Biochem. J. (1969) **114**, 881
Printed in Great Britain

The Biochemical Status of Metabolites of Alkane-Utilizing *Pseudomonas* Organisms

By C. W. BIRD and P. MOLTON
Department of Chemistry, Queen Elizabeth College, London W.8

(*Received* 24 *June* 1969)

Examination of the metabolic products formed by five alkane-utilizing *Pseudomonas* organisms during growth on various alkanes and on glucose as sole carbon sources indicates that many metabolites may be of predominantly synthetic origin rather than intermediary metabolites of alkane breakdown. This conclusion is supported by an examination of the effect of fluoroacetate on such fermentations.

In a previous paper, Bird & Molton (1967) reported the isolation and characterization of the products obtained from *Pseudomonas* X2 grown in continuous culture on *n*-decane as sole carbon source. The products identified were 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, together with small amounts of the amides of *n*-decanoic acid and *n*-valeric acid. Both β-hydroxydecanoic acid and β-hydroxyoctanoic acid thus obtained have the D-configuration, indicating that they are of synthetic rather than degradative origin. This conclusion not only throws doubt on the biochemical status of the other acids formed by *Pseudomonas* X 2, but also on the status of those obtained from other alkane-utilizing *Pseudomonas* organisms.

Reports in the literature of the products formed when various strains of *Pseudomonas* organisms were grown on a number of alkanes as sole carbon sources are briefly summarized in Table 1.

The apparent correlation between substrate and products has been widely interpreted in terms of

Table 1. *Summary of metabolic products formed by alkane-utilizing* Pseudomonas *species*

References: (*a*) Imelik (1948); (*b*) Heringa *et al.* (1961); (*c*) Senez & Azoulay (1961); (*d*) Senez & Konovaltschikoff-Mazoyer (1956); (*e*) Thijsse (1964); (*f*) Ali Khan *et al.* (1964); (*g*) Pomortseva (1957); (*h*) Fredricks (1967); (*i*) Markovetz, Klug & Forney (1967); (*j*) Proctor (1960); (*k*) Romero & Brenner (1966); (*l*) Wagner, Zahn & Buhring (1967); (*m*) Heydeman (1960).

Organism	Substrate	Products identified	Reference
No designation	Heptane	C_5, C_2 and C_1 carboxylic acids	(*a*)
P473	Hexane	C_6, C_4, C_3 and C_2 carboxylic acids	(*b*)
	Heptane	C_7, C_6, C_5, C_4, C_3 and C_2 carboxylic acids	(*b*)
	Heptane	C_7, C_6, C_5, C_4, C_3 and C_2 carboxylic acids, succinic acid	(*d*)
	Hept-1-ene	Hept-6-enoic acid, pent-4-enoic acid, glutaric acid	(*e*)
P. aeruginosa	Heptane	Hept-1-ene	(*c*)
'R'	Heptane	Heptanoic acid, pimelic acid	(*f*)
	Octane	Butyric acid, acetic acid, adipic acid and suberic acid	(*f*)
No designation	Octane	Acetic acid, propionic acid and higher unidentified acids	(*g*)
P. aeruginosa N.C.I.B. 9904	Decane	C_{10}, C_9, C_8 and C_7 acids; 1-, 2-, 3-, 4- and 5-decanols+ 1–5-ketones	(*h*)
P. aeruginosa	Tetradec-1-ene	Tetradecanoic acid and tetradec-13-enoic acid	(*i*)
No designation	Hexadecane	Hexadecanol	(*j*)
P. aeruginosa	Hexadecane	Palmitic acid, palmitoleic acid, stearic acid, oleic acid, β-hydroxylauric acid and β-hydroxymyristic acid	(*k*)
No designation	Hexadecane	Hexadec-1-ene	(*l*)
No designation	Octadecane	Octadecanol	(*m*)

Table 2. *Products from the growth of* Pseudomonas *organisms on various substrates*

Bacteria were grown in shake-flasks in a mineral salts medium with 2% (w/v or v/v) of substrate. The proportions of products relative to the most abundant component in each mixture, as determined by g.l.c. are indicated by: +++, 50–100%; ++, 10–50%; +, 0–10%; −, none detected.

