

Acetyl-CoA Production and Utilization during Growth of the Facultative Methylophilic *Pseudomonas* AM1 on Ethanol, Malonate and 3-Hydroxybutyrate

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(Received 9 December 1975; revised 23 February 1976)

SUMMARY

In *Pseudomonas* AM1, conversion of 3-hydroxybutyrate to acetyl-CoA is mediated by an inducible 3-hydroxybutyrate dehydrogenase, an acetoacetate:succinate coenzyme A transferase (specific for succinyl-CoA) and an inducible β -ketothiolase. Ethanol is oxidized to acetate by the same enzymes as are involved in methanol oxidation to formate. An inducible acetyl-CoA synthetase has been partially purified and characterized; it is essential for growth only on ethanol, malonate and acetate plus glyoxylate, as shown by the growth characteristics of a mutant (ICT54) lacking this enzyme. Free acetate is not involved in the assimilation of acetyl-CoA, and hydroxypyruvate reductase is not involved in the oxidation of acetyl-CoA to glyoxylate during growth on 3-hydroxybutyrate. A mutant (ICT51), lacking 'malate synthase' activity, has been isolated and its characteristics indicate that this activity is normally essential for growth of *Pseudomonas* AM1 on ethanol, malonate and 3-hydroxybutyrate, but not for growth on other substrates such as pyruvate, succinate and C₁ compounds. The growth properties of a revertant (ICT51R) and of a mutant lacking malyl-CoA lyase (PCT57) indicate that an alternative route must exist for assimilation of compounds metabolized exclusively by way of acetyl-CoA.

INTRODUCTION

In *Pseudomonas* AM1, the malate synthase pathway has been proposed as a route for the assimilation of substrates that are metabolized to acetyl-CoA or acetate: these include 3-hydroxybutyrate, ethanol, malonate, lactate and pyruvate (Salem *et al.*, 1973*b*; Dunstan & Anthony, 1973; Anthony, 1975*a*). During operation of this pathway, one molecule of acetyl-CoA (or acetate) is oxidized to glyoxylate [in reactions also occurring as part of the same pathway during growth on C₁ compounds (Dunstan & Anthony, 1973)]. The glyoxylate then condenses with a second molecule of acetyl-CoA in a reaction catalysed by malate synthase to give malate. It has been suggested that the malate synthase activity may result from the sequential action of malyl-CoA lyase and malyl-CoA hydrolase or transferase (Salem, Hacking & Quayle, 1973*a*) and recent results show that this is indeed the case (Cox & Quayle, 1976).

The present paper describes investigations designed to determine the pathways involved in the formation of acetyl-CoA from ethanol, malonate and 3-hydroxybutyrate; to distinguish between acetate and acetyl-CoA as precursors of glyoxylate; and to determine whether or not hydroxypyruvate reductase is involved in the oxidation of acetate to glyoxylate in *Pseudo-*

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monas AM1. The importance of acetyl-CoA synthetase for growth on ethanol and malonate has been demonstrated by the isolation and characterization of a mutant lacking this enzyme, and the importance of malate synthase activity for normal growth of *Pseudomonas* AM1 on ethanol, malonate and 3-hydroxybutyrate has been confirmed by the isolation and study of a mutant lacking this activity. A preliminary report of these two mutants has been published (Taylor & Anthony, 1975).

METHODS

Maintenance, growth and harvesting of bacteria. Methods were as previously described (Anthony, 1975*b*). Carbon sources included in the basal salts medium were used at a concentration of 0.2 % except for: methanol and ethanol (0.4 %); 3-hydroxybutyrate, malonate and acetate (0.1 %); glyoxylate (5 mM).

