The Metabolism of *n*-Decane by a *Pseudomonas*

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The growth of a *Pseudomonas* on *n*-decane was found to produce stearic acid, oleic acid, palmitic acid, palmitoleic acid, decanoic acid, octanoic acid, β-hydroxydecanoic acid, β-hydroxyoctanoic acid, β-hydroxyhexanoic acid and β-hydroxyadipic acid. Small amounts of *n*-decanamide and *n*-valeramide were also isolated. The effects of nitrogen and oxygen limitation on the formation of these products in continuous fermentations is reported.

Though the ability of micro-organisms to utilize alkanes as their sole carbon source has been recognized for over 60 years, the metabolic pathway involved is as yet ill-defined (ZoBell, 1950; Van der Linden & Thijsse, 1965). However, it is generally believed that the initial oxidative attack occurs at the terminal carbon atoms. The contrast with the low specificity observed in chemical attack on alkanes suggested the use of micro-organisms for the selective introduction of oxygen-containing functional groups at the terminal carbon atoms.

As a necessary preliminary to studies in this field we report the isolation and characterization of the normal metabolic products obtained from *Pseudomonas* X2, grown in continuous culture on a mineral-salt medium with *n*-decane as sole carbon source, and the influence of culture conditions on their formation.

EXPERIMENTAL

Isolation and growth of organism. The species of *Pseudomonas*, designated X2, was isolated from Thames Estuary mud by Professor S. J. Pirt by using a medium containing *n*-decane as the sole carbon source. Continuous fermentations were carried out by Mr D. G. MacLennan by using the first stage of the two-stage equipment described by Callow & Pirt (1961), fitted with an automatic oxygen-tension control (MacLennan & Pirt, 1967). A dilution rate of 0·1 $hr.^{-1}$ at 30°, with the pH maintained at 7·1, gave a cell production of 1 g. dry wt./l. In addition to *n*-decane (>99% purity) the medium contained: NaH_2PO_4 (1·56 g./l.), K_2HPO_4 (6·97 g./l.), NH_4Cl (2·0 g./l.), $CaCl_2$ (0·01 g./l.), $Na_2B_4O_7,10H_2O$ (0·1 mg./l.), $MgSO_4,7H_2O$ (0·2 g./l.), $FeSO_4,7H_2O$ (0·5 mg./l.), $MnSO_4,6H_2O$ (0·5 mg./l.), $CuSO_4,5H_2O$ (0·1 mg./l.), $Na_2MoO_4,2H_2O$ (0·1 mg./l.) and EDTA (disodium salt) (0·26 g./l.).

Chromatography. Analytical gas–liquid chromatography was carried out on a Pye Argon Chromatograph at 100° and 150° with a column packing of 10% polyethylene glycol adipate 1500 on 100–120-mesh acid-washed Celite. An Aerograph Autoprep, equipped with a 10 ft. 10% silicone SE–30 on 80–100-mesh Celite column and programmed from 80° to 250°, was used for preparative gas–liquid chromatography.

Thin-layer chromatography was carried out on Merck silica gel G with as solvent system either 27% (v/v) ether in light petroleum (b.p. 40–60°) or, for very polar material, chloroform. The components were detected by exposure to iodine vapour.

Spectra. Nuclear-magnetic-resonance (n.m.r.) spectra were recorded on a Varian HA–100 instrument for CCl_4 solutions with tetramethylsilane as the internal standard. Mass spectra were measured on an A.E.I. M.S.9 instrument.

Isolation of extracellular products. The fermentation liquors (about 190 l.) were collected from continuous cultures of *Pseudomonas* X2 grown with various *n*-decane concentrations. The cells were removed either by continuous centrifugation or, more conveniently, by addition of sodium CM-cellulose (0·6 g./l.). A portion of the liquor was extracted at pH 7 with ether. After removal of the ether, the resulting extract was chromatographed on silica gel. *n*-Decane was eluted with light petroleum (b.p. 40–60°) and the remaining compounds were eluted with methanol. Separation by preparative gas–liquid chromatography yielded *n*-decanamide and *n*-valeramide.

The total fermentation liquor (190 l.) was acidified to pH 2 with H_2SO_4 and extracted with dichloromethane. After drying over Na_2SO_4 and distilling off the dichloromethane, the residue was separated as above into *n*-decane (560 g.) and a brown viscous oil (19·3 g.). After methylation with diazomethane, the oil was separated by preparative thin-layer chromatography into five fractions.

