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Microbial Degradation of Hydrocarbons

CATABOLISM OF 1-PHENYLALKANES BY *NOCARDIA SALMONICOLOR*

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1. *Nocardia salmonicolor* grew on a variety of alkanes, 1-phenylalkanes and 1-cyclohexylalkanes as sole carbon and energy sources. 2. Growth on 1-phenyldodecane in batch culture was diauxic. Isocitrate lyase activity was induced during lag phase, reaching a maximum activity in the first growth phase, during which both the aromatic ring and the side chain were degraded. However, 4-phenylbutyrate, 4-phenylbut-3-enoate, 4-phenylbut-2-enoate, 3-phenylpropionate, cinnamate and phenylacetate accumulated in the growth medium. These compounds disappeared at the onset of diauxic lag and four hydroxylated compounds accumulated; one was 4-(*o*-hydroxyphenyl)but-3-enoate and another was identified as 4-(*o*-hydroxyphenyl)butyrate. These compounds were utilized during the second growth phase. 3. Washed 1-phenyldodecane-grown cells oxidized acetate, cinnamate, 3,4-dihydroxyphenylacetate, homogentisate, *o*-, *m*- and *p*-hydroxyphenylacetate, phenylacetate, and 4-phenylbutyrate rapidly without lag. 4. Extracts of such cells rapidly oxidized homogentisate,3,4-dihydroxyphenylacetate, catechol and protocatechuate. 5. The organism grew readily on 4-phenylbutyrate, phenylacetate, *o*-hydroxyphenylacetate, homogentisate and 3,4-dihydroxyphenylacetate as sole carbon energy sources, but growth was slow on cinnamate and 4-phenylbut-3-enoate. 6. When cinnamate and phenylacetate were sole carbon sources for growth, phenylacetate and *o*-hydroxyphenylacetate respectively were detected in culture supernatants. 4-Phenylbut-3-enoate and 4-phenylbutyrate both yielded a mixture of cinnamate and phenylacetate. 7. It is proposed that 1-phenyldodecane is catabolized by ω-oxidation of the terminal methyl group, side-chain β-oxidation to 4-phenylbutyrate, both β- and α-oxidation to phenylacetic acid, hydroxylation to homogentisate via *o*-hydroxyphenylacetate and ring cleavage to maleylacetoacetate. Catabolism via 3,4-dihydroxyphenylacetate may also occur. 8. Growth on 1-phenylnonane was also diauxic and cinnamic acid, phenylpropionic acid, benzoic acid and hydroxyphenylpentanoic acid accumulated in the medium. Respirometric data and ring-cleavage enzyme activities showed similar patterns to those obtained after growth on 1-phenyldodecane. The results suggest that the main catabolic routes for 1-phenyldodecane and 1-phenylnonane may converge at cinnamate. 9. Possible reasons for diauxie are discussed.

Previous studies have demonstrated that *Nocardia* isolates from soil can utilize 1-phenylalkanes as sole carbon and energy sources for growth (Webley *et al.*, 1955, 1956; Davis & Raymond, 1961). The metabolism of phenylalkanes is important as these compounds are components of many detergents and some herbicides. Webley *et al.* (1955, 1956) obtained evidence that 1-phenylalkanes were degraded by β-oxidation, presumably after ω-oxidation, by a strain of *Nocardia opaca*, yielding cinnamic acid and benzoic acid from compounds with an odd number of carbon atoms, and phenylacetic acid from compounds with an even number of carbon atoms; metabolism of the ring was not demonstrated. Davis & Raymond (1961) showed that an isolate similar to *Nocardia salmonicolor* degraded both the side chain and the aromatic ring of 1-phenylalkanes with an odd number of carbon atoms, but phenylacetic acid accumulated from compounds with an even number of carbon atoms and was not further metabolized. Compounds of this type with a short side chain were unable to support growth, but could be co-oxidized in the presence of an *n*-alkane.

Willetts & Cain (1970, 1972) described the metabolism of 1-phenylundecane-*p*-sulphonate and 1-phenyldodecane-*p*-sulphonate by an unidentified *Bacillus* sp. The proposed route was via the corresponding *p*-hydroxyphenylalkanoic acid, the side chain being degraded by β-oxidation followed by cleavage of the ring in the *ortho* position. A *Vibrio* species has been shown to degrade linear

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alkylbenzenesulphonates partially, attacking only the side chain (Bird & Cain, 1972). Baggi *et al.* (1972) have described the first steps in the metabolism of some 2-, 3- and 4-phenylalkanes by *Pseudomonas* and *Nocardia* isolates. The total degradation of 1-phenylalkanes with an even number of carbon atoms has not been previously reported, and the present paper describes a study of 1-phenyldodecane breakdown by *N. salmonicolor* and compares it with that of 1-phenylnonane. Preliminary reports of part of this work have appeared (Sariaslani *et al.*, 1972; Sariaslani & Higgins, 1973).

Materials and Methods

Micro-organism, source, maintenance and culture

N. salmonicolor N.C.I.B. 9701 was obtained from the National Collection of Industrial Bacteria, Aberdeen, U.K. It was maintained on nutrient–agar slopes [2.8% (w/v) Oxoid; CM3, Oxoid Ltd., London E.C.4, U.K.] and was grown at 30°C in liquid culture in a 16-litre-capacity stirred fermenter (L.H. Engineering, Stoke Poges, Bucks, U.K.) with an air flow rate of 4 litres/min or on a gyrotory shaker in 2-litre conical flasks, plugged with cotton-wool and containing 500ml of growth medium. Hydrocarbon (usually 0.5%, v/v) or alternative carbon source (0.5%, w/v) was added to the mineral-salts medium described by Davis & Raymond (1961), which also contained trace-metal mixture (1 ml/litre) (Bauchop & Elsden, 1960).

Preparation of washed cell suspensions

Organisms were harvested by centrifugation (23000*g*, 1h, 12°C), washed twice with 10mM-sodium phosphate buffer, pH 7.0, and resuspended in the same buffer.

Measurement of cell density

The density of organisms in both growth and suspension media was measured by direct dry weight determination.

Measurement of O_2 uptake by washed cell suspensions

O_2 consumption was recorded at 30°C with a Gilson respirometer (Gilson Medical Electronics, Middleton, Wis., U.S.A.) or an O_2 electrode (Rank Bros., Bottisham, Cambridge, U.K.). Unless otherwise stated incubation mixtures contained sodium phosphate buffer, pH 7.0 (100μmol), cell suspension (approx. 10mg dry wt.) and substrate (100μmol) in a total volume of 3.0ml. Respirometer flasks contained 0.2ml of 20% (w/v) NaOH in the centre well to absorb CO_2.

Preparation of cell-free extracts

Extracts were prepared by sonication of washed cell suspensions for 2min in an MSE sonicator (type 150W) and the resulting suspensions were usually centrifuged at 100000*g* for 1h at 4°C. The pellet fraction was usually discarded. Extracts prepared for assay of β-oxidation enzymes were centrifuged at 38000*g* for 15min and the pellet was discarded.

Assays

Protein assay. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo., U.S.A.) as standard.

Isocitrate lyase (*EC* 4.1.3.1). Isocitrate lyase was assayed as described by Dixon & Kornberg (1959).

Homogentisate 1,2-*dioxygenase* (*EC* 1.13.11.5). Activity in extracts was measured both spectrophotometrically (Chapman & Dagley, 1962) and polarographically. A value of 13500 was used for the ε_{330} of the product, maleylacetoacetate, at pH 7.2 (Knox & Edwards, 1955). Reaction mixtures were incubated at 30°C and contained sodium phosphate buffer, pH 7.0 (260μmol), sodium homogentisate (10μmol) and extract (0.5–2mg of protein) in a total volume of 3.0ml.

3,4-*Dihydroxyphenylacetate* 3,4-*dioxygenase* (*EC* 1.13.11.7), *catechol* 1,2-*dioxygenase* (*EC* 1.13.11.1) *and protocatechuate* 3,4-*dioxygenase* (*EC* 1.13.11.3). Activity in extracts was measured polarographically as described for homogentisate 1,2-dioxygenase, but reaction mixtures contained sodium 3,4-dihydroxyphenylacetate, catechol or sodium protocatechuate (10μmol) respectively, rather than homogentisate.

