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THE MICROBIAL METABOLISM OF C_1- AND C_2-COMPOUNDS

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for the degree of Doctor of Philosophy

Iain J. Taylor

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CONTENTS

Page

CHAPTER 1

A review

1.1	Introduction	1
	PART A The oxidation and assimilation of C_1-compounds	1
1.2	Bacteria capable of growth on C_1-compounds	1
	a) Obligate methylotrophs	3
	b) Facultative methylotrophs	4
	c) Restricted facultative methylotrophs	5
1.3	The oxidation of C_1-compounds	10
	a) Methane oxidation	11
	b) Methanol oxidation	12
	c) Formaldehyde and formate oxidation	15
	d) Oxidation of N-methyl compounds	16
	e) The electron transport chain of methylotrophs	20
1.4	The assimilation of C_1-compounds	21
	a) The ribulose monophosphate cycle	21
	b) The serine pathway	25
1.5	The assimilation of acetyl-CoA produced by the serine pathway	27
	a) Bacteria which form isocitrate lyase	27
	b) Oxidation of acetyl-CoA to glyoxylate in *Pseudomonas* AM1	29
1.6	Distribution of the carbon assimilation pathways	31
	PART B The metabolism of poly-β-hydroxybutyrate	34
1.7	Nature and occurrence of poly-β-hydroxybutyrate	34

Page

1.8 The enzymes of poly-β-hydroxybutyrate biosynthesis 35

1.9 The enzymes of poly-β-hydroxybutyrate degradation 36

a) Extracellular PHB degradation 36

b) Intracellular PHB degradation 38

1.10 Regulation of poly-β-hydroxybutyrate metabolism 40

PART C Regulation and functions of the TCA cycle in bacteria 44

1.11 Functions of the TCA cycle 44

1.12 Bacteria lacking a complete TCA cycle 46

a) Gram-negative facultative anaerobes 46

b) Gram-positive facultative anaerobes 48

c) Autotrophic bacteria 48

d) Methylotrophic bacteria 49

1.13 Regulation of citrate synthase activity 51

a) Bacteria which have a complete TCA cycle 51

b) Bacteria which lack a complete TCA cycle 51

1.14 The molecular weights of citrate synthase from different bacteria 52

CHAPTER 2

Materials and methods

2.1 Materials 55

a) Chemicals 55

b) Preparation of CoA derivatives 57

c) Bacterial strains 57

		Page
2.2	Purification of U-^{14}C-acetate	57
2.3	Media and maintenance of cultures	58
2.4	Growth and harvesting of cultures	59
2.5	Estimation of the dry weight of cells in suspension	59
2.6	Isolation of mutants of _Pseudomonas_ AM1	61
2.7	Characterisation of the growth response of mutant strains	62
2.8	Isolation of revertants	63
2.9	Measurement of oxygen uptake by bacterial suspensions	64
2.10	Preparation of sonic extracts	64
2.11	Protein assay	65
2.12	Enzyme and cytochrome assays	65
	a) Enzymes of β-hydroxybutyrate metabolism	66
	b) Enzymes of the TCA cycle	66
	c) Enzymes of C_1 metabolism	67
	d) Enzymes of C_2 metabolism	67
	e) Enzymes of the glyoxylate bypass	68
	f) Cytochrome assays	68
2.13	Molecular weight and kinetic studies of citrate synthase	68
2.14	Partial purification of acetyl-CoA synthetase from _Pseudomonas_ AM1	70
2.15	Assay of ADP production and adenylate kinase	73
2.16	Amino acid analysis and poly-β-hydroxybutyrate estimation	73

Page

2.17 Bacterial incorporation of ^{14}C-acetate 74

2.18 Chromatographic analysis 74

2.19 Detection of radioactive compounds 75

2.20 Co-chromatography of radioactive compounds 76

2.21 Incorporation of radioactivity from ^{14}C-acetate into growing cultures of bacteria 76

CHAPTER 3

The formation of acetyl-CoA during growth of Pseudomonas AM1 on β-hydroxybutyrate, ethanol and C_3-compounds

3.1 Introduction 78

3.2 The enzymology of β-hydroxybutyrate oxidation to acetyl-CoA 78

a) β-hydroxybutyrate dehydrogenase 79

b) Acetoacetate:succinate CoA transferase 79

c) β-ketothiolase 81

d) Acetoacetyl-CoA reductase 83

3.3 Factors affecting the poly-β-hydroxybutyrate content of *Pseudomonas* AM1 85

3.4 The enzymology of ethanol metabolism to acetyl-CoA 88

a) Alcohol (methanol) dehydrogenase 88

b) Oxidation of acetaldehyde 88

c) Activation of acetate to acetyl-CoA 90

3.5 The significance of acetyl-CoA synthetase induction during growth on β-hydroxybutyrate 92

3.6 Identification of the products of acetyl-CoA synthetase 93

Page

a) Measurement of ADP production 93

b) Separation of adenylate kinase and acetyl-CoA synthetase 96

c) Identification of acetyl-CoA 98

3.7 Characterisation of mutant ICT 54 99

3.8 The metabolism of malonate to acetyl-CoA 102

3.9 Oxidation of lactate and pyruvate to acetyl-CoA 103

3.10 Acetyl-CoA synthetase activities in extracts of *Pseudomonas* AM1 103

3.11 Summary 105

CHAPTER 4

The assimilation of acetyl-CoA during growth of Pseudomonas AM1 on C_1-compounds, β-hydroxybutyrate, ethanol and C_3-compounds