Isolation of mutants ICT54, ICT51 and ICT51R. Mutants ICT54 and ICT51 were isolated by a method based on that of Heptinstall & Quayle (1970), designed to select for mutants able to grow on malate but not on a combination of acetate (0.1 %) plus glyoxylate (5 mM). Bacteria treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) were expressed in malate medium and then treated three times with benzylpenicillin (1000 u./ml medium) in the presence of acetate plus glyoxylate. Mutants isolated by this selection procedure included mutant ICT54 which lacked acetyl-CoA synthetase and mutant ICT51 which lacked malate synthase activity. Mutant ICT51R was isolated on (acetate plus glyoxylate)-agar plates from a streptomycin-resistant derivative of mutant ICT51, by the method described by Taylor & Anthony (1976). All bacterial strains, including mutant 20BL which was a gift from Professor J. R. Quayle, were maintained on succinate-agar slopes at 2 °C.

Preparation of cell-free extracts, determination of protein, and enzyme assays. The growth characteristics of mutant bacteria were determined in liquid media as described by Taylor & Anthony (1976). The methods used for determination of protein and preparation of cell-free extracts were as described by Anthony (1975*b*). All spectrophotometric enzyme assays were carried out using a Pye- Unicam SP1800 recording spectrophotometer at 25 °C. The following enzymes were assayed by published procedures: 3-hydroxybutyrate dehydrogenase, EC. 1.1.1.30 (Schuster & Doudoroff, 1962); acetoacetate:succinate CoA-transferase, EC. 2.8.3.5 (Stern *et al.*, 1956); β -ketothiolase, EC. 2.3.1.9, cleavage reaction (Senior & Dawes, 1973), condensation reaction (Oeding & Schlegel, 1973); acetoacetyl-CoA reductase, EC. 1.1.1.36 (Ritchie, Senior & Dawes, 1971); succinyl-CoA synthetase, EC. 6.2.1.5 (Kaufman, 1955); malyl-CoA lyase (Salem *et al.*, 1973*a*); acetokinase, EC. 2.7.2.1 (Rose, 1955); CoA,NAD-dependent aldehyde dehydrogenase (Dawes & Foster, 1956). Malate synthase activity was assayed by the method of Dixon & Kornberg (1959).

Assay and partial purification of acetyl-CoA synthetase. Acetyl-CoA synthetase (EC. 6.2.1.1) was assayed by the hydroxamate method of Jones & Lipmann (1955) except that the incubation temperature was 40 °C instead of 30 °C. Acetyl-CoA synthetase activity was partially purified by the following method. A crude sonic extract of bacteria grown on 3-hydroxybutyrate was dialysed against 20 mM-phosphate buffer pH 7.0 and treated with protamine sulphate (1.8 %, w/v) at 2 °C until precipitation was complete. Precipitated protein and nucleic acids were removed by centrifuging. The supernatant liquid was heated at 60 °C for 15 min, precipitated protein was again removed by centrifuging, and the supernatant was treated at 20 °C with solid ammonium sulphate to obtain a fraction that precipitated at between 52 % and 75 % ammonium sulphate saturation. The precipitate was dissolved in 20 mM-phosphate buffer pH 7.0 to give a protein concentration of about 10 mg ml⁻¹. This fraction, which was free of succinyl-CoA synthetase activity, was further

purified, to remove adenylate kinase, by gel-filtration on a column (1.5 × 23 cm) of Sephadex G150. The column was equilibrated with 20 mM-phosphate buffer and proteins were eluted with the same buffer at room temperature.

Crude extracts, and partially purified extracts containing adenylate kinase activity, catalysed the production of ADP in an assay mixture containing (μmol , in a total volume of 3.0 ml): coenzyme A, 4; ATP, 2; sodium acetate, 20; phosphate buffer pH 7.4, 100; MgCl_2 , 10; reduced glutathione, 10; NADH, 2.5; phosphoenolpyruvate, 5; pyruvate kinase and lactate dehydrogenase, 10 units each; and acetyl-CoA synthetase fraction, 50 to 200 μg protein (added to start the reaction). Adenylate kinase was assayed by the above method except that acetate and coenzyme A were replaced by AMP (1 μmol). Acetyl-CoA was identified as a product of acetyl-CoA synthetase by coupling its formation to NADH production in the presence of NAD^+ , malate, malate dehydrogenase and citrate synthase. The assay mixture contained (μmol , in 2.0 ml): Tris-HCl buffer pH 7.4, 200; sodium malate, 20; sodium acetate, 20; coenzyme A, 0.1; ATP, 10; MgCl_2 , 10; NAD^+ , 1; and acetyl-CoA synthetase fraction, 50 μg . Malate dehydrogenase (20 units) was added, and E_{340} was monitored for 2 to 3 min before the addition of citrate synthase (5 units). The rate of production of NADH in the absence of citrate synthase was subtracted from the final rate and was equivalent to approximately 120 nmol acetyl-CoA produced/min per mg protein.