β-Oxidation enzymes. Butyryl-CoA dehydrogenase (EC 1.3.99.2) and thiolase (acetyl-CoA acetyltransferase, EC 2.3.1.9) were assayed by the methods of Mahler (1955) and Lynen & Ochoa (1953) respectively.

Detection of acidic intermediates accumulating in the growth medium by t.l.c. Organisms were removed from the medium by centrifugation (23000*g*, 1h, 12°C). Samples (500ml) of supernatant were then acidified with HCl to pH3 and extracted three times with equal volumes of diethyl ether. The ethereal extracts were combined and dried over anhydrous $MgSO_4$ before removal of the ether under decreased pressure. The residue was then taken up in 5ml of diethyl ether and portions were applied to silica-gel t.l.c. plates (Eastman–Kodak, Kirkby, Liverpool, U.K.). Chromatograms were developed for non-hydroxylated aromatic acids in the solvent system light petroleum (b.p. 60–80°C)–acetic acid (49:1, v/v) and for hydroxylated aromatic acids in the system light petroleum (b.p. 60–80°C)–diethyl ether–formic

acid (usually 45:5:1, by vol.). Aromatic acids were detected on chromatograms with 0.4% (w/v) Bromocresol Green and hydroxylated aromatic compounds were detected with 1% (v/v) ethanolic Gibbs' reagent (2,6-dichloro-*p*-benzoquinone-4-chlorimine) followed by saturated aq. $NaHCO_3$.

Assay of non-hydroxylated aromatic acids accumulating in the growth medium. Ether extracts of culture supernatants were subjected to column chromatography (30cm × 2.5cm) on silicic acid (Mallinckrodt; Eastman–Kodak), the eluting solvent being light petroleum (b.p. 60–80°C)–acetic acid (49:1, v/v). The eluted fractions containing non-hydroxylated acids were combined, evaporated to dryness and the residue was taken up in a measured volume of methanol. The concentration of each component in the mixture was then calculated from the u.v. spectrum, by using values for the extinction coefficients for each compound at three wavelengths determined from spectra of the separate pure components. The values used for the molar extinction coefficients of 4-phenylbutyrate, 4-phenylbut-3-enoate and cinnamate were 3.12×10^3, 1.13×10^4 and $1.86\times10^4\,l\cdot mol^{-1}\cdot cm^{-1}$ (217nm), 1.32×10^2, 2.03×10^4 and $1.28\times10^4\,l\cdot mol^{-1}\cdot cm^{-1}$ (252nm) and 3.3×10^1, 2.75×10^3 and $2.40\times10^4\,l\cdot mol^{-1}\cdot cm^{-1}$ (273nm) respectively.

Alternatively ether extracts were treated with diazomethane and the methyl esters were determined by g.l.c.

Isolation and purification of aromatic acids from the growth medium

Acidified culture supernatants were extracted with diethyl ether as described under 'Detection of acidic intermediates accumulating in the growth medium by t.l.c.', the extracted compounds dissolved in the minimum volume of diethyl ether and applied to a column (30cm×2.5cm) of silicic acid (Mallinckrodt, 100 mesh). Elution with light petroleum (b.p. 60–80°C)–diethyl ether–formic acid (45:5:1, by vol.) resulted in the separation of the non-hydroxylated acids from the hydroxylated ones. Non-hydroxylated-acid fractions were combined, concentrated by removing solvent under decreased pressure and applied to a similar column, which was eluted with light petroleum (b.p. 60–80°C)–acetic acid (49:1, v/v). The components were completely separated by several passages through this column. Fractions containing pure components were combined, evaporated to dryness and the isolated intermediates recrystallized from light petroleum (b.p. 60–80°C).

Fractions containing hydroxylated aromatic acids were combined, solvent was removed under decreased pressure and the mixture was reapplied to a column (30cm × 2.5cm) of silicic acid, the components being separated by elution with the solvent system light petroleum (b.p. 60–80°C)–diethyl ether–formic acid (the proportions being varied stepwise between 45:5:1 and 25:25:1, by vol.). Components were recrystallized from light petroleum (b.p. 60–80°C)–benzene (approx. 100:1, v/v) or from chloroform–carbon tetrachloride (approx. 100:1, v/v).

Chemicals

Catechol, 2,6-dichloro-*p*-benzoquinone-4-chlorimine, decanoic acid, phenylacetic acid and 4-phenylbutyric acid were obtained from BDH Chemicals, Poole, Dorset, U.K. Cinnamic acid and sodium benzoate were purchased from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K., and *o*-, *m*- and *p*-hydroxyphenylacetic acids, 3,4-dihydroxyphenylacetic acid, acetoacetyl-CoA and palmitoyl-CoA were from Sigma Chemical Co., St. Louis, Mo., U.S.A. 1-Phenyldodecane and 1-phenylnonane were obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. 4-Phenylbut-3-enoic acid was synthesized by the method of Linstead & Williams (1926).

Spectral analyses

I.r. spectra were recorded in Nujol with a Perkin–Elmer model 457 spectrophotometer and u.v. spectra were obtained with a Unicam SP.800 recording spectrophotometer. An AEI MS902 mass spectrometer was used to record mass spectra and n.m.r. spectra were obtained with a Perkin–Elmer R10 instrument. A Jeol JNM PS100 n.m.r. spectrometer was used for high-resolution spectra. The solvent for n.m.r. spectroscopy was deuterochloroform with tetramethylsilane as internal standard except where stated otherwise.

Gas–liquid chromatography

Phenolic acids were converted into the methyl esters by treatment with diazomethane (de Boehr & Backer, 1954), and were separated by using a pre-packed coiled glass column (1.83m × 2.0mm internal diam. containing 3% (w/v) methylsilicone gum (SE 30) on Gas Chrome Q (100–120 mesh) (Pye Unicam, Cambridge, U.K.) or a similar column containing 10% (w/v) polyethylene glycol adipate on Diatomite C (100–120 mesh). A Pye model 104 gas chromatograph fitted with a flame-ionization detector was used. Conditions were as follows: carrier gas (N_2); flow rate, 30–50ml/min; oven temperature, 110–180°C; detector temperature, 225°C. Quantitative measurements were made with standard methyl esters and a Honeywell Electronic 15 potentiometric recorder fitted with a disc integrator (Disc Instruments Inc., Santa Ana, Calif., U.S.A.).

Table 1. *Ability of N. salmonicolor to use various hydrocarbons as substrates for growth*

The organism was grown on *n*-dodecane and 0.2ml portions of this culture were used to inoculate samples (50ml) of growth medium containing hydrocarbon (liquid, 0.2–0.4%, v/v; solid, 0.2–0.4%, w/v). Cultures were incubated in screw-cap bottles (500ml) for 48h at 30°C on a gyratory shaker, and were scored for growth visually. For gaseous potential substrates, growth was tested by incubating cultures in similar bottles with cottonwool plugs in anaerobic jars containing hydrocarbon–air mixtures (1:1, v/v).

