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Microbial Metabolism of C_1 and C_2 Compounds

THE ROLE OF ACETATE DURING GROWTH OF *PSEUDOMONAS* AM1 ON C_1 COMPOUNDS, ETHANOL AND β-HYDROXYBUTYRATE

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Pseudomonas AM1 grows on β-hydroxybutyrate and methanol at similar rates. β-Hydroxybutyrate is not metabolized by way of the glyoxylate bypass, but is assimilated by the novel route (with acetate as an intermediate) that operates during growth of this organism on ethanol. Evidence from short-term labelling experiments indicates that acetate, which is a possible intermediate in the assimilation of C_1 compounds, is rapidly metabolized to glycine during growth of *Pseudomonas* AM1 on methanol.

Pseudomonas AM1 is a pink bacterium capable of growth on a variety of carbon sources including C_1 compounds, ethanol, malonate and β-hydroxybutyrate. Ethanol and β-hydroxybutyrate are usually assimilated in micro-organisms by way of the glyoxylate bypass (Kornberg, 1959). By contrast it has been shown that in *Pseudomonas* AM1 ethanol is metabolized by a novel pathway and that acetate, glycollate, glyoxylate and malate are probably intermediates in this pathway (Dunstan *et al.*, 1972*a*). The results in the present paper indicate that this novel route also operates during growth of *Pseudomonas* AM1 on β-hydroxybutyrate and malonate. It has previously been suggested that some of the reactions of this pathway are also essential for growth on C_1 compounds (Dunstan *et al.*, 1972*b*). C_1 compounds are assimilated by the condensation of a C_1 unit with glycine to give serine, which is then converted into glycolytic intermediates (Large *et al.*, 1962; Large & Quayle, 1963). The glycine is regenerated by a cyclic route in which a product of the serine pathway is cleaved to two C_2 compounds (Salem *et al.*, 1972). These compounds are precursors of glyoxylate (or are glyoxylate itself), which is transaminated to glycine (Harder & Quayle, 1971). Evidence has previously been presented that acetate (and possibly glycollate) may serve as precursors of glyoxylate in this pathway (Dunstan *et al.*, 1972*b*). The work presented in the present paper supports this conclusion.

A preliminary account of part of this work has been published (Anthony & Dunstan, 1972; Dunstan & Anthony, 1972).

Experimental

Materials

Chemicals. All chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., except the following: [U-^{14}C]acetate (sodium salt) (specific radioactivity 56mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; DL-isocitrate (trisodium salt, allo-free) was from Calbiochem Ltd., London W.1, U.K.; phenylhydrazine hydrochloride was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; NE 250 liquid scintillator was from Nuclear Enterprises Ltd., Edinburgh 11, U.K.; hydroxypyruvate (lithium salt) was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; CoA from yeast (grade 1, free acid), NAD^+, NADH, $NADP^+$ and NADPH were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Acetyl-CoA was prepared by the method of Srere (1969).

Bacterial strains. Pseudomonas AM1 (N.C.I.B. 9133) was obtained from the National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, U.K.

Methods

Maintenance and growth of the organism. The maintenance and growth of cultures of *Pseudomonas* AM1 were as described by Dunstan *et al.* (1972*a*). The basal growth medium, without any added carbon source, is referred to as 'salts medium'. Carbon sources included in growth media were used at a concentration of 0.2%, except for methanol (0.4%) and sodium glyoxylate (5mM).

Purification of [U-^{14}C]acetate, measurements of oxygen uptake by whole cell suspensions and determination of growth responses of mutants. These methods were as described by Dunstan *et al.* (1972*a*,*b*).

