2,4-dinitrophenylhydrazone derivative in growing cultures and whole-cell suspensions metabolizing benzoate under anaerobic conditions. It may have originated from compound (V) (see Scheme 1).

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