Type of hydrocarbon	Hydrocarbons supporting heavy growth	Hydrocarbons supporting poor growth	Hydrocarbons that were not growth substrates
n-Alkane	*n*-Heptane	*n*-Octane	Methane
	n-Decane		Ethane
	n-Undecane		*n*-Propane
	n-Dodecane		*n*-Pentane
	n-Octadecane		*n*-Nonane
	n-Tetradecane		
Aromatic	1-Phenylpentane	1-Phenylbutane	Benzene
	1-Phenylheptane	1-Phenylhexane	Toluene
	1-Phenylnonane	1-Phenyloctane	Ethylbenzene
	1-Phenyldecane		*p*-Cymene
	1-Phenylundecane		
	1-Phenyldodecane		
	1-Phenyltridecane		
	1-Phenyltetradecane		
	1-Phenylpentadecane		
Alicyclic			Cyclohexane
	1-Cyclohexylbutane		
	1-Cyclohexylhexane		1-Cyclohexylpropane
	1-Cyclohexyloctane		1-Cyclohexylheptane
	1-Cyclohexylnonane		
	1-Cyclohexyldecane		

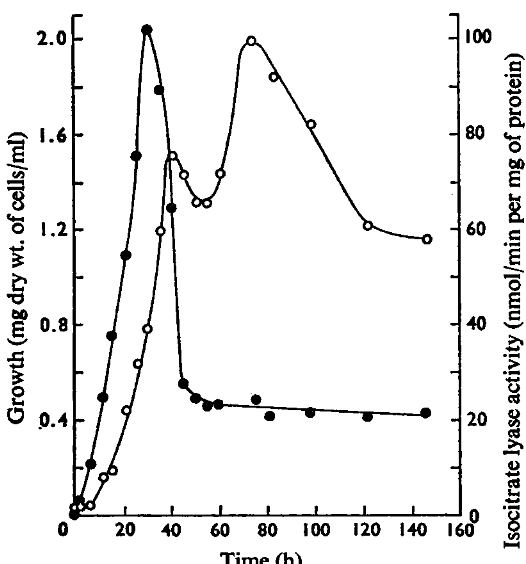


Fig. 1. *Growth of N. salmonicolor on 1-phenyldodecane and isocitrate lyase activity in cell-free extracts*

The organism was grown in a 16-litre-capacity stirred fermenter (air flow rate 5litres/min, impeller speed 800rev./min). Growth and enzyme activity were measured as described in the Materials and Methods section. Growth (○); isocitrate lyase activity (●).

Gas–liquid chromatography–mass spectrometry

Separation of components was effected as described under 'Gas–liquid chromatography' except that the carrier gas was helium. The mass spectrometer was an AEI MS30.

Results and Discussion

Growth of N. salmonicolor on hydrocarbons

The micro-organism was found to grow on a wide range of alkanes, 1-phenylalkanes and 1-cyclohexylalkanes as sole sources of carbon and energy. Table 1 shows the results of a survey of potential growth substrates. The catabolism of two of the phenylalkanes supporting growth was subjected to detailed examination.

Growth of N. salmonicolor on 1-phenyldodecane

Organisms were harvested from a nutrient-broth culture (3litres), washed in 10mM-sodium phosphate buffer, pH7.0, resuspended in the same buffer aseptically and used as an inoculum for 15litres of 1-phenyldodecane–mineral-salts medium. The growth curve and isocitrate lyase activity during growth are shown in Fig. 1. There was a 6h lag phase during which rapid induction of isocitrate lyase

occurred. Exponential growth followed with a mean generation time of 6h. There was a marked diauxie and the isocitrate lyase activity reached a maximum value of 100nmol/min per mg of protein just before the onset of diauxic lag. The specific activity then decreased rapidly and was maintained at a low value during the lag and second phase of growth. This suggested that the *n*-alkane side chain was degraded by β-oxidation and yielded acetyl-CoA units during the first phase of growth. It is well known that the glyoxylate cycle serves an anaplerotic role when many microbes grow on acetate as the sole carbon and energy source (Kornberg, 1966). The involvement of the cycle in microbial *n*-alkane catabolism, as evidenced by increased isocitrate lyase activity during growth on these compounds, has been previously reported (Trust & Millis, 1970).

During the first growth phase, four or sometimes five acidic compounds accumulated in the growth medium and their chromatographic properties are summarized in Table 2. Compounds 1, 2, 3 and 4 accumulated sequentially and a trace of compound 5 could be detected in most samples by g.l.c. Compound 5 accumulated in larger amounts when a dense suspension of organisms harvested during the first growth phase was resuspended and incubated in complete 1-phenyldodecane medium (Table 2). Routine examination of culture supernatants from other experiments by g.l.c. showed that compound 5 represented a greater proportion of acids accumulating when the air supply was restricted. The accumulated compounds disappeared abruptly from the medium at the beginning of diauxic lag. For identification purposes, they were extracted from culture supernatants from the first growth phase, purified and crystallized as described in the Materials and Methods section, except for compounds 3, 5 and 6, which were detected and identified by g.l.c.–mass spectrometry after methylation.

The disappearance of the above compounds at the beginning of diauxic lag was accompanied by the accumulation of similar quantities of acidic compounds, which gave positive reactions with Gibbs' reagent, indicating that they were ring-hydroxylated. At least four hydroxylated acids were present and they were utilized during the second growth phase. The chromatographic properties of three of these compounds are also summarized in Table 2.

Identification of intermediates accumulating during the first growth phase

Culture supernatant (10litres) from mid-first exponential phase was extracted as described in the Materials and Methods section and the following compounds were isolated.

Compound 1. White crystals (m.p. 51–53°C) were obtained from light petroleum (b.p. 60–80°C), the yield being 16mg. The mass spectrum showed a molecular-ion peak at *m/e* 164. Major fragmentation peaks were apparent at *m/e* 146, 118 and 104 (base peak), representing successive loss of H_2O, CO and a CH_2 fragment. There was also a major peak at $m/e = 91$ caused by a tropylium ion, showing the presence of an aromatic ring. This is consistent with an aromatic compound containing a C_4 side chain terminating in a carboxyl group. The n.m.r. spectrum gave a broad signal at 11.45p.p.m. attributable to a single carboxyl proton; five aromatic protons appeared as a sharp peak at 7.20p.p.m. The protons of three side-chain methylene groups appeared as two distorted triplets, one at 2.62p.p.m. ($J = 6.0$Hz), the other at 2.32p.p.m. ($J = 5.8$Hz) and a complex multiplet centred at 11.95p.p.m. Both spectra are consistent with the metabolite being 4-phenylbutyric acid and were identical in all respects with those of authentic 4-phenylbutyric acid (literature m.p. 52°C).

Compound 2. White crystals (m.p. 84–87°, yield 10mg) were obtained from light petroleum (b.p. 60–80°C). The mass spectrum gave a molecular-ion peak at *m/e* 162. The major fragmentation pathway involved loss of H_2O, CO and H_2 giving peaks at *m/e* 144, 116 (base peak) and 114 (metastable at 112.1). The presence of an aromatic ring was demonstrated by a tropylium ion represented by a peak at *m/e* 91. The spectrum is indicative of an aromatic compound with a C_4-unsaturated side chain, the terminal group being carboxyl. An alternative fragmentation pattern involving loss of the carboxyl group by cleavage in the C_1–C_2 position, giving an intense peak at *m/e* 117, suggests that the double bond lies in the C_3–C_4 position. This fragmentation is confirmed by the presence of a metastable species at *m/e* 84.5. The mass spectrum indicates that the compound is 4-phenylbut-3-enoic acid. The presence of a double bond conjugated with the aromatic ring is also indicated by a u.v.-absorption maximum at 252nm ($\varepsilon = 2.03 \times 10^4 l \cdot mol^{-1} \cdot cm^{-1}$). The literature m.p. for 4-phenylbut-3-enoic acid is 87–88°C, but 65°C for the corresponding 2-enoic acid, confirming the identity as 4-phenylbut-3-enoic acid.

Compound 3. In this case extracts of culture supernatants were treated with diazomethane, and then subjected to combined g.l.c.–mass spectrometry. A component was detected with a mass spectrum similar to but not identical with that of the methyl ester of 4-phenylbut-3-enoate. In both cases there was a molecular-ion peak at $m/e = 176$. The two spectra differed primarily in the relative abundances of the peaks at *m/e* 117 and 144. The ratio

$$\frac{\text{abundance at } m/e \text{ 117}}{\text{abundance at } m/e \text{ 144}}$$

was 20 and 2.8 respectively for the methyl esters of compound 3 and 4-phenylbut-3-enoate. This strongly

Table 2. *Chromatographic properties of some 1-phenyldodecane catabolites formed by N. salmonicolor during the first growth phase and at the onset of diauxic lag*

Culture supernatant was extracted as described in the Materials and Methods section and samples (0.25 ml) were applied to silica-gel t.l.c. plates. Compounds 1,2,3 and 4 were present in extracts from the first growth phase. For the detection and isolation of compound 5, organisms were harvested aseptically from the first growth phase and resuspended in complete 1-phenyldodecane growth medium (5%, v/v, of hydrocarbon) at high cell density (15 mg/ml). Cultures [500 ml in 2-litre conical flasks] were incubated on a gyrotary shaker at 30°C for 3 h before removal of the cells by centrifugation and extraction of the culture supernatants. Ether extracts were chromatographed as described above. Compound 6 was present in trace amounts in extracts from the first growth phase. Compounds 7, 8 and 9 were extracted from diauxic lag-phase supernatants. The development of chromatographs and staining procedures are described in the Materials and Methods section. G.l.c. was also carried out as described in the Materials and Methods section and under the following conditions: column temperature, 180°C; injector temperatures, 240°C; detector temperature, 225°C; N_2 carrier gas flow rate, 35 ml/min (methyl-silicone gum), 45 ml/min (polyethylene glycol adipate). ND, not determined.