Bacterial incorporation of [U-^{14}C]acetate. Cultures of bacteria growing on methanol were harvested in the mid-exponential phase of growth by centrifuging at 10000*g* for 15min at room temperature, and suspended in salts medium to a concentration of 3mg dry wt./ml. A portion (4ml) of this suspension

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was incubated aerobically at 30°C in the presence of 2μmol of non-radioactive sodium acetate. After 10min 80μCi of purified [U-^{14}C]acetate was added; 1ml samples were withdrawn at suitable time-intervals, immediately transferred to 3ml of boiling ethanol and left for 3min. The ethanolic suspensions were placed in a water bath at 50°C for a further 15min and then centrifuged. The supernatant fluid was decanted and the pellet resuspended in 1ml of boiling aq. 20% (v/v) ethanol. Insoluble material was removed by centrifugation and the combined supernatant fluids were evaporated to dryness under reduced pressure at 40°C.

Chromatography and radioautography. The residue obtained from the procedure described above was suspended in 0.1–0.2ml of aq. 20% (v/v) ethanol and spotted on to Whatman no. 1 chromatography paper (46cm×57cm). The radioactive compounds were separated by two-dimensional chromatography; the solvent used for the first dimension was phenol–NH_3 [200ml of water-saturated phenol (400g of phenol/100ml of water) plus 1ml of aq. NH_3 (sp.gr. 0.88)] and for the second dimension the solvent was butan-1-ol–acetic acid–water (12:3:5, by vol.) The solvent was run to the end of the paper in both cases. After drying of the chromatogram the radioactive compounds were located by radioautography, then eluted and counted for radioactivity as described by Dunstan *et al.* (1972*a*).

Identification of radioactive compounds. The identity of the radioactive compounds was confirmed by co-chromatography with authentic standards. A suitable portion of the radioactive sample was evaporated to dryness together with an authentic sample (50μg of carboxylic acid, 10μg of amino acid). The residue was then dissolved in 0.05ml of aq. 20% (v/v) ethanol and spotted on to Whatman no. 1 chromatography paper. The carboxylic acids were chromatographed in butan-1-ol–acetic acid–water (12:3:5, by vol.) and in ethanol–NH_3 (sp.gr. 0.92)–water (16:1:3, by vol.). The amino acids were chromatographed in the phenol solvent described above and in butan-1-ol–acetone–water–diethylamine (10:10:5:2, by vol.).

After drying of the chromatogram the radioactive compounds were located by radioautography. The chromatograms were then stained, by using acridine (0.1% solution made up in aq. 99.5% ethanol) for the carboxylic acids and ninhydrin (0.5% solution made up in acetone) for the amino acids. When the positions of the radioactive compound and the authentic standard coincided in both solvents the two substances were assumed to be identical.

Enzyme assays. Sonic extracts were prepared as described by Dunstan *et al.* (1972*a*). Enzymes were assayed spectrophotometrically in a Unicam SP.1800 or a Unicam SP.500 apparatus at room temperature; 10mm light-path silica cells were used. The reference cell contained water. The following enzymes were assayed, by using published methods: citrate synthase (EC 4.1.3.7) (Srere, 1969); isocitrate dehydrogenase (EC 1.1.1.42) (Ochoa, 1955*a*); malate dehydrogenase (EC 1.1.1.37) (Ochoa, 1955*b*); malate synthase (EC 4.1.3.2) (Dixon & Kornberg, 1959); isocitrate lyase (EC 4.1.3.1) (Kornberg, 1965); β-hydroxybutyrate dehydrogenase (Shuster & Doudoroff, 1962). Protein was assayed by the method of Lowry *et al.* (1951); crystalline bovine serum albumin (fraction V) was used as a standard.

Results

Metabolism of β-hydroxybutyrate

During a survey of potential growth substrates it was found that *Pseudomonas* AM1 grows well on β-hydroxybutyrate (the mean generation time is 7–8h compared with 7h on methanol and 10h on ethanol and on malonate). Whole cells of *Pseudomonas* AM1 grown on β-hydroxybutyrate rapidly oxidize this compound. The rate of oxygen uptake by whole cells is 50μl/h per mg dry wt. (after correction for endogenous oxygen uptake) and extracts of such cells contain β-hydroxybutyrate dehydrogenase. This enzyme is inducible, the specific activity in β-hydroxybutyrate-grown cells being 2–3 times that in cells grown on other carbon sources (Table 1). A cytochrome *c*-deficient mutant of *Pseudomonas* AM1 (Anthony & Dunstan, 1971) is capable of growth on β-hydroxybutyrate (but not on methanol or ethanol), indicating that cytochrome *c* is not essential for β-hydroxybutyrate oxidation.