Organism	X2				X4				X5		P473				'R'			
Substrate	Decane	Nonane	Decane +1mM-fluoro-acetate	Glucose	Decane	Nonane	Decane +1mM-fluoro-acetate	Glucose	Decane	Glucose	Decane	Nonane	Decane +1mM-fluoro-acetate	Glucose	Decane	Nonane	Decane +1mM-fluoro-acetate	Glucose
Products																		
Heptanoate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Octanoate	+	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+
Nonanoate	+	++	−	−	−	−	−	−	−	+	+	−	−	+	−	−	−	−
Decanoate	+++	+++	+	++	++	+	+++	−	−	++	+	−	−	+++	−	−	−	−
Undecanoate	−	++	−	−	−	−	−	−	−	−	−	−	−	+++	−	−	+	−
Laurate	+	++	−	+	+++	−	−	−	−	−	+	−	−	−	−	−	−	−
Myristate	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Palmitate +palmitoleate	+++	++	+++	++	−	+++	++	+++	+	−	+	+	−	+	−	−	−	−
Stearate +oleate	+	++	−	++	−	−	++	−	+	−	−	−	−	−	−	−	−	−
Propionamide	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Butyramide	−	−	−	−	−	−	++	−	−	−	−	−	−	−	−	−	−	−
Valeramide	+	−	+++	−	−	−	−	−	−	−	−	+	−	+++	−	−	−	−
Hexanamide	−	−	−	−	+	−	−	−	−	+	+	−	+	−	−	−	−	−
Heptanamide	−	−	−	−	+	−	−	−	+++	−	−	−	−	−	−	−	−	−
Octanamide	−	−	−	−	+	−	−	−	−	−	+++	+++	+++	+	−	−	−	−
Nonanamide	+++	+	−	−	−	−	−	−	−	−	+	+	−	−	−	+++	−	−
Decanamide	−	−	+++	−	+	−	−	−	+++	−	+++	−	+	+++	−	−	++	−
α-Hydroxyvalerate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−
β-Hydroxybutyrate	−	++	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
β-Hydroxyoctanoate	+++	+++	−	−	−	−	−	−	−	++	+	+	−	+	−	−	−	−
β-Hydroxydecanoate	+++	+++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
β-Hydroxylaurate	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
α-Oxohexanoate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−
α-Oxo-octanoate	−	−	−	−	+++	−	−	−	−	−	+	−	−	+++	−	−	−	−
α-Oxoglutarate	−	−	−	−	−	−	−	−	−	−	+++	+	−	−	−	−	−	−
Succinate	−	−	−	−	−	−	−	+++	−	−	−	−	−	−	−	−	−	−
Glutarate	+++	++	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	++
Adipate	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	++	+++	−
Pimelate	−	−	−	−	−	−	+	−	−	−	+++	−	++	−	−	++	++	+++
Suberate	−	−	−	−	−	−	++	−	−	−	+++	−	−	+++	+	−	++	+++
Azelaiate	+	−	−	−	−	−	−	−	+++	+	+	−	−	+++	−	−	++	−
Sebacate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+++	+++	+++	+++
Propanol	−	−	−	−	+++	−	−	−	−	−	−	−	−	−	−	−	−	−
Decanol	−	−	−	−	−	−	−	−	−	−	+++	−	−	−	−	−	−	−
Decan-2-one	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Butane-2,3-diol	+++	−	−	−	−	−	−	−	+++	−	−	−	−	−	−	−	−	−
Diacetyl	−	−	−	−	−	−	−	−	−	+++	−	−	−	−	−	−	−	−

alkane degradation via terminal or $\alpha\omega$-diterminal oxidation, followed by successive β-oxidations of the resulting carboxylic acids.

The work reported in this paper shows that most acids formed by alkane-utilizing *Pseudomonas* organisms are synthetic in origin and thus provides no evidence of degradative metabolic pathways.