Fraction 1, R_F 0·77 (5·7 g.), was separated by preparative gas–liquid chromatography into seven further fractions, 1*a*–1*g*. Fractions 1*a*–1*c* comprised only minor amounts of material of which the major component was identified by gas–liquid chromatography as methyl octanoate. Fraction 1*d* (20 mg.) was methyl decanoate. At least ten components were present in fraction 1*e* (5 mg.), but a mass spectrum showed no *m/e* peaks at 214 and 242 indicative of methyl laurate and methyl myristate respectively. Fraction 1*f* (0·25 g.) was a mixture of methyl palmitate and methyl palmitoleate. A 1:1 composition was indicated both by gas–liquid chromatography and an n.m.r. spectrum that showed one olefinic proton (τ 4·68) for every three methoxyl

protons (τ6·40). The two esters were separated by thin-layer chromatography on silica gel made up with 0·1N-$AgNO_3$. Fraction 1*g* (0·53g.) was a mixture of methyl stearate and methyl oleate. The n.m.r. spectra showed this to be a 1:3 mixture and this was separated into its components by thin-layer chromatography on $AgNO_3$-impregnated silica gel.

Fraction 2, R_F0·25 (3·1g.), was separated by preparative gas–liquid chromatography into the two major components, methyl β-hydroxyoctanoate and methyl β-hydroxydecanoate.

Fraction 3, R_F0·18 (0·14g.), and fraction 4, R_F0·12 (0·60g.), contained too many components for effective separation.

Fraction 5, R_F0 (0·18g.), on preparative gas–liquid chromatography, yielded dimethyl β-hydroxyadipate (about 1mg.). A portion (8l.) of the acidified fermentation liquor, which had already been extracted with dichloromethane, was continuously extracted with ether for a week, giving an orange oil (1·1g.). After methylation with diazomethane, the oil was further separated by thin-layer chromatography, with 5% (v/v) methanol in $CHCl_3$, into three fractions with R_F values 0–0·1 (20mg.), 0·1–0·2 (20mg.) and 0·6–1·0 (1·0g.). The last fraction was separated by preparative gas–liquid chromatography into methyl β-hydroxyhexanoate (20mg.) and methyl β-hydroxyoctanoate (50mg.).

Characterization and identification of products

n-*Decanamide.* Retention time was identical with that of an authentic specimen. The mass spectrum shows peaks at *m/e* 171 (2·9), 113 (1), 112 (1·4), 97 (5·2), 83 (6·9), 73 (9), 72 (40), 69 (9·9), 59 (100), 55 (16·5), 44 (14·8) and 41 (22·6).

n-*Valeramide.* Retention time was identical with that of an authentic specimen. The mass spectrum shows peaks at *m/e* 101 (1·5), 99 (2·2), 83 (7·4), 73 (12·4), 72 (19·5), 69 (9·9), 59 (100), 57 (20), 55 (21·1), 44 (16) and 41 (28·1).

Methyl decanoate. The retention time and i.r. spectrum were identical with published data. The mass spectrum shows peaks at *m/e* 186, 155, 143, 129, 101, 87 and 74 (base peak). (Found: C, 70·6; H, 11·7. Calc. for $C_{11}H_{22}O_2$: C, 71·0; H, 11.8%).

Methyl palmitate. The retention time and mass spectrum, *m/e* 270 and 74 (base peak), were identical with those of an authentic specimen.

Methyl palmitoleate. The retention time and mass spectrum, *m/e* 268 and 74 (base peak), were identical with those of an authentic specimen.

Methyl oleate. The retention time and mass spectrum, *m/e* 296 and 74 (base peak), were identical with those of an authentic specimen.

Methyl stearate. The retention time and mass spectrum, *m/e* 298 and 74 (base peak), were identical with those of an authentic specimen.

Methyl β-hydroxydecanoate. This had $[\alpha]_D^{18}$ $-16·6\pm1°$ (*c* 0·98 in $CHCl_3$) (literature $[\alpha]_D^{20}$ $-18·3\pm1°$; Cartwright, 1957) (Found: C, 65·1; H, 11·1. Calc. for $C_{11}H_{22}O_3$: C, 65·3; H, 11·0%). The n.m.r. spectrum showed (numbers of protons in parentheses): τ6·18 (1) multiplet, 6·41 (3), 6·98 (1), 7·65 (1), 7·73 (1) doublet, 8·73 (12) multiplet and 9·12 (3) triplet. The mass spectrum shows peaks at *m/e* 202, 201, 184, 171, 153, 152, 129, 128, 127, 103 (base peak), 74 and 71.