The product of β-hydroxybutyrate oxidation is presumably acetoacetate, which is then further oxidized by way of acetyl-CoA and the tricarboxylic acid cycle. As shown in Table 1, a number of enzymes of the cycle are present at relatively high activities in extracts of β-hydroxybutyrate-grown *Pseudomonas* AM1.

The key enzymes of the glyoxylate bypass have been measured in extracts of β-hydroxybutyrate-grown cells (Table 1). Malate synthase was present at a fairly high activity, but only trace amounts of isocitrate lyase were detected. The specific activity of this enzyme is too low to account for the growth rate on β-hydroxybutyrate, and it is therefore unlikely to be involved in the assimilation of this substrate.

A number of mutants of *Pseudomonas* AM1 have been isolated (Dunstan *et al.*, 1972*b*) and some of their growth properties are shown in Table 2. Mutants PCT57 and PCT64, which are blocked in reactions specifically involved in the metabolism of C_1 compounds, are able to grow on β-hydroxybutyrate and on malonate. Mutant PCT48, however, is unable to grow on C_1 compounds, ethanol, malonate or β-hydroxybutyrate unless either glyoxylate or glycollate is provided in the medium. Dunstan *et al.* (1972*b*) suggested that this mutant is unable to convert

Table 1. *Specific activities of enzymes of the glyoxylate bypass and the tricarboxylic acid cycle in Pseudomonas AM1*

Experimental details are given in the Experimental section.

	Specific activity (μmol of substrate used/h per mg of protein)			
Growth substrate ...	Succinate	Methanol	β-Hydroxybutyrate	Ethanol
Isocitrate lyase	0.0*	0.0*	0.03	0.04
Malate synthase	2.3*	1.17*	4.6	1.75
Citrate synthase	7.01*	4.03*	7.8	3.1
Malate dehydrogenase	97.0	36.0	192.0	194.0
Isocitrate dehydrogenase	18.0	16.0	34.0	10.0
β-Hydroxybutyrate dehydrogenase	6.6	6.0	17.5	9.4

* Values of Large & Quayle (1963).

Table 2. *Growth responses of mutants of Pseudomonas AM1*

Growth responses were determined in liquid media as described in the Experimental section. The relative amounts of growth observed after three serial subcultures in liquid medium are represented by values 0–5 (based on visual estimates). Mutant PCT48 is unable to convert acetate into glycollate (Dunstan *et al.*, 1972*b*). Mutants PCT57 and PCT64 are unable to form serine from C_1 compounds (Dunstan *et al.*, 1972*b*). Mutant PCT76 lacks soluble cytochrome *c* (Anthony & Dunstan, 1971).

	Growth response			
Growth substrate Strain ...	Wild-type	PCT48	PCT57, PCT64	PCT76
Succinate	4	4	4	4
Methanol	4	0	0	0
Methanol+glycollate	5	3	–	–
Methanol+glyoxylate	5	3	–	–
Ethanol	2	0	2	0
Ethanol+glycollate	3	2	–	–
Ethanol+glyoxylate	3	2	–	–
β-Hydroxybutyrate	4	0	4	4
β-Hydroxybutyrate+glycollate	5	3	–	–
β-Hydroxybutyrate+glyoxylate	5	3	–	–
Malonate	2	0	2	2
Malonate+glycollate	3	2	–	–
Malonate+glyoxylate	3	2	–	–

acetate into glycollate. This reaction appears, therefore, to be involved in the metabolism of C_1 compounds, ethanol, malonate and β-hydroxybutyrate. The evidence from enzymic studies and from the growth responses of mutants suggests, therefore, that β-hydroxybutyrate and ethanol are assimilated by the same route in *Pseudomonas* AM1, both substrates being oxidized to acetate, which is then metabolized to malate, probably by way of glycollate and glyoxylate (Scheme 1).

