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BIOTRANSFORMATION OF HYDROCARBONS AND RELATED COMPOUNDS BY WHOLE ORGANISM SUSPENSIONS OF METHANE-GROWN *METHYLOSINUS TRICHOSPORIUM* OB 3b

I. J. Higgins, R. C. Hammond, F. S. Sariaslani, D. Best, M. M. Davies, S. E. Tryhorn and F. Taylor.
Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

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SUMMARY: Whole organisms of *Methylosinus trichosporium* OB 3b partially metabolise a wide range of compounds including *n*-alkanes, alkenes, aromatic, alicyclic and terpenoid hydrocarbons, alcohols, phenol, pyridine and ammonia. The reactions involve oxidations, dechlorinations and condensations; most of them are probably initiated by a broad specificity methane mono-oxygenase. Rates of oxidation of ethane and propene are of the same order as those for methane. Catalytic activities are quite stable in organisms stored under appropriate conditions. The findings differ substantially from those recently described for *Methylococcus capsulatus* (Bath), whole organisms of which show very restricted biotransformation capacity.

INTRODUCTION: The few cell-free methane mono-oxygenase activities examined to date are not specific for methane (1) and the enzymes of *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* oxygenate a particularly wide range of compounds including *n*-alkanes, *n*-alkenes, ethers, alicyclic and aromatic substances (2-4). However, these compounds are not growth substrates for methanotrophic bacteria and therefore it is of interest to determine whether this lack of specificity is expressed *in vivo*, and if so to attempt to assess the metabolic implications. In the case of *M.capsulatus* (Bath) only five compounds tested other than methane were oxidised by whole cell suspensions and three of these were oxidised extremely slowly. However, twelve compounds were oxidised in the presence of formaldehyde which may act as a source of reducing power for the mono-oxygenase. Even so, the range of compounds oxidised was far more restricted than for the cell-free system, 2-butene being the largest hydrocarbon molecule to be attacked (5). *Mts. trichosporium* OB 3b differs substantially from *M.capsulatus* (Bath), the two

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species being representatives of the two main subdivisions of obligate methanotrophs. They differ morphologically, use fundamentally different carbon incorporation pathways and may contain fundamentally different methane mono-oxygenases although this has been disputed (3,6,7). We have therefore examined the ability of whole organism suspensions of *Mts. trichosporium* to oxidise or otherwise modify a wide range of compounds. Ethane oxidation by this species has previously been studied (8).

METHODS: *Mts. trichosporium* OB 3b was grown in continuous culture on a methane-air mixture as previously described (6). Steady-state organisms were harvested using an Alfa Laval continuous flow centrifuge, washed once with sodium phosphate buffer (20 mM, pH 7.0) and resuspended in the same buffer. Incubations were routinely carried out in sealed, conical flasks (250 ml) having a side-arm and centre-well. The main compartment contained sodium phosphate buffer (20 ml, 20 mM pH 7.0 containing 5 mM $MgCl_2$ - incubation buffer) and 70-80 mg dry wt of cells. Usually the substrate (2-3 ml) was contained in the centre-well but phenol and ammonium chloride were added directly to the suspension at 0.5 and 25 mM final concentrations respectively. Hydrophobic solids were added as fine powders. Gaseous substrates (1-10 ml) were injected into sealed flasks. Incubations with other substrates were done under both air and methane/air (1/1, v/v). Flasks were incubated for 15-24h on a gyrotary shaker at 30°C. For liquid and solid substrates, organisms were removed by centrifugation (12000g, 10 min, 10°C), washed with incubation buffer and the resulting supernatant and washings were extracted with equal volumes of diethyl ether, before drying the extracts over anhydrous $MgSO_4$ and concentrating on a rotary evaporator. The remaining aqueous phase was then adjusted to pH3 with 1N HCl, re-extracted, treated as above and methylated with diazomethane. When pyridine was the test compound, the suspension medium after removal of organisms was extracted twice with half volumes of dichloromethane, the extracts pooled and treated according to Colby *et al* (2). The product, pyridine-N-oxide was identified using a t.l.c. technique (2). For gaseous substrates the complete incubation medium was used for subsequent product identification and assay.