Incorporation of [$U\text{-}^{14}\text{C}$]acetate into wild-type Pseudomonas AM1, and mutants ICT54 and 2OBL, grown on 3-hydroxybutyrate. Cultures of bacteria were incubated with [$U\text{-}^{14}\text{C}$]acetate for 1 min and radioactive compounds were extracted with boiling aqueous ethanol according to the method of Dunstan, Anthony & Drabble (1972a). After this time, the distribution of radioactivity amongst the non-volatile ethanol-soluble components of *Pseudomonas* AM1 and mutant 2OBL had reached equilibrium.

Chromatographic analysis of radioactive compounds. Ethanolic suspensions containing radioactive compounds were evaporated to dryness under reduced pressure at 35 °C, resuspended in 0.01 M-HCl (0.5 ml) and applied to Dowex columns (10 × 0.6 cm, H^+ form). Neutral and acidic compounds were eluted with water (10 ml) and basic compounds with 2 M- NH_4OH (4.0 ml). These fractions were evaporated to dryness, dissolved in 20 % (v/v) ethanol (0.3 ml) and spotted on to Whatman no. 1 chromatography paper (dimensions 20 × 57 cm). The acidic fractions were run in ethanol/ammonia/water (16:1:3, by vol.), and compared with a mixture of standard carboxylic acids (30 μg each of citric, malic, succinic, fumaric and glycollic acids) run on either side of the test samples. Basic compounds were separated in phenol/ammonia/water (200 ml water-saturated phenol:1 ml NH_3) and compared with a standard mixture of the following amino acids (6 μg each): alanine, glycine, serine, aspartic and glutamic acids.

After drying the chromatograms, strips containing the standard amino or carboxylic acids were cut off and stained. Carboxylic acids were stained with acridine (0.1 %, w/v) in 99.5 % ethanol, and appeared as yellow spots which fluoresced under u.v. light. Amino acids were stained with ninhydrin (0.5 %, w/v) in acetone: after drying, the colour was developed for a few minutes at 110 °C and amino acids appeared as blue/purple spots.

Radioactive compounds were detected with a Dunnschicht II chromatogram scanner. Areas of the chromatogram corresponding to peaks of radioactivity were cut out, placed in water (2.0 ml) and shaken for 5 min. The paper was removed and a sample of the eluant was counted in NE 250 scintillant. All counting was carried out in a Philips liquid scintillation analyser programmed for automatic quench correction.

The position of the radioactive peaks compared with the positions of the standards suggested the identity of the labelled compounds. This was confirmed by co-chromatography

with authentic standards in the original solvent and in a second solvent. The second solvent was butanol/acetic acid/water (12:3:5, by vol.) for both amino and carboxylic acids.

Incorporation of radioactivity from [U-¹⁴C]acetate into growing cultures of bacteria. Cultures (200 ml) were shaken at 30 °C in Erlenmeyer flasks (500 ml), fitted with side arms for assessment of growth by following the extinction at 650 nm. The medium contained 3-hydroxybutyrate (0.1 %), to which 2 μ Ci of [U-¹⁴C]acetate and non-radioactive acetate (final concentration, 2 mM) were added once the cells had started to grow exponentially. Samples containing 0.2 to 0.6 mg dry weight equivalent of organisms were withdrawn at intervals throughout growth and collected on a membrane filter, previously washed with water and sodium acetate. The bacteria were washed with acetate (2 \times 5 ml, 0.1 M) and water (2 \times 5 ml), transferred on the filters to scintillation vials, and dissolved in NE 250 scintillant (10 ml) for estimation of radioactivity. Incorporation of radioactivity is expressed as specific activity (d.p.m./ μ g dry wt bacteria).