Methyl β-hydroxyoctanoate. This had $[\alpha]_D^{18}$ $-18·5\pm1°$ (*c* 1·0 in $CHCl_3$) (literature $[\alpha]_D$ $-24°$; Lemieux & Giguere, 1951) (Found: C, 62·0; H, 10·5. Calc. for $C_9H_{18}O_3$: C, 62·1; H, 10·4%). The n.m.r spectrum showed (numbers of protons in parentheses) τ6·13 (1) multiplet, 6·38 (3), 7·00 (1), 7·67 (1), 7·71 (2) doublet, 8·65 (8) multiplet and 9·11 (3) triplet. The mass spectrum shows peaks at *m/e* 174, 173, 156, 143, 125, 124, 103 (base peak), 74 and 71.

Methyl β-hydroxyhexanoate. The thin-layer-chromatographic R_F value was characteristic of hydroxy ester. The mass spectrum shows peaks at *m/e* 146 (0·2), 145 (1·5), 128 (1·7), 125 (1·8), 124 (0·9), 117 (2·9), 116 (3·6), 115 (43), 103 (64), 74 (46), 71 (55), 61 (29), 59 (40), 55 (41), 44 (15), 43 (100), 42 (15), 41 (29), 40 (3), 39 (15), 31 (17) and 29 (19).

Dimethyl β-hydroxyadipate. The mass spectrum shows peaks at *m/e* 191 (0·3), 171 (33), 170 (19), 149 (24), 139 (20), 126 (22), 112 (19), 87 (44), 74 (72), 69 (37), 59 (21), 57 (69), 56 (25), 55 (65), 43 (59), 41 (100), 39 (26), 29 (74) and 27 (27). The gas–liquid-chromatographic retention time is different from that of dimethyl β-hydroxy-β-methylglutarate.

RESULTS

In view of the dangers inherent in relying solely on chromatographic techniques for identifying the metabolic products of *Pseudomonas* X 2, all of those present in adequate amounts in the culture medium have been isolated and further characterized.

In addition to stearic acid, oleic acid, palmitic acid, palmitoleic acid and decanoic acid, which were all isolated as their methyl esters, small amounts of octanoic acid were detected by gas–liquid chromatography in the appropriate fraction. However, no evidence could be obtained for the presence of lauric acid and myristic acid.

Gas–liquid-chromatographic examination of the crude extracts indicated the presence of small amounts of methyl esters in the fermentation medium. *n*-Decanamide and *n*-valeramide were isolated and identified by their mass spectra and gas–liquid-chromatographic retention times. The latter criterion is particularly important in this case as the parent mass peaks of amides are notoriously difficult to identify, although the characteristic ions, *m/e* 59, $CH_2{:}C(OH)\cdot\overset{+}{N}H_2$ and *m/e* 44, $\overset{+}{C}O\cdot NH_2$, are readily observed.

Three β-hydroxy acids, namely D-β-hydroxydecanoic acid, D-β-hydroxyoctanoic acid and β-hydroxyhexanoic acid, were isolated also as their methyl esters. Their structures follow from their mass spectra (e.g. Fig. 1). The weak parent peak and strong $(M-1)^+$ peak is characteristic of hydroxy compounds as is also the formation of ions $(M-18)$ corresponding to loss of water. The methyl ester grouping is indicated by the ion *m/e* 74, $CH_2{:}C(OH)\cdot O\cdot\overset{+}{C}H_3$, and this in conjunction with the ion *m/e* 103, $HO{:}\overset{+}{C}H\cdot CH_2\cdot CO\cdot O\cdot CH_3$, places the hydroxyl group at the β-position. This assign-

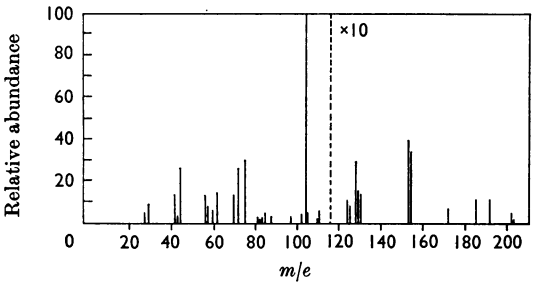

Fig. 1. Mass spectrum of methyl β-hydroxydecanoate.