Most products were detected by gas chromatography using a coiled glass column (2.1 m x 4 mm i.d.) containing methylsilicone gum (SE30, 3% on Diatomite CQ, 100-120 mesh) in a Pye-Unicam 204 instrument fitted with flame ionisation detectors (nitrogen flow rate, 40-60 ml/min; oven temperature, 80-150°C). Aqueous samples were examined using the same system but containing a column of chromosorb 101 (nitrogen flow rate, 40 ml/min; oven temperature, 120-150°C). Most product identification was done using a Pye 104 gas chromatograph linked via a membrane separator to an AEI MS20 mass spectrometer. G.l.c. conditions were as described above except the carrier gas was helium (flow rate, 30 ml/min). Product mass spectra were compared with those obtained for authentic samples where available. Hydroxylamine was detected by a colourimetric method (9). In control experiments, cell suspensions which had not been exposed to test compounds were extracted and examined by these techniques. For all test compounds, control flasks without cell suspension were incubated, extracted

and examined for any non-biological transformations. For selected substrates (ethylbenzene, 1-phenylheptane, propene) organisms were harvested aseptically and incubations were carried out under rigorous aseptic conditions.

Initial rates of ethane and propene oxidation were determined polarographically using a Rank oxygen electrode. Rates of product formation in incubation mixtures were determined by gas chromatography as described above.

RESULTS AND DISCUSSION: Whole organisms of *Mts. trichosporium* oxidise a wide range of compounds including *n*-alkanes, alkenes, aromatic, alicyclic and terpenoid hydrocarbons, alcohols, phenol, pyridine and ammonia. A number of examples are listed in Table 1. Only a few compounds tested did not yield products. These were either difficult to present to the bacterium being hydrophobic solids (e.g. anthracene, biphenyl, anthroquinones) or probably toxic (e.g. 1-chloro-pentane, 1-chloro-octane, nitroaniline). Some of these transformations are single step reactions reflecting only the lack of substrate specificity of methane mono-oxygenase e.g. benzene $\longrightarrow$ phenol, propene $\longrightarrow$ propene oxide. Others are more complex, in some cases reflecting more than one point of attack by the oxygenase on an individual substrate and also involving non-oxygenative reactions presumed catalysed by other enzymes. For example, of the products formed from ethylbenzene, benzyl alcohol and p-hydroxy-ethylbenzene would be immediate products of methane mono-oxygenase activity. However, phenylacetic acid is probably formed from benzyl alcohol by two oxidoreductase activities, perhaps catalysed by methanol and formaldehyde dehydrogenase enzymes. Benzoic acid is probably derived from phenylacetic acid by an α-oxidation mechanism which could again involve methane mono-oxygenase.

The products formed from phenylheptane indicate that the bacterium can degrade the alkane side-chain, initiation of this process probably being due to lack of specificity of methane mono-oxygenase. Since only C-odd products are formed, it is probable that the side chain is β-oxidised to acetate. Extensive carbon incorporation and oxidation of acetate has been demonstrated in the closely related species, *Methanomonas methanooxidans* (10).

TABLE 1. COMPOUNDS ATTACKED BY WHOLE ORGANISM SUSPENSIONS OF *METHYLOSINUS TRICHOSPORIUM* AND PRODUCTS GENERATED.

Compound	Products	Comments
Ammonia	Hydroxylamine	
Benzene	Phenol	
Bicyclohexyl	Nil	
Butadiene	3,4-Epoxy-1-butene	)
But-2-ene	2,3-Epoxybutane	) Configuration not determined.
m-Chlorophenol	Nil	Two non-chlorinated products. One is a phenol with a heptanoic acid side chain.
m-Chlorotoluene	Benzyl alcohol, benzyl epoxide, *m*- or ρ-methyl benzyl alcohol	Other unidentified products with alkanoic acid side chains on aromatic nuclei.
m-Cresol	*m*- and ρ-Hydroxybenzaldehyde	
o-Cresol	5-Methyl-1,3-benzene diol	
Cyclohexane	Cyclohexanol, 3-hydroxy-cyclohexanone	
Cyclohexanol	3-Hydroxycyclohexanone	
n-Decane	Nil	
Ethane	Acetaldehyde, acetate, acetone	
Ethylbenzene	Benzoic acid, benzyl alcohol, ρ-hydroxy ethylbenzene, phenylacetic acid.	