Chemicals. All chemicals were obtained from BDH except: coenzyme A (lithium salt) was from PL Biochemicals Inc., Milwaukee, U.S.A.; diketone (acetoacetic anhydride), Dowex 50W(H⁺-form), NADP⁺, NAD⁺, NADPH, NADH, citrate synthase, malate dehydrogenase, pyruvate kinase and lactate dehydrogenase were from Sigma; glyoxylic acid monohydrate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) were from Koch-Light; and Sephadex G150 was from Pharmacia.

Acetyl-CoA and acetoacetyl-CoA were prepared by the reaction of coenzyme A with the respective anhydrides of acetate and acetoacetate according to the methods of Srere (1969) and Simon & Shemin (1953) respectively. [U-¹⁴C]acetate from The Radiochemical Centre, Amersham, Buckinghamshire, was purified by the method of Dunstan *et al.* (1972*a*). (2*S*)-4-Malyl-CoA was a gift from Professor J. R. Quayle.

NE 250 scintillation fluid was obtained from Nuclear Enterprises Ltd, Beenham, Berkshire.

RESULTS

The formation of acetyl-CoA during growth of Pseudomonas AM1 on 3-hydroxybutyrate, ethanol and malonate

The interconversion of 3-hydroxybutyrate and acetyl-CoA. The first enzyme involved in 3-hydroxybutyrate oxidation is an NAD⁺-specific 3-hydroxybutyrate dehydrogenase which was induced between 2.5 and 10-fold during growth of *Pseudomonas* AM1 on 3-hydroxybutyrate compared with its level during growth on other substrates (Table 1).

The specific activities of the second enzyme, acetoacetate:succinate CoA-transferase, showed much less variation with carbon source than 3-hydroxybutyrate dehydrogenase (Table 1). The transferase was assayed by following the disappearance of acetoacetyl-CoA on addition of succinate to the reaction mixture. The following compounds were inactive as replacements for succinate in extracts prepared from bacteria grown in 3-hydroxybutyrate or methanol: malate, acetate, propionate, glycollate and formate. As these compounds were not activated by transfer of coenzyme A from acetoacetyl-CoA, it can be concluded that, in the direction of acetoacetate activation, the enzyme is specific for succinyl-CoA.

The final enzyme for the oxidation of 3-hydroxybutyrate to acetyl-CoA is an inducible β -ketothiolase (Table 1). The ratio of specific activities was constant whether assayed in the direction of acetoacetyl-CoA cleavage or acetyl-CoA condensation, the rate of cleavage being about four times that of condensation. The constant ratio demonstrates that one enzyme is involved in both directions and suggests that β -ketothiolase is also involved in poly-3-hydroxybutyrate biosynthesis in *Pseudomonas* AM1. The polymer is the only carbon

Table 1. *Specific activities of enzymes of 3-hydroxybutyrate metabolism in extracts of Pseudomonas AMI grown on various carbon sources*

Bacteria were harvested in the mid-exponential phase of growth and enzymes were assayed as described in Methods. Enzyme levels are expressed as nmol/mg protein per min, and are the average of results from at least two separate extracts.

Enzyme	Growth substrate				
	3-Hydroxybutyrate	Succinate	Methanol	Ethanol	Lactate
3-Hydroxybutyrate dehydrogenase	350	146	66.5	33.2	145
Acetoacetate: succinate CoA-transferase	91.5	70.5	41.3	36.0	95.0
β -Ketothiolase (condensation)	310	87.0	75.4	81.5	126
Acetoacetyl-CoA reductase	320	356	196	166	183

and energy reserve in this bacterium and forms between 3 and 8% of its dry weight during growth on methanol, ethanol, malonate, 3-hydroxybutyrate, lactate and succinate (Taylor, 1975). The substrate for polymerization of 3-hydroxybutyrate is not the free acid but 3-hydroxybutyryl-CoA, which is produced by reduction of acetoacetyl-CoA. The acetoacetyl-CoA reductase catalysing this reaction was shown to be present in extracts of *Pseudomonas AMI* and it had similar specific activities on all growth substrates (Table 1). The rate with NADH was 10 times that measured with NADPH.