Compounds isolated			T.l.c. properties		G.l.c. properties of methyl esters	
No.	Name	Formula	R_F in light petroleum (b.p. 60–80°C)–acetic acid (49:1, v/v)	R_F in light petroleum (b.p. 60–80°C)–diethyl ether–formic acid (85:15:2, by vol.)	Retention time on methyl-silicone gum (min)	Retention time on polyethylene glycol adipate (min)
1	4-Phenylbutyric acid	C_6H_5–$[CH_2]_3$—CO_2H	0.75	0.95	6.3	4.7
2	4-Phenylbut-3-enoic acid	C_6H_5–CH═CH—CH_2—CO_2H	0.71	0.95	8.4	9.3
3	4-Phenylbut-2-enoic acid (tentative identification)		ND	ND	ND	7.9
4	*trans*-Cinnamic acid	C_6H_5–CH═CH—CO_2H	0.67	0.95	6.3	7.0
5	Phenylacetic acid	C_6H_5–CH_2—CO_2H	0.54	0.95	2.8	2.5
6	3-Phenylpropionic acid	C_6H_5–$CH_2CH_2CO_2H$	ND	ND	ND	3.7
7	4-(*o*-Hydroxyphenyl)butyric acid	C_6H_4(OH)–$[CH_2]_3$—CO_2H	0.04	0.59	ND	ND
8	*trans*-4-(*o*-Hydroxyphenyl)-but-3-enoic acid	C_6H_4(OH)–CH═CH—CH_2CO_2H	0.04	0.37	ND	ND
9	6-(*o*-Hydroxyphenyl)-hydroxyhexanoic acid (tentative identification)		0.04	0.18	ND	ND

suggests that compound 3 is 4-phenylbut-2-enoate, since the position of the double bond in this compound would favour cleavage of the carbon–carbon bond in the C_1–C_2 position. Compound 3 is tentatively identified therefore as 4-phenylbut-2-enoate on the basis of this mass spectrum and the comparison with that of compound 2.

Compound 4. White crystals (m.p. 130–134°C, yield 10mg) were obtained from light petroleum (b.p. 80–100°C). The i.r. spectrum showed the presence of an aromatic ring (absorption band at $1630cm^{-1}$), strong absorption bands associated with a carboxyl group (1680, $1220cm^{-1}$), and the presence of a *trans* double bond was indicated by absorption due to C–H bending vibrations at $980cm^{-1}$. The mass spectrum showed a molecular-ion peak at *m/e* 148, the fragmentation pattern being consistent with cinnamic acid. The n.m.r. spectrum indicates that the isomer is *trans*, the olefinic protons being represented by doublets, both with $J = 16.2$Hz, at 6.48 and 7.86 p.p.m. Authentic *trans*-cinnamic acid (literature m.p. 133–134°C) gave i.r., n.m.r., u.v. and mass spectra identical with those of the compound isolated.

Compound 5. This compound was isolated as white crystals (yield 3mg) from diethyl ether extracts of 500ml of growth medium after incubation with a dense suspension of washed cells (see the legend to Table 2). The g.l.c. properties after methylation were identical with those of phenylacetic acid methyl ester. The melting point of the free acid crystals from light petroleum (b.p. 60–80°C) was 72°C and a mixed melting point with authentic phenylacetic acid (literature m.p. 77°C) showed no depression. The mass spectrum of the isolated compound was identical with that of authentic phenylacetic acid. This compound was also present in small amounts in ether extracts of growth medium from cultures growing on 1-phenyldodecane in the impeller-agitated fermenter (3litre culture volume), and the concentration increased to approx. 0.5mM when the air flow rate was restricted to 0.1litre/min. The compound was identified in these extracts by g.l.c.–mass spectrometry of the methyl ester, which had a mass spectrum identical with that of authentic methyl phenylacetate.

Compound 6. In this case extracts of culture supernatants were treated with diazomethane and a component was detected by g.l.c. which had retention times identical with those of the methyl ester of 3-phenylpropionic acid on both methylsilicone gum (SE 30) and polyethylene glycol adipate columns. A mass spectrum of the compound obtained by combined g.l.c.–mass spectrometry showed a molecular-ion peak at *m/e* 150 and the fragmentation pattern and major peak ratios were identical with those obtained for authentic methyl 3-phenylpropionate, confirming the identification.

Identification of hydroxylated compounds accumulating at the onset of diauxic lag

Culture supernatant (16litres) from the diauxic lag period was extracted as described in the Materials and Methods section and the following compounds were isolated.

Compound 7. White crystals (m.p. 64°C, yield 90mg) were obtained from benzene–light petroleum (b.p. 60–80°C) (1:100, v/v). The mass spectrum showed a molecular-ion peak at *m/e* 180. The major fragmentation pathway involves the loss of H_2O, CO and the CH_2–CH radical to give peaks at *m/e* 162, 134 and 107 (base peak), suggesting a C_4 side chain terminating in a carboxyl group, substituted in the C-4 position with a monohydroxylated aromatic ring. The n.m.r. spectrum showed a broad peak at 5.7p.p.m. attributable to the carboxylic and phenolic protons. The aromatic protons were represented by signals at 7.00p.p.m. (two superimposed doublets, $J = 7.4$Hz) attributable to protons in the C-3 and C-6 positions, and also a signal at 6.76p.p.m. (two superimposed triplets, $J = 7.4$Hz) interpreted as being due to the protons in the C-4 and C-5 positions. This indicates that the hydroxyl group is in the *ortho* position. The side-chain protons gave signals at 2.68p.p.m. (triplet, $J = 7.7$Hz), 2.44p.p.m. (triplet, $J = 6.2$Hz) and 1.96p.p.m. (multiplet, J values 7.7 and 6.2Hz) interpreted as being due to three methylene groups. The spectral data are consistent with the compound being 4-(*o*-hydroxyphenyl)butyric acid [m.p. for the authentic compound is reported as 65–67°C; Mitsui *et al.* (1962)].

Compound 8. Creamy-white crystals (m.p. 73–75°C, yield 6mg) were obtained from benzene–light petroleum (b.p. 60–80°C). The mass spectrum showed a molecular-ion peak at *m/e* 178. The main fragmentation pattern involves the sequential loss of carboxyl group, H_2 and C_2H_2 to give peaks at *m/e* 133, 131 (base peak) and 105 (metastable at *m/e* 83.1). This spectrum indicates an aromatic compound with a C_4-unsaturated side chain, the terminal group being carboxyl, and also shows hydroxyl substitution in the ring. Since the main pattern of fragmentation involves loss of carboxyl group, the double bond is most likely in the C_3–C_4 position. An n.m.r. spectrum of the sodium salt of the acid was obtained with ^2H_2O as solvent and tetramethylsilane as external standard. The methylene protons were represented by signals at 3.22p.p.m. ($J = 6.0$Hz) and the presence of a *trans* double bond was indicated by olefinic protons represented by a quartet at 6.20p.p.m. ($J = 16.0$Hz) and a doublet at 6.83p.p.m. ($J = 16.0$Hz). Four aromatic protons were represented by multiplets in the region of 6.63p.p.m. (protons in positions C-4 and C-5) and 7.42p.p.m. (protons in positions C-3 and C-6). This n.m.r. spectrum suggests that the hydroxyl group is in the *ortho* position.