EXPERIMENTAL

General. The composition of the inorganic salts medium, continuous culture methods and the analytical g.l.c. method were reported previously (Bird & Molton, 1967). For the continuous fermentations an ammonium chloride concentration of 1·15g./l. was used.

Organisms. The isolation of *Pseudomonas* X2 was described previously (Bird & Molton, 1967). Organisms X4 and X5 were isolated in a similar way from river mud and laboratory tap water respectively. The remaining two organisms designated P473 and 'R' have been the subjects of papers from other laboratories (Heringa, Huybregtse & Van der Linden, 1961; Ali Khan, Hall & Robinson, 1964) and were kindly made available to us.

Shake-flask cultures. Sterile inorganic salts medium (25ml.) and the substrate (0·5g. or 0·5ml.) were shaken for 2–3 days in a 250ml. conical flask at 30° with a 1ml. inoculum of decane-grown organism. The purity (> 99·5%) of the alkane substrates was carefully checked by g.l.c. The extent of growth was estimated by measuring extinctions on an EEL colorimeter. Cells were removed from the cultures by centrifugation; the fermentation liquor was acidified to pH2 with H_2SO_4 and extracted with an equal volume of dichloromethane by shaking for 5min. The organic phase was dried over Na_2SO_4, methylated with diazomethane and examined by analytical g.l.c.

Large batch cultures. These were grown in a 5l. or 10l. round-bottomed flask fitted with a flange head, with facilities for the separate addition of medium, substrate, inhibitor and inoculum, control of air and nitrogen supplies, and continuous stirring, all under completely sterile conditions. The whole apparatus was maintained at 27° in a constant-temperature bath.

RESULTS AND DISCUSSION

At the outset of the investigation the five *Pseudomonas* organisms were grown in shake-flask cultures with alkanes of different chain-lengths and glucose as sole carbon source. The products that accumulated are recorded in Table 2. The organisms P473 and 'R' were grown on *n*-decane and *n*-nonane for comparison, although they were originally selected for their ability to utilize shorter-chain alkanes. For any one organism the general pattern of products is remarkably independent of the substrate. This is most clearly shown by comparing the products formed during growth on alkanes and on glucose. With the exception of *Pseudomonas* 'R' most organisms accumulate fatty acids and/or their amides up to C_{10}. Palmitic acid and stearic acid are fairly common products, but acids of intermediary chain length are less frequently found.

Although β-hydroxy acids and dicarboxylic acids are formed to some extent by most organisms, it was found that *Pseudomonas* X2 forms relatively large quantities of the former product whereas *Pseudomonas* 'R' forms the latter. The similarity of the pattern of products formed from different hydrocarbons and glucose points to their being products of biosynthesis rather than being intermediary metabolites of alkane breakdown.

The effect of fluoroacetate on the products formed by *Pseudomonas* X2 when grown in batch culture on *n*-decane, *n*-nonane and glucose is noteworthy. Fluoroacetate (1mM) caused approx. 50% inhibition of growth under these conditions. Since the effect

Table 3. *Effect of inhibitors on product formation by* Pseudomonas *X2 in batch cultures*

Details of culture conditions and key are given in Table 2.

Substrate	Decane (1%)	Decane (1·3%)	Nonane (1·3%)	Glucose (1%)
Inhibitor	Fluoride (1mM)	Fluoro-acetate (1mM)	Fluoro-acetate (1mM)	Fluoro-acetate (1mM)
Products				
Ethanol	+++	−	−	−
Hexanoate	++	−	−	−
Heptanoate	++	−	−	−
Octanoate	++	+++	++	−
Nonanoate	++	+++	+	−
Decanoate	++	+++	+	−
Undecanoate	+	−	++	−
Laurate	++	+	++	−
Tridecanoate	−	+++	++	−
Myristate	++	+++	+	−
Pentadecanoate	++	−	++	−
Palmitate + palmitoleate	++	+++	++	−
Heptadecanoate	−	−	+++	−
Stearate + oleate	++	+	++	−
Nonadecanoate	−	−	++	−
Arachidate	−	+	−	−
α-Oxobutyrate	−	+	++	−
α-Oxohexanoate	++	−	−	−
α-Oxodecanoate	−	−	++	+++
α-Hydroxyvalerate	−	+++	++	−
β-Hydroxybutyrate	−	+++	−	+
β-Hydroxyhexanoate	−	+++	−	+++
β-Hydroxyoctanoate	++	+++	−	+++
β-Hydroxydecanoate	−	+++	++	+++
Valeramide	−	+	+	−
Hexanamide	−	−	++	−
Heptanamide	−	−	+	−
Octanamide	−	+	+	−
Decanamide	−	+++	+	−
Glutarate	−	+	+	+++
Adipate	−	+	−	+
Sebacate	−	−	−	+++
Undecan-1,11-dioate	−	−	−	+
Dodecan-1,12-dioate	−	−	−	+