The oxidation of ethanol to acetyl-CoA. In *Pseudomonas AMI*, ethanol is oxidized to acetaldehyde by the methanol dehydrogenase which interacts with the cytochrome chain at the level of cytochrome *c* (Anthony, 1975*b*; Widdowson & Anthony, 1975).

No nicotinamide-nucleotide-linked aldehyde dehydrogenase could be detected in extracts of ethanol-grown *Pseudomonas AMI* which contained no NAD⁺, CoA-dependent acetaldehyde dehydrogenase, as is found in ethanol-grown *Hyphomicrobium x* (Attwood & Harder, 1974). Acetaldehyde is thus presumably oxidized to the level of free acetate by the NAD(P)⁺-independent aldehyde dehydrogenase which leads to reduction of the cytochrome chain at the level of cytochrome *b* (Widdowson & Anthony, 1975). The low activity of this enzyme probably accounts for the acetaldehyde that accumulates during the rather slow growth of *Pseudomonas AMI* on ethanol. The activation of the acetate produced by acetaldehyde oxidation is catalysed by acetyl-CoA synthetase and is discussed below.

The metabolism of malonate to acetyl-CoA. *Pseudomonas AMI* grows on malonate with a mean generation time of 7 to 10 h, and whole cells oxidized this substrate with a Q_{O_2} of approx. $20 \mu\text{l h}^{-1}$ (mg dry wt)⁻¹. No malonyl-CoA synthetase activity could be detected when malonate was used instead of acetate in the usual acetyl-CoA synthetase assay (see Methods). This result, together with the observation that a mutant lacking acetyl-CoA synthetase is unable to grow on malonate (see below), suggests that malonate is metabolized by decarboxylation to free acetate which is then activated to acetyl-CoA in a reaction catalysed by acetyl-CoA synthetase. This contrasts with the route in *Pseudomonas fluorescens* in which malonyl-CoA is decarboxylated to acetyl-CoA, the malonyl-CoA being formed by a specific malonyl-CoA synthetase (Wolfe, Ivler & Rittenberg, 1954).

The role of acetyl-CoA synthetase in growth of Pseudomonas AMI

In *Pseudomonas AMI*, there is an acetyl-CoA synthetase which is induced sevenfold during growth on 3-hydroxybutyrate compared with its level during growth on methanol (Table 2).

Table 2. Specific activities of acetyl-CoA synthetase, 'malate synthase' and malyl-CoA lyase in extracts of *Pseudomonas* AM1, and mutants ICT51, ICT54 and ICT51R

Extracts were prepared and assayed as described in Methods. Enzyme activities are expressed as nmol substrate used or product formed/mg protein per min.

Growth substrate	Acetyl-CoA synthetase			'Malate synthase'		Malyl-CoA lyase		
	WT	ICT54	ICT51	WT	ICT51	WT	ICT51	ICT51R
Methanol	13.4	1.5	16.3	44	1.9	840	920	1320
Ethanol	20.6	—	—	42	—	—	—	—
3-Hydroxybutyrate	94.6	ND	—	35	—	210	—	170
Lactate	39.6	0.7	—	45	3.4	130	160	240
Malonate	84.5	—	—	26	—	—	—	—
Succinate	—	—	—	—	—	200	160	120
Malate	15.0	1.4	15.0	25	2.0	—	—	—

ND, Not detected; —, not assayed; WT, wild type.

No acetate kinase was detected in extracts of *Pseudomonas* AM1 grown on any substrate. Assay of the acetyl-CoA synthetase in crude extracts suggested that the products of the reaction might be acetyl-CoA and ADP instead of the usual AMP and pyrophosphate. Such an enzyme would be able to function in the direction of acetate formation from acetyl-CoA, and free acetate might thus be important in the assimilation of acetyl-CoA produced during 3-hydroxybutyrate oxidation. To investigate this further, the enzyme was purified and characterized, and a mutant lacking the enzyme was isolated.