Role of acetate in the assimilation of C_1 compounds

A study of the growth responses of mutants of *Pseudomonas* AM1 has indicated that acetate may be an intermediate in the synthesis of glyoxylate (and hence glycine) from C_1 compounds (Dunstan *et al.*, 1972*a*,*b*). If this is the case, then glycine should be an early intermediate in the metabolism of acetate by methanol-grown *Pseudomonas* AM1. This prediction was tested by a short-term labelling experiment with [U-^{14}C]acetate. In this type of experiment cells are incubated with radioactive substrate and samples are removed at short time-intervals. The ethanol-soluble intermediates are extracted, separated, counted for radioactivity and identified. The proportion of the total radioactivity incorporated into early intermediates (or compounds in rapid equilibrium with early intermediates) will either be constant or will decrease with time. Compounds containing an increasing

Table 3. *Distribution of ^{14}C among the non-volatile labelled components of the ethanol-soluble fractions of methanol-grown Pseudomonas AM1 incubated with [U-^{14}C]acetate*

Washed organisms (12mg dry wt.) were preincubated aerobically in 4ml of salts medium with 2μmol of acetate at 30°C for 10min. [U-^{14}C]Acetate (4μmol; 80μCi of ^{14}C) was added at zero time and 1ml samples were removed at suitable time-intervals into 3ml of boiling ethanol. The precipitates were extracted with 1ml of aq. 20% (v/v) ethanol and the combined extracts analysed by two-dimensional chromatography. The radioactive compounds were eluted, counted and identified as described in the Experimental section. Correction was made for quenching and for the background counts of vials. The chromatogram background radioactivity was 40–90c.p.m.; it is given for reference but was not used for correction purposes. A blank sample containing boiled cells and radioactive substrate was treated in an identical manner with the experimental samples; no radioactive compounds were detected in the blank sample after chromatography and radioautography.

	Radioactivity in compound (c.p.m.)						
Time (s) / Compound ...	Glutamate	Compound 'B'	Glycine	Malate	'Succinate+ fumarate'	Others	Total
7	750	2680	1210	2600	480	9730	17450
15	1560	7580	3050	10960	3950	29420	56520
30	10560	10540	6840	24480	8120	43970	104510
60	24240	15140	7880	22600	6510	74020	150390

proportion of the total radioactivity are later intermediates. The results of such an experiment using [U-^{14}C]acetate and methanol-grown *Pseudomonas* AM1 are shown in Table 3 and Fig. 1. The proportion of radioactivity incorporated into malate, 'succinate plus fumarate' (which were not distinguished) and glutamate increased over the first 30s, indicating that they were not early intermediates in the metabolism of acetate by methanol-grown *Pseudomonas* AM1. By contrast, the proportion of radioactivity incorporated into glycine was approximately constant throughout the time-course of the experiment. The proportion of radioactivity incorporated into an unidentified substance (compound 'B') decreased during the experiment. It appears, therefore, that glycine and compound 'B' are early intermediates (or are in equilibrium with early intermediates) in the metabolism of acetate by methanol-grown *Pseudomonas* AM1.

These labelling patterns contrast with those obtained with [U-^{14}C]acetate and ethanol-grown organisms. In this experiment, glycollate, malate and compound 'B' were early intermediates (Dunstan *et al.*, 1972*a*). The low activity of serine–glyoxylate aminotransferase present in ethanol-grown cells would account for the failure to detect radioactive glycine in this experiment. It is notable that radioactivity was not detected in glycollate when methanol-grown organisms were used; this may be because the pool size of glycollate is very small as a result of rapid metabolism to glyoxylate and glycine. Compound 'B' is formed from acetate by both methanol- and ethanol-grown cells; all attempts to identify this substance have been unsuccessful, but it may have an important role in the metabolism of acetate by *Pseudomonas* AM1.