4.1 Introduction 106

4.2 The malate synthase pathways 106

4.3 Characterisation of mutant ICT 5 110

4.4 Characterisation of mutant ICT 51 114

4.5 Properties of mutant ICT 51R 116

4.6 Properties of mutant PCT 57 119

4.7 The involvement of glycollate during acetyl-CoA assimilation by the malate synthase pathway 120

4.8 Metabolism of U-^{14}C-acetate by β-hydroxybutyrate-grown wild-type *Pseudomonas* AM1 and mutant 20 BL 123

4.9 Metabolism of U-^{14}C-acetate by β-hydroxybutyrate-grown ICT 54 126

Page

4.10 Incorporation of ^{14}C-acetate into cultures of *Pseudomonas* AM1 and mutant ICT 54 growing on β-hydroxybutyrate 127

4.11 Summary and discussion 129

CHAPTER 5

Properties of a mutant of Pseudomonas AM1 lacking 2-oxoglutarate dehydrogenase: a biochemical basis for obligate methylotrophy

5.1 Introduction 133

5.2 Growth and oxidative properties of mutant ICT 41 134

5.3 Activities of TCA cycle enzymes in mutant ICT 41 138

5.4 Properties of revertants of mutant ICT 41 143

5.5 Amino acid accumulation by mutant ICT 41 143

5.6 Summary and discussion 149

CHAPTER 6

Regulation of the tricarboxylic acid cycle and malate synthase of Pseudomonas AM1

6.1 Introduction 153

6.2 Activities of TCA cycle enzymes during growth of *Pseudomonas* AM1 on various substrates 153

6.3 Regulation of citrate synthase activity 155

6.4 Regulation of isocitrate dehydrogenase activity 158

6.5 Regulation of malate synthase activity 160

6.6 Summary and discussion 166

Page

CHAPTER 7

Unsolved problems and areas for further investigation

7.1 Introduction 172

7.2 Unsolved problems of metabolism of C_1-compounds by *Pseudomonas* AM1 172

7.3 Areas for further investigation 174

7.4 Mutants unable to oxidise methanol 176

Summary of mutant properties 178

REFERENCES 180

ABSTRACT

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THE MICROBIAL METABOLISM OF C_1- AND C_2-COMPOUNDS

by IAIN JOHN TAYLOR

In the facultative methylotroph, Pseudomonas AM1, the malate synthase pathway has been proposed as a route for the assimilation of growth substrates metabolised to acetate or acetyl-CoA; these include β-hydroxybutyrate, ethanol, malonate, lactate and pyruvate. In this study, the enzymes involved in the metabolism of these compounds to acetyl-CoA have been identified and the role of malate synthase during acetyl-CoA assimilation has been examined by the isolation of mutants lacking the enzyme. The growth properties of one mutant (ICT 51) indicated that malate synthase activity is required during assimilation of β-hydroxybutyrate, ethanol and malonate but not for growth on C_1-compounds, lactate or pyruvate. However, results with two further mutants lacking malate synthase activity (ICT 51R, PCT 57) suggested that an alternative to the malate synthase pathway may operate under certain conditions.

Another mutant of Pseudomonas AM1 lacking acetyl-CoA synthetase (ICT 54) grew on all substrates tested except ethanol and malonate indicating that free acetate is the precursor of acetyl-CoA during the assimilation of these substrates. These results also demonstrated that acetyl-CoA synthetase is not required for growth of Pseudomonas AM1 on C_1-compounds, lactate, pyruvate or β-hydroxybutyrate despite the observation that this enzyme is induced during growth on the latter compound.

Radioactive labelling experiments with ^{14}C-acetate and whole cells of mutant ICT 54 and mutant 20 BL (lacking hydroxypyruvate reductase) showed that acetyl-CoA (not acetate) is the precursor for oxidation to glyoxylate and that the intermediate formation of glycollate is probably not involved.