Partial purification and characterization of acetyl-CoA synthetase. Crude extracts of *Pseudomonas* AM1 grown on 3-hydroxybutyrate catalysed the formation of coenzyme A esters from acetate, propionate and succinate, but not from 3-hydroxybutyrate, malonate, glycollate or malate. The relative rates were 70, 38, and 76 nmol min⁻¹ mg⁻¹ for acetate, propionate and succinate, respectively, in the standard acetyl-CoA synthetase assay. The acetyl-CoA synthetase was partially purified (sixfold) from these extracts by a procedure involving heating at 60 °C, fractionating with ammonium sulphate, and gel-filtration on Sephadex G150 (see Methods). The ammonium sulphate fractionation removed succinyl-CoA synthetase and the final stage removed adenylate kinase. Using the purified preparation, it was shown that ADP was only produced during the acetyl-CoA synthetase reaction when adenylate kinase was added to the reaction mixture. The rate of ADP formation was then twice the rate of production of acetyl-CoA. Thus, in crude extracts, AMP, the product of the acetyl-CoA synthetase reaction gives rise to ADP by reaction with ATP catalysed by adenylate kinase. Acetyl-CoA was shown to be the product of the acetyl-CoA synthetase reaction by coupling its formation to NADH production in the presence of NAD⁺, malate, malate dehydrogenase and citrate synthase. The formation of propionyl hydroxamate, catalysed by crude extracts, in the acetyl-CoA synthetase reaction mixture containing propionate as substrate has not been investigated further; but the catalyst is probably acetyl-CoA synthetase, as found with acetyl-CoA synthetases from other sources (Jencks, 1962).

Isolation and characterization of a mutant lacking acetyl-CoA synthetase. In order to elucidate the role of acetyl-CoA synthetase, a mutant (ICT54) lacking this enzyme (Table 2) was isolated by selecting for ability to grow on malate but not on a combination of acetate

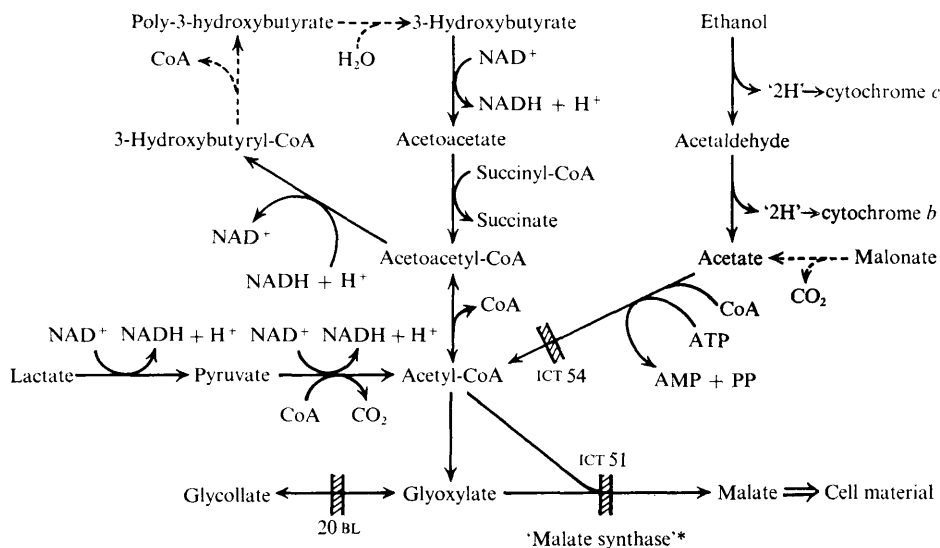


Fig. 1. Pathways for assimilation of ethanol, malonate, 3-hydroxybutyrate, pyruvate and lactate in *Pseudomonas AM1*. Hatched bars indicate metabolic lesions in mutants. Reactions shown as dotted lines have not been investigated. * The 'malate synthase' activity is catalysed by malyl-CoA lyase and malyl-CoA hydrolase (Cox & Quayle, 1976).