The spectral and chromatographic evidence indicates that the compound is 4-(*o*-hydroxyphenyl)-but-3-enoic acid.

Compound 9. Creamy-yellow micro crystals (m.p. 94–96°C, yield 1.5mg) were obtained from benzene–light petroleum (b.p. 60–80°C). The compound is tentatively identified as a 6-(*o*-hydroxyphenyl)-hydroxyhexanoic acid on the basis of its chromatographic properties and a mass spectrum. There was a molecular-ion peak at *m/e* 224 and the fragmentation pattern was consistent with a hydroxylated aromatic ring (hydroxylated tropylium ion at *m/e* 107) attached to a C_6 side chain, terminating in a carboxyl group and bearing a hydroxyl substituent.

Quantification of intermediates accumulating during the first growth phase

The accumulation of 4-phenylbutyric acid and cinnamic acid during growth on 1-phenyldodecane is shown in Fig. 2. The former compound accumulates first, reaching a maximum concentration of 2.5mM, followed by cinnamic acid (maximum concentration, 1.0mM). The maximum amount of 4-phenylbut-3-enoate in the medium occurred after 30h and was only 10μM. The amount of phenylacetic acid accumulating was similarly very low under these conditions of high aeration. About 14% of the growth substrate accumulates as these acids, but at the onset of diauxie there is only a trace of 1-phenyldodecane remaining in the medium, suggesting that both the aromatic ring and the alkane side chain are being metabolized simultaneously during the first growth phase. However, the fact that aromatic compounds (mainly 4-phenylbutyrate and cinnamate) accumulate suggests that the rate of catabolism of the aromatic ring is limited by the rate at which 4-phenylbutyrate is converted into phenylacetate and further metabolized, at least under conditions of high aeration. It appears that under conditions of low aeration the further metabolism of phenylacetate becomes rate-limiting.

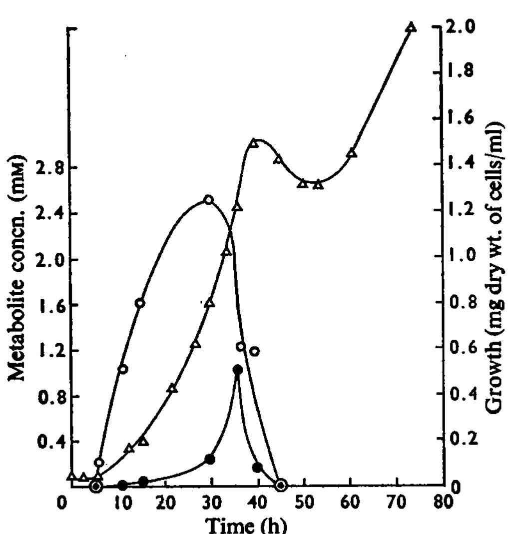


Fig. 2. *Growth of N. salmonicolor showing accumulation of 4-phenylbutyrate and cinnamate*

The organism was grown as described in the legend to Fig. 1 with 1-phenyldodecane as carbon source. Culture supernatants were extracted with diethyl ether, the accumulated acids separated and assayed spectrophotometrically as described in the Materials and Methods section. 4-Phenylbutyrate concn. (○); cinnamate concn. (●), growth (△).

Respirometric studies with washed cell suspensions

The results of experiments comparing the ability of intact cells to oxidize possible intermediates after

Table 3. *Oxidation of possible intermediates in* 1-*phenylalkane catabolism by washed cell suspensions of N. salmonicolor*

Suspensions of the organism were prepared from first-growth-phase cultures with 1-phenyldodecane or 1-phenylnonane [0.5% (v/v) of phenylalkane] as substrate and from mid-exponential-phase cultures growing on 2.5% (w/v) nutrient broth. Values for O_2 uptake were obtained from respirometric experiments as described in the Materials and Methods section. Incubation mixtures contained substrate (100μmol), sodium phosphate buffer, pH 7.0 (100μmol), and cell suspension (8 and 6mg dry wt. of 1-phenylalkane-grown cells and nutrient-broth-grown cells respectively). O_2 uptake was measured for 1h in all cases. Values are corrected for the endogenous respiration and are initial rates. ND, not determined.

	O_2 uptake (μl of O_2/h per mg dry wt.) after growth on substrate shown		
	1-Phenyldodecane	1-Phenylnonane	Nutrient broth
Acetate	100	21	8
Benzoate	1	2	0
Catechol	5	12	11
Cinnamate	43	13	0
3,4-Dihydroxyphenylacetate	24	7	0
Homogentisate	50	16	8
o-Hydroxyphenylacetate	36	4	0
m-Hydroxyphenylacetate	24	7	0
p-Hydroxyphenylacetate	19	5	0
Mandelate	0	0	0
cis,cis-Muconate	0	ND	0
Phenylacetate	74	16	6
4-Phenylbutyrate	163	ND	22
1-Phenyldodecane	30	ND	20
Protocatechuate	0	6	0

growth on 1-phenyldodecane or on nutrient broth are shown in Table 3. The initial rates of oxidation of acetate, cinnamate, homogentisate, *o*-, *m*- and *p*-hydroxyphenylacetate, phenylacetate and 4-phenylbutyrate are very much higher for 1-phenyldodecane-grown cells than for nutrient broth-grown cells. Benzoate, catechol, mandelate, *cis,cis*-muconate and protocatechuate were not readily oxidized.

Enzyme activities in cell-free extracts

Attempts were made to demonstrate cell-free oxygenation of phenylacetate and *o*-, *m*- and *p*-hydroxyphenylacetate. These substrates did not stimulate O_2 uptake measured polarographically in the presence of NADH or NADPH with or without added FAD. A variety of procedures were used for the preparation of extracts and various potential stabilizing agents and possible cofactors were tested. Difficulties in demonstrating cell-free oxidation of these compounds, particularly phenylacetate in micro-organisms grown on phenylacetate, has been previously reported (Blakley *et al.*, 1967). A search in extracts for enzymes capable of cleaving the aromatic ring revealed *ortho*-cleavage activities for catechol and protocatechuate in cells grown on nutrient broth. After inoculation of such cells into 1-phenyldodecane medium, protocatechuate 3,4-dioxygenase activity remained constant at about 40nmol/min per mg of protein, whereas catechol 1,2-dioxygenase activity was decreased from 92 to 27nmol/min per mg of protein. In contrast, activities of both homogentisate 1,2-dioxygenase and 3,4-dihydroxyphenylacetate 3,4-dioxygenase were induced by growing on 1-phenyldodecane. Fig. 3 shows induction of these enzymes during the first growth phase. Homogentisate 1,2-dioxygenase and 3,4-dihydroxyphenylacetate 3,4-dioxygenase activities were respectively 16- and 10-fold higher after growth on 1-phenyldodecane than after growth on nutrient broth. The product of homogentisate 1,2-dioxygenase activity had an u.v. spectrum identical with that described previously for maleylacetoacetate, the product of mammalian homogentisate 1,2-dioxygenase (Knox & Edwards, 1955).

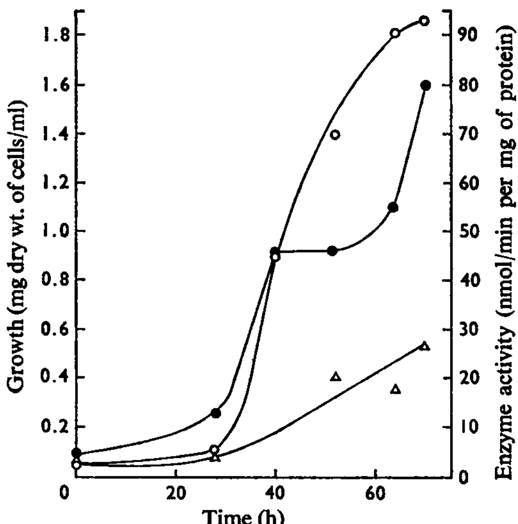


Fig. 3. *Induction of homogentisate* 1,2-*dioxygenase activity and* 3,4-*dihydroxyphenylacetate* 3,4-*dioxygenase activity during growth of N. salmonicolor on* 1-*phenyldodecane*

The medium (16litres) was inoculated at zero time with organisms from 3litres of a nutrient-broth culture after harvesting and washing aseptically. The culture was grown in a stirred fermenter at 30°C (air flow rate 5litres/min; impeller speed 600rev./min) and samples were taken for dry-weight and enzyme determinations at timed intervals during the first growth phase. Extracts were prepared and oxygenase activities measured as described in the Materials and Methods section. Growth (○); homogentisate1, 2-dioxygenase (●); 3,4-dihydroxyphenylacetate 3,4-dioxygenase (△).