Therefore, it is highly probable that at least some methanotrophs can derive carbon and energy from complex compounds which cannot alone support growth.

m- and ρ- Hydroxybenzaldehyde were formed from *m*- cresol in similar amounts. In the case of the latter compound, a shift of the hydroxyl group has apparently occurred and therefore the oxidations of *m*- chlorophenol and *m*- chlorotoluene were examined to attempt to clarify the mechanism. Surprisingly, all products from these compounds had been dechlorinated. In

Table 1. (continued)

Compound	Products	Comments
Ethene	Ethene oxide	
Hexadecane	Hexadecan-1-ol	Other Unidentified products
Isopropyl benzene	p-Hydroxy isopropyl benzene	
Limonene	Nl	
p-Menthane	Nl	
1-Methylnaphthalene	Nl	
2-Methylnaphthalene	Nl	
n-Pentanol	Nl	
Phenol	Catechol, 1,4-dihydroxybenzene	
1-Phenyldecane	Nl	
1-Phenylheptane	1-Hydroxy-1-phenylheptane, 1-phenyl-heptan-7-al, cinnamic acid, benzoic acid	
1-Phenylnonane	Nl	
Propene	Propene oxide	
Pyridine	Pyridine-N-oxide	
Styrene	Styrene epoxide	
Toluene	Benzoic acid, p-hydroxytoluene	
p-Toluidine	Nl	
m-Xylene	Nl	One product has long alkanoic acid side chain.

Nl = not identified.

addition, some products from these two compounds and from *m*- xylene had acquired a methyl group or an alkanoic acid side chain. It is possible that both these dechlorination and condensation activities are a manifestation of the mechanism of methane mono-oxygenase. It has been suggested that the mechanism involves free radicals (11) and dimethyl ether is known to be generated during methane oxidation although it is thought not to be an intermediate, thus supporting this contention (1).

Most of the transformations in Table 1 have been demonstrated in the presence of methane but methane was not present during oxidation of gaseous compounds. In other cases, omission of methane sometimes altered the relative amounts but not the nature of products formed. With the exception of ethane and propene, rates of oxidation have not been recorded as they are usually determined in this experimental system by vapour diffusion rates. Hence the amounts of liquid substrates in extracts of suspension medium are extremely low, in most cases undetected by the procedures used. However, ethane and propene showed initial oxidation rates of about 100% and 30-50% respectively of the rate with methane (30-70 n mol per mg dry wt of organisms/min). Propene oxide accumulated in incubation mixtures up to 5-10 mM. Ethane was oxidised to acetaldehyde and acetate in the absence of methane and acetone accumulation was also noted as previously reported (8).

Organisms stored after harvesting for 200h without methane at $4^{o}C$ or with methane at $30^{o}C$ showed 25 and < 10% oxidising activity loss respectively. At $30^{o}C$ in the absence of methane activity loss was complete within 40-60h. Oxidising activity is retained on storage in liquid nitrogen or at $-15^{o}C$.

These results contrast sharply with those obtained with *M.capsulatus* (Bath), whole organisms of which show a very restricted range of non-growth substrate oxidations even in the presence of formaldehyde as a source of reducing power for the mono-oxygenase. Hydrocarbons with more than four carbon atoms were not oxidised (5). Also, in cases where further oxidation of the product does not occur and methane is absent, there is no exogenous source of reducing power for the mono-oxygenase. In *Mts. trichosporium* it is presumably derived from endogenous metabolites or storage materials. This again is distinct from findings with *M. capsulatus* (Bath) in which even ethane is not oxidised to products in the absence of formaldehyde. Whether these apparently major differences in the behaviour of whole organisms reflect physiological and/or morphological differences between the two species or

fundamental differences in the enzymic mechanisms involved remains to be resolved (3,6,7). However, it is possible that some of these differences merely reflect the type of incubation procedure used.

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