Pseudomonas AM1 was shown to possess a complete tricarboxylic acid cycle during growth on all substrates. Some enzymes (isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase and malate dehydrogenase) had lower specific activities during growth on C_1-compounds although a more important site of regulation of TCA cycle activity was at the level of enzyme inhibition. The NADP-specific isocitrate dehydrogenase was subject to concerted inhibition by a combination of glyoxylate and oxaloacetate and the citrate synthase (M.Wt.250,000) was inhibited by NADH. No inhibition of citrate synthase was found with 2-oxoglutarate.

Results with a mutant (ICT 41) lacking 2-oxoglutarate dehydrogenase provided evidence that the lack of this enzyme may be a sufficient biochemical basis for the obligate methylotrophy of some bacteria and demonstrated the importance of a complete TCA cycle for growth of *Pseudomonas* AM1 on non-C_1-compounds. This mutant grew only on C_1-compounds and oxidised pyruvate, lactate, β-hydroxybutyrate, acetoacetate, acetate and 2-oxoglutarate at very low rates. The absence of 2-oxoglutarate dehydrogenase activity from mutant ICT 41 was probably due to an inactive E_2 component (dihydrolipoamide transsuccinylase).

CHAPTER 1

1.1 Introduction

This Chapter consists of three main Parts; the first is a brief review of the literature concerning the oxidation and assimilation of C_1-compounds (fully reviewed recently by Quayle, 1972 and Anthony, 1975a). This Part specifically deals with the metabolism of reduced C_1-compounds and thus excludes autotrophs which are capable of utilising CO_2 as a sole source of carbon (reviewed by Kelly, 1971). The second Part describes the metabolism and functions of the endogenous energy reserve poly-β-hydroxybutyrate found in many bacteria including those capable of growth on C_1-compounds. The final Part of this Chapter is a review of the functions and regulation of the tricarboxylic acid cycle with particular reference to those bacteria which lack a complete oxidative cycle.

PART A

The oxidation and assimilation of C_1-compounds

1.2 Bacteria capable of growth on C_1-compounds

These bacteria are known as methylotrophs and are able to grow non-autotrophically at the expense of compounds containing one or more carbon atoms but containing no carbon - carbon bonds (Colby and Zatman, 1972). Throughout this thesis the term 'C_1-compound' is used not only for compounds containing one carbon atom, but also for compounds containing more than one carbon atom providing they contain no C - C bonds (Table 1.1).

Until recently, the only known methylotrophs were

Table 1.1 Substrates used for methylotrophic growth

Compounds containing one carbon atom		Compounds containing more than one carbon atom	
methane	CH_4	dimethyl ether	$(CH_3)_2O$
methanol	CH_3OH	dimethylamine	$(CH_3)_2NH$
methylamine	CH_3NH_2	trimethylamine	$(CH_3)_3N$
formaldehyde	HCHO	tetramethylammonium	$(CH_3)_4N^+$
formate	HCOOH	trimethylamine N-oxide	$(CH_3)_3NO$
formamide	$HCONH_2$	trimethylsulphonium compounds	$(CH_3)_3S^+$

bacteria but a number of methylotrophic yeasts have now been isolated. These include species of *Candida*, *Kloekera*, *Torulopsis*, *Pichia* and *Hansensula* some of which have been discussed in a review by Cooney and Levine (1972) which concentrates on the use of methanol as a suitable substrate for industrial fermentations by both yeasts and bacteria.

Methylotrophs have long been divided into two major groups: *obligate methylotrophs* which utilise C_1-compounds as their unique growth substrates upon which their growth is absolutely dependent, and *facultative methylotrophs* which have the added ability to grow on a variety of other organic compounds (Colby and Zatman, 1972). A third group of methylotrophs has recently become recognised and referred to as '*restricted facultative*' *methylotrophs*. This group of methylotrophs constitutes a group of bacteria with a very restricted range of growth substrates which differ from obligate methylotrophs in their capacity to grow on a few multicarbon (or non-C_1) compounds (Colby and Zatman, 1975a).

a) *Obligate methylotrophs*

Until 1966, all known obligate methylotrophs could grow on methane and methanol, but not on any other carbon source (except dimethyl ether). Examples of these obligate methane-utilisers included *Pseudomonas methanica* (Leadbetter and Foster, 1958), *Methylococcus capsulatus* (Foster and Davis, 1966) and *Methanomonas methanooxidans* (Stocks and McCleskey, 1964). In 1970, Whittenbury, Phillips and Wilkinson isolated over 100 different strains of obligate

methane-utilisers and divided them into five Genera on the basis of morphology, type of resting stage formed and membrane structure (Table 1.2). This method of classification was later extended by Lawrence and Quayle (1970) to include *Pseudomonas methanica* (*Methylomonas* group), *Methanomonas methanooxidans* (*Methylosinus* group) and *Methylococcus capsulatus* (*Methylococcus* group). All the groups were shown to possess complex internal membrane structures of two different types. Type I bacteria (*Methylomonas*, *Methylococcus* and *Methylobacter*) have bundles of disc-shaped vesicles while Type II bacteria (*Methylosinus* and *Methylocystis*) have a series of peripheral membranes surrounding the cytoplasm.