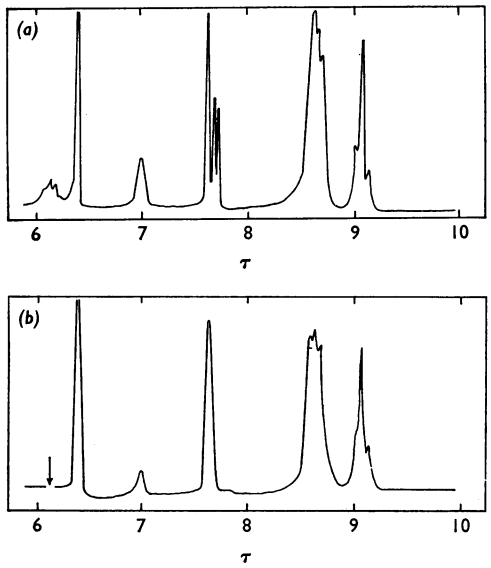

Fig. 2. (*a*) Nuclear-magnetic-resonance spectrum of methyl β-hydroxydecanoate at 100Mcyc./sec. (*b*) Spectrum of methyl β-hydroxydecanoate when irradiated at the frequency indicated by arrow.

ment is further supported by the n.m.r. spectrum (Fig. 2*a*), which, in addition to absorptions at τ 6·13 (O–H), 6·38 (CH_3–O), 7·00 (C–H), 8·65 ($[CH_2]_n$) and 9·11 (R–CH_3), shows a singlet (7·67) and doublet (7·71) attributable to a methylene adjacent to the carbonyl group and unsymmetrically coupled with the tertiary C–H group (τ 6·13). On irradiation at this frequency the methylene bands collapsed to a singlet (Fig. 2*b*). As only minute quantities of dimethyl β-hydroxyadipate were obtained, its characterization rests on the mass spectrum, which shows the expected features (see above). The alternative identification as dimethyl β-hydroxy-β-methylglutarate is excluded by a comparison of the gas–liquid-chromatography retention times with an authentic sample.

The effect of varying the growth conditions was examined to see whether intermediary primary metabolites could be accumulated. The results relevant to the present work are recorded in Table 1. The values quoted are the areas of the gas–liquid-chromatographic peaks of the individual compounds relative to methyl β-hydroxydecanoate, and indicate how the relative proportions of products are affected by fermentation conditions. Comparison of columns 1 and 2 in Table 1 shows that increasing the concentration of *n*-decane results in a greatly increased production of fatty acids relative to hydroxy acids.

The experiments with oxygen limitation were carried out with the amount of ammonium chloride supplied adjusted so as to cause a 25% inhibition of cell growth at the highest oxygen partial pressure with a 2% *n*-decane concentration. This ensured that *n*-decane was always present in excess. No appreciable qualitative changes occurred in the products formed. Comparison of columns 2 and 3 in Table 1 shows that nitrogen limitation causes a very marked decrease in the proportions of C_{16} and C_{18} acids and an increase in the amounts of C_8 and C_{10} acids. Lowering the oxygen partial pressure results in a general decrease in acid formation with the exception of β-hydroxy acids. A changeover from nitrogen to oxygen limitation of growth occurs at an oxygen partial pressure between 5·5 and 3 mm. Hg, and is accompanied by a modest increase in fatty acid production.

DISCUSSION

The production of stearic acid, oleic acid, palmitic acid and palmitoleic acid by *Pseudomonas* X2 is unexceptional, although previous workers have not noted their formation. The presence of these acids indicates the operation of normal fatty acid synthesis in this organism. As the lower acids with even numbers of carbon atoms are presumably their precursors, all of them would be expected to be present, and hence the absence of lauric acid and myristic acid is surprising. This, and the presence of decanoic acid and octanoic acid, accompanied by the corresponding β-hydroxy acids, would appear at first sight to support the currently accepted metabolic pathway via terminal oxidation of the alkane (*n*-decane) to the carboxylic acid, followed by successive β-oxidations. However, as the same products are obtained when the organism is grown on *n*-nonane such deductions are ill-founded. In addition, the D-configurations of the β-hydroxy acids are in better accord with a synthetic origin. It therefore seems unlikely that β-hydroxyadipic acid is derived from a diterminal oxidative degradation.