Further metabolism of phenylacetic acid

At this stage in the investigation it seemed likely that 1-phenyldodecane was being metabolized via phenylacetate, and the enzymic evidence indicated that this compound was probably being further metabolized through either homogentisate or 3,4-dihydroxyphenylacetate or both these compounds. Catabolism via 3,4-dihydroxyphenylacetate has been previously reported for phenylacetate (Dagley & Wood, 1965) and for *p*-hydroxyphenylacetate (Adachi *et al.*, 1964). Blakley *et al.* (1967) showed that a pseudomonad growing on phenylacetate metabolized the substrate through *p*-hydroxyphenylacetate and 3,4-dihydroxyphenylacetate. More recently Blakley (1972) has shown that an unidentified soil micro-organism metabolizes *p*-hydroxyphenylacetate via homogentisate and this reaction has been studied in cell-free systems. The fact that some fungi and bacteria catabolize phenylacetate via homogentisate has been known for some time (Kluyver & Van Zijp, 1951; Dagley *et al.*, 1953; Kunita, 1955). It was important then in the present case to determine which isomer of hydroxyphenylacetate was formed from phenylacetate. It seemed possible that the accumulation of *ortho*-hydroxylated compounds at the end of the first growth phase reflected the lack of substrate specificity of an *ortho* hydroxylase for phenylacetic acid. However, since it was not possible to identify unambiguously an hydroxyphenylacetate during growth on 1-phenyldodecane, or detect an *ortho* hydroxylase in extracts, washed nutrient-broth-

grown cells were inoculated into medium containing phenylacetate (0.2%, w/v) as the sole carbon source and samples were taken during growth in the stirred fermenter (culture volume 12 litres; air flow rate 4 litres/min). Samples were extracted and chromatographed as described in the Materials and Methods section and during the lag phase one hydroxylated aromatic compound accumulated. The colour reaction with Gibbs' reagent was characteristic of a compound hydroxylated in the *ortho* position. For identification, culture supernatant (3 litres) was extracted with diethyl ether and applied to a silicic acid column as described in the Materials and Methods section. The phenylacetic acid was eluted with the solvent system used for non-hydroxylated acids and then the hydroxylated compound was eluted with light petroleum (b.p. 60–80°C)–diethyl ether–formic acid (45:5:1, by vol.). The compound was obtained as white crystals (yield 1.5 mg) after two recrystallizations from light petroleum (b.p. 80–100°C). The mass spectrum gave a molecular-ion peak at *m/e* 152. The main fragmentation pathway involved loss of H_2O and CO, giving peaks at *m/e* 134 and 106 (base peak). An alternative fragmentation pathway involving loss of carboxyl group gave a peak at *m/e* 107. The spectrum was identical in all respects with that of authentic *o*-hydroxyphenylacetate. The melting point of the compound was 139–141°C (literature m.p. is 144–146°C), confirming the identification.

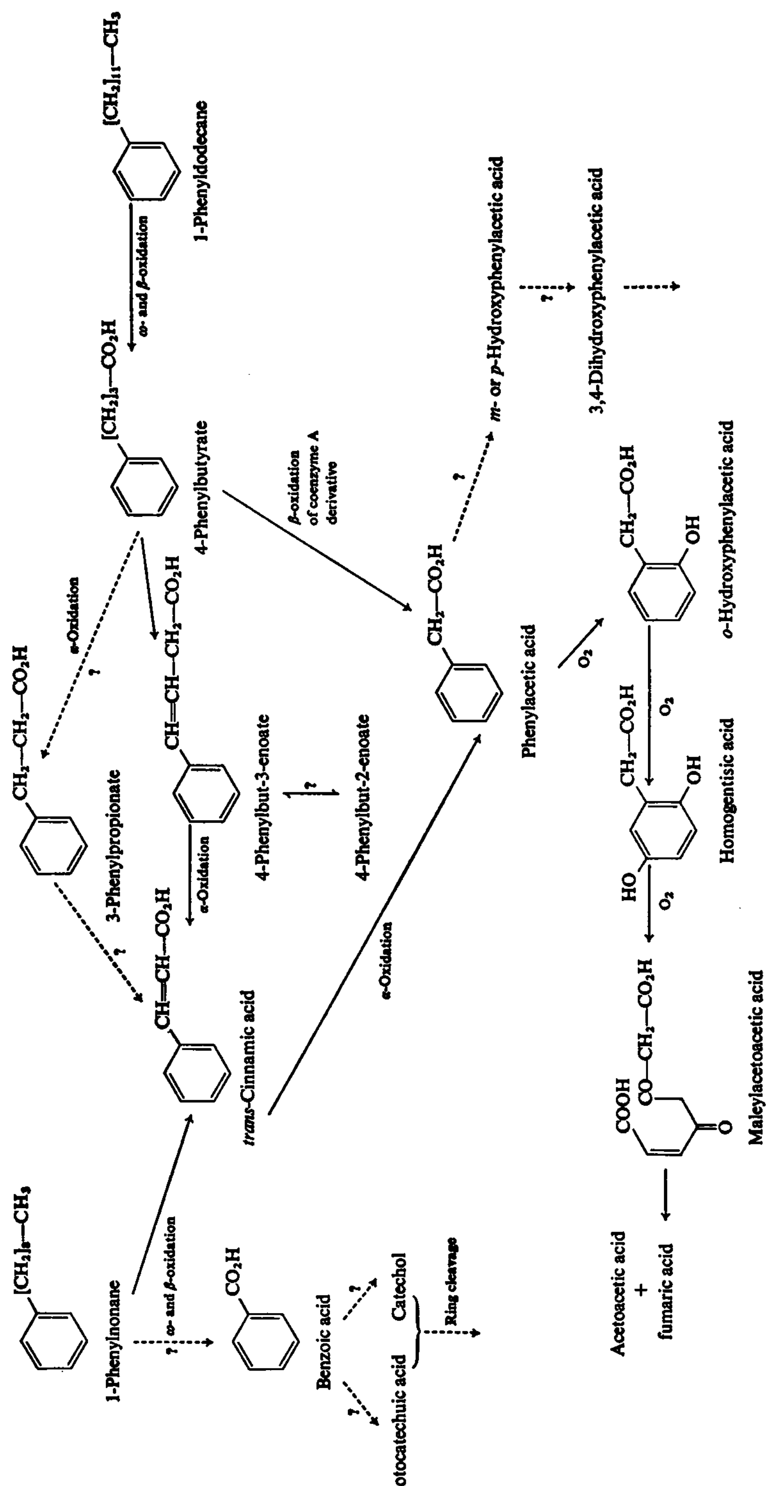
Formation of *o*-hydroxyphenylacetate from phenylacetate, together with the fact that homogentisate 1,2-dioxygenase is induced when the organism is grown on phenylacetate, is consistent with the catabolism of phenylacetate via the *o*-hydroxy derivative and homogentisate to maleylacetoacetate, although it has not proved possible to detect homogentisate in culture supernatants. There is a report in the literature of phenylacetate being converted into homogentisate via *o*-hydroxyphenylacetate during the catabolism of phenylalanine by some species of bacteria (Kunita, 1955). However, washed cell suspensions of 1-phenyldodecane-grown organisms are adapted to metabolize 3,4-dihydroxyphenylacetate as well as homogentisate, and similarly extracts of these organisms also oxidize both compounds. Either the *meta* or the *para* isomer of hydroxyphenylacetate would be the expected precursor of 3,4-dihydroxyphenylacetate, but the formation of these compounds from phenylacetate was not detected and hydroxylated compounds accumulating when 1-phenyldodecane was the growth substrate were always the *ortho* isomers. It is possible that adaptation to metabolize 3,4-dihydroxyphenylacetate reflects gratuitous induction resulting from regulatory linkage, but there also remains the possibility that some phenylacetate may be metabolized via this isomer.