plus glyoxylate. Its succinyl-CoA synthetase activity was the same as that in wild-type *Pseudomonas AM1*. This mutant was unable to grow on ethanol, malonate or acetate plus glyoxylate but grew well on C_1 compounds, 3-hydroxybutyrate, lactate, pyruvate, succinate or malate. These growth characteristics indicate that the relatively high levels of acetyl-CoA synthetase measured during growth of wild-type *Pseudomonas AM1* on 3-hydroxybutyrate do not indicate an essential role for the enzyme during this growth. The failure of mutant ICT54 to grow on ethanol or malonate indicates that free acetate is an intermediate in the oxidation of these substrates (Fig. 1).

In bacteria grown on 3-hydroxybutyrate, the incorporation of radioactive acetate into metabolic intermediates by the mutant ICT54 was only 2% of that incorporated by the wild type (Table 3). This result was confirmed by growing bacteria on 3-hydroxybutyrate in the presence of radioactive acetate for five generations: after this time, the specific activity (d.p.m./ μg dry wt) of mutant ICT54 was only 15% of that measured with wild-type *Pseudomonas AM1*. Thus, acetate must be converted to acetyl-CoA before growth on ethanol, malonate or acetate plus glyoxylate can occur; and, during growth on 3-hydroxybutyrate, there is no route for oxidation of acetate to glyoxylate without the intermediacy of acetyl-CoA.

Demonstration that hydroxypyruvate reductase is not involved in the malate synthase pathway for assimilation of 3-hydroxybutyrate

It has been suggested that glycollate may be an intermediate in the oxidation of acetate to glyoxylate in *Pseudomonas AM1*, with the oxidation of glycollate to glyoxylate being catalysed by hydroxypyruvate reductase (Dunstan, Anthony & Drabble, 1972*b*). However, a mutant (20BL) lacking hydroxypyruvate reductase grew at the same rate as the wild-type bacteria on 3-hydroxybutyrate but had no detectable hydroxypyruvate reductase activity. Thus if glycollate is an intermediate, its oxidation to glyoxylate cannot be catalysed by

Table 3. Distribution of radioactivity among components of the ethanol-soluble fraction of *Pseudomonas* AM1, and mutant ICT54 grown on 3-hydroxybutyrate and incubated with [U - ^{14}C]acetate

Washed organisms were incubated with 2.5 mM-acetate as described in Methods. [U - ^{14}C]acetate ($5 \mu\text{Ci ml}^{-1}$) was added, and after 1 min incubation the ethanol-soluble radioactive compounds were isolated, counted and identified. A correction was made for quenching and for the background counts of the vials. The limit of detection by the chromatogram scanner under the conditions used was approximately 300 d.p.m.

Radioactive compound	Radioactivity (d.p.m.)		
	Wild type	ICT 54	20BL
Malate	9750	1300	10044
Succinate	6670	} 760	6740
Fumarate	13290		9010
Glycollate	6350	ND	2810
Citrate	7400	ND	6500
Aspartate	16810	ND	12920
Glutamate	404380	7680	260390
Alanine	44770	ND	20630
Others	—	1230	—
Total	502920	10970	322600
% wt incorporation	100	2.1	62.0
glycollate/malate	0.65	—	0.28

ND, No radioactive peaks detected.

hydroxypyruvate reductase. The demonstration that the [^{14}C]glycollate:[^{14}C]malate ratio was lower in mutant 20BL than in wild-type bacteria incubated with [^{14}C]acetate (Table 3) supports the suggestion that some of the glycollate in experiments with wild-type *Pseudomonas* AM1 may arise by reduction of glyoxylate catalysed by hydroxypyruvate reductase (Anthony, 1975a).