We have been unable to determine whether any

Table 1. *Effect of growth conditions on product formation by* Pseudomonas *X* 2

Bacteria were grown in defined medium (see the Experimental section), which was modified in the oxygen limitation experiments by adjusting the NH_4Cl concentration so as to cause a 25% decrease in cell production at 29·2 mm. Hg oxygen partial pressure, with a 2% *n*-decane concentration. The proportions of products relative to β-hydroxydecanoate are based on gas–liquid-chromatographic peak areas.

	Proportions of products relative to β-hydroxydecanoate						
	Concn. of *n*-decane		Partial pressure of O_2				
Products	1%	2%	29·2 mm.	13·7 mm.	5·5 mm.	3 mm.	1·0 mm.
Dichloromethane extract (g. of products/l. of medium supernatant).....	0·73	1·3	0·93	0·13	0·13	0·30	0·17
Octanoate	0·56	0·37	1·91	1·24	0·72	1·70	1·13
Decanoate	4·4	8·5	3·41	7·96	4·28	1·93	9·01
Palmitate + palmitoleate	102	570	27·9	7·01	4·87	9·01	10·9
Stearate + oleate	120	460	24·6	3·72	3·35	3·60	11·3
β-Hydroxyoctanoate	23·3	37	79·4	73·0	65·8	62·1	63·0
β-Hydroxydecanoate	100	100	100	100	100	100	100

of these compounds are formed by both degradative and synthetic pathways. The initial increase in the proportions of C_{10} and C_8 acids observed in fermentations conducted under nitrogen limitation conditions (cf. columns 2 and 3 in Table 1), when the amounts of C_{16} and C_{18} acids have fallen sharply, would support a contribution from a degradative pathway. It is noteworthy that Thijsse & Van der Linden (1963) found that chloramphenicol, which also limits cell growth, promotes the accumulation of propionic acid by a *Pseudomonas* growing on *n*-heptane.

The possible presence of hydroxy acids, which subsequently lactonize, in the fermentation liquors of another *Pseudomonas* sp. utilizing *n*-heptane has been noted (Ali Khan, Hall & Robinson, 1964), and another isolate growing on *n*-hexadecane accumulates β-hydroxymyristic acid and β-hydroxylauric acid (Romero & Brenner, 1966). In neither case was it established whether these were degradative or synthetic products, although a strain of *Pseudomonas* growing on glycerol forms glycolipids containing D-β-hydroxydecanoic acid (Bergström, Theorell & Davide, 1947; Jarvis & Johnson, 1949). The isolation of β-hydroxyadipic acid from natural sources has not been reported previously, whereas β-oxoadipic acid is a well-known intermediate in the biological degradation of aromatic hydrocarbons.

A further noteworthy feature is the formation of *n*-decanamide and *n*-valeramide. Small amounts of methyl esters of the accumulated acids have been detected by gas–liquid chromatography in the original extracts. Since we have found that the growth of *Pseudomonas* X 2 on decane is inhibited by decanoic acid in concentrations of 1 mM, it is tempting to regard amide and ester formation as detoxification mechanisms.

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REFERENCES

Ali Khan, M. Y., Hall, N. N. & Robinson, D. S. (1964). *Leeuwenhoek ned. Tijdschr.* **30**, 417.

Bergström, S., Theorell, H. & Davide, H. (1947). *Ark. Kemi Min. Geol.* **23A**, no. 13.

Callow, D. S. & Pirt, S. J. (1961). *J. appl. Bact.* **24**, 12.

Cartwright, N. J. (1957). *Biochem. J.* **67**, 663.

Jarvis, F. G. & Johnson, M. J. (1949). *J. Amer. chem. Soc.* **71**, 4124.

Lemieux, R. U. & Giguere, J. (1951). *Canad. J. Chem.* **29**, 678.

MacLennan, D. G. & Pirt, S. J. (1967). *J. gen. Microbiol.* **45**, 289.

Romero, E. M. & Brenner, R. R. (1966). *J. Bact.* **91**, 183.

Thijsse, G. J. E. & Van der Linden, E. C. (1963). *Leeuwenhoek ned. Tijdschr.* **29**, 89.

Van der Linden, A. C. & Thijsse, G. J. E. (1965). *Advanc. Enzymol.* **27**, 469.

ZoBell, C. E. (1950). *Advanc. Enzymol.* **10**, 443.