Route of 1-phenyldodecane catabolism

The results suggest that 1-phenyldodecane is catabolized by the routes shown in Scheme 1. Tests for growth on the postulated intermediates revealed that the organism grows readily on 4-phenylbutyrate, phenylacetate, *o*-hydroxyphenylacetate, homogentisate, or 3,4-dihydroxyphenylacetate, but only poorly on cinnamate and 4-phenylbut-3-enoate. The latter two compounds clearly are intermediates in the degradation of 1-phenyldodecane, since they are further utilized after accumulating in the medium, and cell suspensions from the first growth phase readily oxidize them.

Supporting evidence for this catabolic scheme comes from studies in which the organism was grown on likely catabolic intermediates as the sole carbon and energy sources. The isolation of *o*-hydroxyphenylacetate as a metabolite of phenylacetate has been reported above. When 4-phenylbutyrate served as growth substrate, adaption from nutrient broth was slow (lag phase 20 h) and during this phase phenylacetate accumulated in the medium to a concentration, measured by g.l.c., of 3.0 mM (i.e. approx. 8% of the initial growth-substrate concentration); small amounts of cinnamate were also detected. Culture supernatants from mid-exponential phase contained four hydroxylated aromatic acids; one of these was identified as 4-(*o*-hydroxyphenyl)butyric acid and another as 4-(*o*-hydroxyphenyl)but-3-enoic acid. Growth was slow, stationary phase being reached after about 120 h. In similar experiments when 4-phenylbut-3-enoate was the growth substrate, cinnamate and phenylacetate accumulated, and when cinnamate was the substrate phenylacetate was detected in culture supernatants.

Referring again to Scheme 1, the question arises whether that part of the pathway that reflects the free acids that accumulate in the medium is the major catabolic route for 1-phenyldodecane. More specifically, are the two α-oxidation steps on the major catabolic route? The alternative possibility is that most of the substrate is metabolized via 4-phenylbutyryl-CoA by β-oxidation to phenylacetate. It is likely that 4-phenylbutyrate accumulates in the medium as a result of the β-oxidation enzyme system having a decreased affinity for 4-phenylbutyryl-CoA, probably resulting from steric hindrance owing to the proximity of the aromatic ring. Evidence for this comes from studies of the kinetics of butyryl-CoA dehydrogenase, albeit in crude extracts of 1-phenyldodecane-grown organisms, when the apparent K_m values for palmitoyl-CoA and 4-phenylbutyryl-CoA were found to be 6 and 88 μM respectively. This suggests that 4-phenylbutyryl-CoA is a less effective substrate than



Scheme 1. *Proposed pathways of* 1-*phenyldodecane and* 1-*phenylnonane catabolism by N. salmonicolor*

Table 4. *Effect of growth substrate on β-oxidation-enzyme activities in cell-free extracts of N. salmonicolor*

Extracts were prepared from cells harvested during the exponential phase (first exponential phase for cells grown on 1-phenyldodecane) as described in the Materials and Methods section. The substrate concentration in growth media was 0.5% (v/v) for 1-phenyldodecane and 0.5% (w/v) for the other substrates. Butyryl-CoA dehydrogenase and thiolase activities were measured as described in the Materials and Methods section.

Growth substrate	Specific activity of butyryl-CoA dehydrogenase (nmol of dichlorophenol-indophenol reduced/min per mg of protein)	Specific activity of thiolase (nmol of aceto-acetyl-CoA utilized/min per mg of protein)
1-Phenyldodecane	107	117
Succinate	38	30
4-Phenylbutyrate	20	14
3-Phenylpropionate	45	8
Phenylacetate	33	11

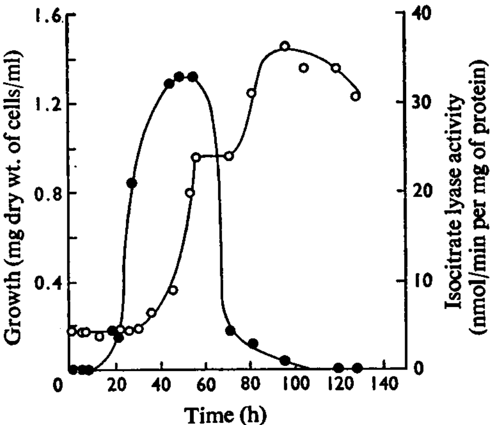


Fig. 4. *Growth of N. salmonicolor on 1-phenylnonane and isocitrate lyase activities in cell-free extracts*

The experimental procedure was identical with that described in the legend to Fig. 1 except that the growth substrate was 0.5% (v/v) 1-phenylnonane. Growth (○); isocitrate lyase activity (●).

palmitoyl-CoA for this component of the β-oxidation enzyme complex.

Given that 4-phenylbutyrate does accumulate for the reasons discussed above, then the other compounds found in the growth medium during the first growth phase may well be components of a 'salvage mechanism' for 4-phenylbutyrate. Table 4 shows the specific activities in cell-free extracts of two of the β-oxidation enzymes (butyryl-CoA dehydrogenase and thiolase) after growth on various substrates. Growth on 1-phenyldodecane led to a marked increase in the activities of these enzymes compared with growth on succinate, but activities were not increased by growing on any of the remaining substrates. The fact that growth on 4-phenylbutyrate does not lead to an increase in the activities of these enzymes is consistent with the hypothesis that the free acid is metabolized primarily by α-oxidation via the other free acid intermediates detected, rather than by activation to the coenzyme A derivative followed by β-oxidation.

The detection of both isomers of 4-phenylbutenoate in the culture medium is of considerable interest, since the coenzyme A derivative of the 2-enoate, but not its isomer, would be the expected β-oxidation intermediate. The 3-enoate is the favoured isomer on chemical grounds, since there is the possibility of resonance stabilization between the π electrons of the side-chain double bond and those of the aromatic ring; it is presumably formed by isomerization of the 2-enoate.

Further work, particularly at the enzymic level, is necessary to establish the pathway in Scheme 1 unambiguously. The main weakness in the scheme for 1-phenyldodecane catabolism is the position of phenylacetate as a major catabolite. It is not possible at present to obtain further evidence on this point, chiefly because of the difficulty of detecting the relevant enzymes in cell-free systems.

Catabolism of 1-phenylnonane by N. salmonicolor

Growth on this odd-carbon-atom substrate and isocitrate lyase activities in cell-free extracts showed a similar pattern to that obtained with 1-phenyldodecane (compare Fig. 4 with Fig. 1). With 1-phenylnonane the diauxie was less pronounced and the maximum isocitrate lyase activity was lower, possibly reflecting the smaller proportion of 1-phenylnonane that would be converted into acetate compared with 1-phenyldodecane. Analysis of ether extracts of culture supernatants for non-hydroxylated aromatic acids showed that one such compound accumulated in large amounts. Culture supernatant (8 litres) from late first exponential phase was extracted and subjected to column chromatography as described in the Materials and Methods section and the component was isolated as white feathery crystals from benzene–light petroleum (b.p. 40–60°C). The mass spectrum was identical with that of *trans*-cinnamic acid and the yield was 5.0 g, representing the conversion of 17% of the growth substrate into this product.

When ether extracts of the above culture supernatant were treated with diazomethane and subjected

to g.l.c.–mass spectrometry (see the Materials and Methods section), trace amounts of two methyl esters other than methyl cinnamate were detected. These had mass spectra identical with those of methyl 3-phenylpropionate and methyl benzoate respectively.