of fluoroacetate may have been due to the release of fluoride ion (Goldman, 1965), a comparative experiment entailing growth on decane in the presence of 1 mM-fluoride was included (Table 3). Comparison of the products identified suggests that the effect with fluoroacetate is not due to fluoride ion; for example, characteristic of the fluoride-inhibited cultures was the production of appreciable amounts of ethanol, which was absent from the fluoroacetate-inhibited cultures. In addition, the formation of β-hydroxy acids was diminished and neither amides nor dicarboxylic acids were formed in the presence of fluoride.

Again the close similarity of the products formed from nonane and decane in the presence of fluoroacetate points to their being of predominantly synthetic origin.

The present results indicate that many of the products excreted into the medium by alkane-metabolizing *Pseudomonas* organisms could well be derived from synthetic rather than degradative pathways. Although some contribution of acids from degradative pathways to the general pool cannot be excluded on present evidence, clearly deductions about metabolic degradative pathways based merely on the presence of such products in the culture fluids could be ill-founded.

Another criterion frequently used to establish metabolic pathways in this field is that of simultaneous adaptation. This assumes that growth of an organism on an intermediary metabolite will occur without lag in oxygen uptake, since the appropriate metabolic enzymes will be immediately available. Experiments of this type with *Pseudomonas* X2 have thrown some doubt on the validity of this approach. For example, *Pseudomonas* X2 grows much more rapidly on glucose than on *n*-decane or *n*-decanol as the substrate. Conversely *n*-decanoic acid not only fails to support growth but also inhibits growth of the organism on *n*-decane.

The foregoing difficulties do not appear to have been generally appreciated in the field of alkane metabolism, although parallels are available in other biochemical fields.

REFERENCES

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

Bird, C. W. & Molton, P. (1967). *Biochem. J.* **104**, 987.

Fredricks, K. M. (1967). *Leeuwenhoek ned. Tijdschr.* **33**, 41.

Goldman, P. (1965). *J. biol. Chem.* **240**, 3434.

Heringa, J. W., Huybregtse, R. & Van der Linden, A. C. (1961). *Leeuwenhoek ned. Tijdschr.* **27**, 51.

Heydeman, M. T. (1960). *Biochim. biophys. Acta*, **42**, 557.

Imelik, B. (1948). *C. R. Acad. Sci., Paris*, **227**, 1178.

Markovetz, A. J., Klug, M. J. & Forney, F. W. (1967). *J. Bact.* **93**, 1289.

Pomortseva, N. V. (1957). *Dokl. Akad. Nauk S.S.S.R.* **117**, 896.

Proctor, M. H. (1960). *Biochim. biophys. Acta*, **42**, 559.

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

Senez, J. C. & Azoulay, E. (1961). *Biochim. biophys. Acta*, **47**, 307.

Senez, J. C. & Konovaltschikoff-Mazoyer, M. (1956). *C. R. Acad. Sci., Paris*, **242**, 2873.

Thijsse, G. J. E. (1964). *Biochim. biophys. Acta*, **84**, 195.

Wagner, F., Zahn, W. & Buhring, U. (1967). *Angew. Chem. (int. Ed.)*, **6**, 359.