The role of malate synthase in the growth of Pseudomonas AM1 on 3-hydroxybutyrate, ethanol and malonate

In an attempt to confirm the proposed role for malate synthase during growth of *Pseudomonas* AM1 on 3-hydroxybutyrate, ethanol and malonate, mutant ICT51 which lacked malate synthase activity (Table 2) was isolated by the same procedure as that described for mutant ICT54. Mutant ICT51 grew on the same substrates as wild-type bacteria (including C_1 compounds, pyruvate, lactate and succinate) except for ethanol, malonate or 3-hydroxybutyrate, thus demonstrating a requirement for malate synthase activity during growth of *Pseudomonas* AM1 on these three substrates. The virtual absence of the enzyme from mutant ICT51 grown on methanol confirms that malate synthase activity is not required for methylotrophic growth despite its presence in wild-type *Pseudomonas* AM1 on all growth substrates (Table 2). A revertant of mutant ICT51 was isolated and was able to grow on ethanol, malonate, 3-hydroxybutyrate and acetate plus glyoxylate. Unexpectedly, extracts of the revertant grown on these compounds contained no detectable malate synthase activity.

DISCUSSION

Our results show that ethanol, malonate and 3-hydroxybutyrate are assimilated in *Pseudomonas* AM1 by the routes shown in Fig. 1. The pathway for oxidation of 3-hydroxy-

butyrate to acetyl-CoA is essentially similar to that found in other bacteria (Senior & Dawes, 1973; Oeding & Schlegel, 1973). The oxidation of ethanol to acetate is catalysed by the same enzymes that catalyse methanol oxidation to formate; and is followed by the acetyl-CoA synthetase reaction to give acetyl-CoA. This route contrasts with that found in another methylotroph growing on ethanol (*Hyphomicrobium* x) in which an NAD-linked dehydrogenase catalyses ethanol oxidation to acetaldehyde which may then be oxidized to acetyl-CoA by a CoA, NAD⁺-dependent acetaldehyde dehydrogenase or by the sequential action of an NAD⁺-linked acetaldehyde dehydrogenase and acetyl-CoA synthetase (Attwood & Harder, 1974).

The oxidation of pyruvate to acetyl-CoA by *Pseudomonas* AM1 is catalysed by a typical pyruvate dehydrogenase, and the necessity for complete oxidation of acetyl-CoA by enzymes of the TCA cycle during growth on multicarbon compounds has been demonstrated previously (Taylor & Anthony, 1976).

The most difficult results to interpret are those involving measurements of 'malate synthase' activity. The preceding paper (Cox & Quayle, 1976) shows that this activity in *Pseudomonas* AM1 is due to the combined action of malyl-CoA lyase (catalysing condensation of acetyl-CoA and glyoxylate) and malyl-CoA hydrolase, and that mutant ICT51 has an altered hydrolase activity. Our results with this mutant suggest that 'malate synthase' activity is usually essential for growth of *Pseudomonas* AM1 on ethanol, malonate and 3-hydroxybutyrate, but not for growth on pyruvate and lactate. Pyruvate is assimilated by decarboxylation to acetyl-CoA but not by carboxylation to C₄ compounds, and it has been suggested that the 'malate synthase' pathway operates for synthesis of C₄ compounds during growth on pyruvate and lactate (Salem *et al.*, 1973*b*). The results with revertant ICT51R, which has the growth properties of wild-type bacteria but has not regained wild-type malyl-CoA hydrolase activity (Cox & Quayle, 1976) and hence 'malate synthase' activity, suggest that there is an alternative route for the assimilation of acetyl-CoA in revertant ICT51R and this might also operate during growth of *Pseudomonas* AM1 on pyruvate and lactate (see Taylor, 1975). The alternative route might also operate during growth on ethanol, malonate and 3-hydroxybutyrate of mutant PCT57, which grows on all substrates except C₁ compounds but lacks malyl-CoA lyase (Salem, Hacking & Quayle, 1974) and hence 'malate synthase' activity (Cox & Quayle, 1976). The nature of the alternative route(s), and the regulatory factors determining whether or not it operates during growth of mutants and of wild-type *Pseudomonas* AM1, are not known.

We thank Professors K. A. Munday and M. Akhtar for their encouragement in this work and the Science Research Council for a grant to C. Anthony.

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