In addition a hydroxylated aromatic acid was isolated from 1-phenylnonane culture supernatant (8 litres). The compound was obtained after column chromatography (see the Materials and Methods section) as yellow creamy crystals (m.p. 67–69°C, yield 8mg) from benzene–light petroleum (b.p. 60–80°C). The reaction with Gibbs' reagent was characteristic of a compound hydroxylated in the *ortho* position. The mass spectrum gave a molecular-ion peak at *m/e* 194 and the main fragmentation pattern involved sequential loss of H_2O, CO and the radicals $-CH_2-CH_2$ and CH to give peaks at *m/e* 176, 148, 120 (base peak) and 107 (hydroxylated tropylium ion). The mass spectrum indicates that the compound is a hydroxyphenylpentanoic acid, and it is tentatively identified as the *o*-hydroxyphenylpentanoic acid on the basis of the colour reaction with Gibbs' reagent.

The capacity of first-growth-phase 1-phenylnonane-grown organisms to oxidize possible catabolic intermediates is shown in Table 3. The pattern of results is very similar to that obtained for 1-phenyldodecane-grown organisms, although in most cases the absolute rates of O_2 uptake are only 25–30% of those obtained after growth on 1-phenyldodecane. The only notable difference between the two series of results is that 1-phenylnonane-grown organisms can oxidize protocatechuate.

These results then suggest that the routes for degradation of 1-phenyldodecane and 1-phenylnonane may converge at cinnamate (Scheme 1), at least with respect to the proportions of the substrates that accumulate in the culture medium. Data for ring-cleavage enzyme activities in cell-free extracts after growth on 1-phenylnonane show rather low activities (see Table 5). However, activities towards homogentisate, protocatechuate, catechol and 3,4-dihydroxyphenylacetate were increased 5-, 16-, 10- and 2.5-fold respectively after growth on 1-phenylnonane rather than on succinate. In addition the pattern of activities is clearly different from that resulting from growth on benzoate, when there was specific induction of high catechol 1,2-dioxygenase activity. This suggests that 1-phenylnonane is not catabolized primarily via benzoate, although since this compound does accumulate in the culture medium it may be a component of a minor pathway; however, washed cell suspensions oxidize this compound only very slowly. The accumulation of 3-phenylpropionate and large amounts of cinnamate is reminiscent of accumulation of the analogues, 4-phenylbutyrate and the 4-phenylbutenoates, during growth on 1-phenyldodecane, and presumably is due to the same phenomenon, i.e. that the coenzyme A derivatives of these compounds are poor substrates for the β-oxidation enzyme system. The formation of benzoate, however, indicates that the system probably can function to a limited extent with these substrates. Free cinnamic acid is most probably catabolized via phenylacetate because phenylacetate was detected in the culture medium during growth on cinnamate, and it is clear that the micro-organism can further metabolize the latter compound. Catabolism of cinnamate via phenylacetate has not been reported previously, however; the two main routes that have been described are via β-oxidation to benzoate (Webley *et al.*, 1955) and via 3-phenylpropionate, followed by hydroxylation and ring cleavage (Coulson & Evans, 1959; Blakley & Simpson, 1964).

Table 5. *Effect of growth substrate on ring-cleavage oxygenase activities in cell-free extracts of N. salmonicolor*

Extracts were prepared from cells harvested during the exponential phase (first exponential phase for cells grown on 1-phenylalkanes) as described in the Materials and Methods section. The substrate concentration in growth media was 0.5% (v/v) for phenylalkanes, 0.5% (w/v) for phenylacetate and benzoate and 1.0% (w/v) for succinate. The homogentisate 1,2-dioxygenase and 3,4-dihydroxyphenylacetate 3,4-dioxygenase activities were measured polarographically as described in the Materials and Methods section. Catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase activities were measured as described for homogentisate 1,2-dioxygenase except that reaction mixtures contained catechol (10μmol) and sodium protocatechuate (10μmol) respectively, rather than sodium homogentisate.

	Specific activity (nmol of O_2/min per mg of protein) with the substrate shown			
Growth substrate	Homogentisate	Protocatechuate	Catechol	3,4-Dihydroxyphenylacetate
1-Phenyldodecane	80	41	27	27
Phenylacetate	33	42	33	14
1-Phenylnonane	3.5	7.9	9.0	1.0
Benzoate	1.8	1.4	97	4.8
Succinate	0.7	0.5	0.9	0.4

Accumulation of hydroxylated intermediates

A particularly interesting aspect of growth on 1-phenyldodecane is the exact coincidence of the onset of diauxic lag with the disappearance of non-hydroxylated acids and the accumulation of hydroxylated ones. It was evident from t.l.c. that a similar event occurred during growth on 1-phenylnonane. To investigate this phenomenon further, washed cell suspensions were prepared from first-growth-phase cells grown on 1-phenyldodecane. Incubation of these suspensions for 1–3h in 0.1 M-potassium phosphate buffer, pH 7.0, led to the excretion from the cells of the same (judging from t.l.c.) four hydroxylated aromatic acids that accumulate during diauxic lag. In addition some exogenously supplied compounds were hydroxylated under these conditions, e.g. benzoate was hydroxylated to protocatechuate, the product being identified by t.l.c. co-chromatography with authentic protocatechuate, the staining reaction with Gibbs' reagent and g.l.c. of the methyl ester (conditions as described in Table 2; retention times 13.8min on methylsilicone gum, 12.4min on polyethylene glycol adipate). This accumulation of hydroxylated compounds could be inhibited by adding sodium alkanoates (see below).

At the onset of diauxie the amounts of hydroxylated compounds formed are approximately equivalent to the amounts of non-hydroxylated acids that disappear from the culture medium, judging from t.l.c. and g.l.c. data. The formation of the former compounds may well reflect lack of substrate specificity of a hydroxylase for phenylacetate, as discussed above. However, it is necessary to explain why this hydroxylation of accumulated compounds occurs so abruptly when before the onset of diauxie, the hydroxylated acids are not detectable. A simple explanation would be that the supply of the normal substrate for the enzyme (probably phenylacetate) ceases or is greatly diminished at the onset of diauxie and so the enzyme becomes free to function with the accumulated analogues in the culture medium, but the true explanation may well be more complex than this and further studies are necessary to establish the reasons for this phenomenon.

Explanation of the diauxic effect

There are several possible reasons for diauxie; the simplest is that it represents an adaptation period during which enzymes are synthesized, which are required to metabolize completely the free acids that have accumulated in the medium during the first growth phase. Against this explanation, however, is the fact that washed cell suspensions prepared from first-growth-phase organisms readily oxidize the accumulated acids (Table 3). This suggests that the first-growth-phase organisms can metabolize these compounds and that they accumulate as a result of a simple rate effect, their rate of further metabolism being slower than their rate of formation.

An alternative but more complex possibility would be that diauxie occurs as a result of an imbalance in intermediary metabolism. When diauxie begins, the *n*-alkane side chain of the original substate has, to a large extent, been degraded and so the amount of acetate feeding into the central pathways of metabolism will be very much decreased, yet the organism still has a highly active glyoxylate cycle as shown by the high isocitrate lyase activity. The same situation pertains when cells are harvested, washed and resuspended in buffer, when hydroxylated acids are excreted. Under both these conditions the glyoxylate cycle may operate at an activity far in excess of that required to handle the amount of acetate being formed. This may lead to a metabolic imbalance resulting from competition for acetate between malate synthase and citrate synthase. There are two lines of evidence to support this hypothesis, the first being the extremely rapid decrease in isocitrate lyase activity (5.5%/h) at the end of the first growth phase when 1-phenylalkanes serve as growth substrates (Figs. 1 and 4). The second line of evidence comes from experiments in which washed first-growth-phase organisms were incubated in 0.1 M-potassium phosphate buffer, pH 7.0, containing either sodium octanoate, sodium decanoate or sodium dodecanoate at 5 mM. These additions completely inhibited accumulation of hydroxylated aromatic acids. Further, ultrasonication of cell suspensions after incubation followed by ether extraction and t.l.c. and g.l.c. analyses showed that there were no free aromatic acids, hydroxylated or otherwise, remaining inside the cells. This indicates that the inhibition of excretion was not due to an effect on cell permeability. These sodium alkanoates are all ready sources of acetyl-CoA and it may be that their addition 'corrects' the imbalance in intermediary metabolism.

Further studies are necessary to establish unambiguously the reason(s) for diauxie.

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