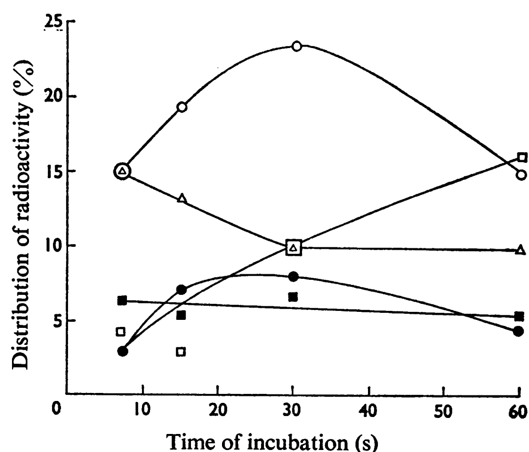
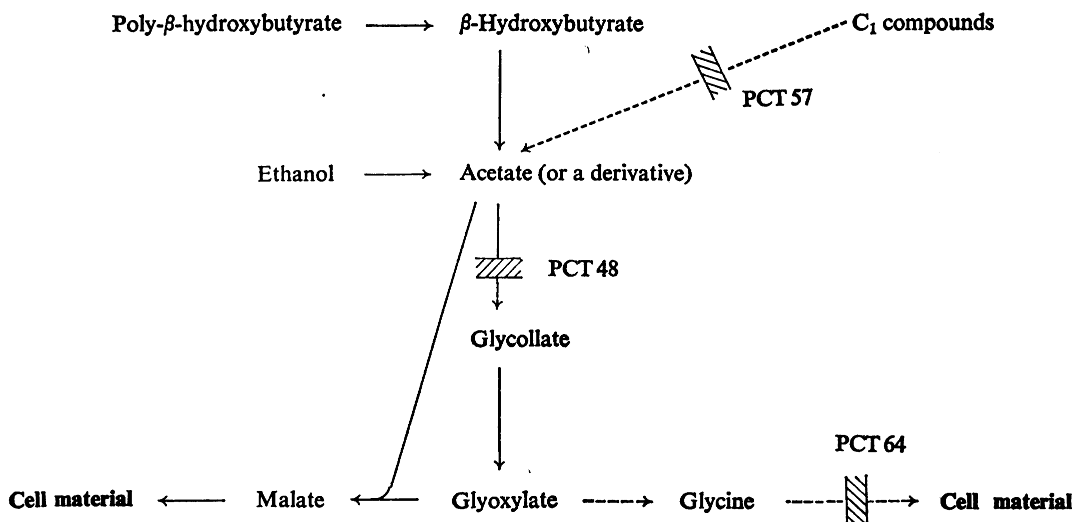


Fig. 1. *Variation with time of the percentage distribution of radioactivity incorporated from [U-^{14}C]acetate into non-volatile components of the ethanol-soluble fraction of methanol-grown Pseudomonas AM1*

For experimental details see Table 3 and the text. ○, Malate; △, compound 'B'; ●, 'succinate plus fumarate'; ■, glycine; □, glutamate.

Discussion

It can be concluded from the results presented above that, during growth of *Pseudomonas* AM1 on β-hydroxybutyrate, ethanol or malonate, acetate is metabolized to malate; by contrast, during growth



Scheme 1. *Role of acetate in the related pathways for assimilation of β-hydroxybutyrate, C_1 compounds and ethanol by Pseudomonas AM*1

Broken lines indicate series of reactions specifically involved in the metabolism of C_1 compounds. Metabolic lesions in the designated mutants are indicated by hatched bars.

on methanol, acetate metabolism is diverted to the production of glycine.

Poly β-hydroxybutyrate is the major storage compound in *Pseudomonas* AM1, and may constitute up to 8% of the dry weight. It is probable that the pathway proposed in Scheme 1 provides a route for the generation of biosynthetic intermediates from these endogenous reserves.

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