More recently obligate methylotrophs have been isolated which cannot utilise methane as a carbon source. Such bacteria include Bacterium C2A1, Bacterium 4B6 (Colby and Zatman, 1973), Organism W1 (Dahl, Mehta and Hoare, 1972) and *Methylomonas* M-15 (Sahm and Wagner, 1975) (Table 1.2). These organisms differ morphologically from the methane-utilisers in that they do not possess internal membrane ultrastructures.

No obligate methylotroph so far described is capable of growth on formate, and none grow anaerobically with nitrate as terminal oxidant instead of oxygen.

b) Facultative methylotrophs

Table 1.3 gives a list of bacteria capable of both methylotrophic growth and typical heterotrophic growth on compounds such as carbohydrates, ethanol, lactate and

malate. All the facultative methylotrophs are Gram-negative and, with the exception of *Hyphomicrobium*, are able to grow on the following compounds as well as the C_1-compounds shown: ethanol or acetate (or both), pyruvate and lactate, succinate and other dicarboxylic acids and at least one carbohydrate. The pink pseudomonads are the bacteria most frequently encountered and these are usually capable of growth on oxalate. *Hyphomicrobium* X, a stalked, budding bacterium is included in Table 1.3 although this is an atypical facultative methylotroph. It can only grow non-methylotrophically at the expense of ethanol, acetate and β-hydroxybutyrate and growth on the latter compound is extremely slow with a doubling time of 35 hours (Attwood and Harder, 1974). These growth properties suggest that *Hyphomicrobium* X may be classified with the restricted facultative methylotrophs discussed below. Another unusual feature of this organism is its ability to grow anaerobically with nitrate as a terminal oxidant (Attwood and Harder, 1972).

No facultative methylotroph is capable of growth on methane and the well-defined internal membrane structures found in methane-oxidisers have not been observed in any facultative methylotroph so far described.

c) Restricted facultative methylotrophs

Recently Zatman and co-workers have described the isolation of several strains which, like *Hyphomicrobium* X, were only capable of non-methylotrophic growth on a very restricted range of compounds (Colby and Zatman, 1975a).

Table 1.2 Substrates supporting the growth of obligate methylotrophs (from Anthony, 1975a)

Organism	Substrates supporting growth	Reference
*Methane-utilisers (Type I):	methane, methanol	Quayle, 1972
Methylomonas		
(*Pseudomonas methanica*)		
Methylobacter		
Methylococcus		
*Methane-utilisers (Type II):	methane, methanol	Quayle, 1972
Methylocystis		
Methylosinus		
(*Methanomonas methano-oxidans*)		
Obligate methylotrophs unable to use methane :		
Bacterium 4B6	methylamine (not methanol or formate	Colby and Zatman, 1973
Bacterium C2A1	methylamine, methanol (not formate)	Colby and Zatman, 1973
Organism W1	methylamine, methanol (not formate)	Dahl *et al.*, 1972
Methylomonas M-15	methanol (not methylamine or formate)	Sahm and Wagner, 1975

*The generic names given are those suggested by Whittenbury *et al.*, (1970); the names in parentheses refer to previously described methane utilisers now included in these genera.

In addition to those compounds listed dimethyl ether supports growth of all the methane-utilisers and dimethylamine and trimethylamine supports growth of Bacterium C2A1 and Bacterium 4B6. All the bacteria in this table use the RMP pathway of formaldehyde fixation except the Type II methane-utilisers which use the serine pathway.

Table 1.3 Substrates supporting growth of facultative methylotrophs (from Anthony 1975a)

Organism	Compounds with one carbon atom	Compounds with more than one carbon atom	References
Hyphomicrobium sp.	methylamine, methanol, formate	tmn (not dmn)	Harder *et al.*, 1973
Pink pseudomonads*:			
Pseudomonas AM1	methylamine, methanol, formate	dmn, tmn	Peel and Quayle, 1961
Pseudomonas M27	methylamine, methanol, formate	(not dmn)	Anthony and Zatman, 1964
Pseudomonas 3A2	methylamine, methanol, formate	dmn, tmn, tmo	Colby and Zatman, 1973
Non-pigmented pseudomonads:			
Pseudomonas aminovorans	methylamine, formate (not methanol)	dmn, tmn, tmo	Eady *et al.*, 1971
Pseudomonas MS	methylamine (not methanol or formate	dmn, tmn, tms	Kung and Wagner, 1970
Pseudomonas MA	methylamine (not methanol or formate)	(not dmn, tmn)	Shaw, Tsai and Stadtman, 1966
Pseudomonad C	methanol, formate (not methylamine)	-	Steiglitz and Mateles, 1973
Non-pigmented, Gram-negative non-motile bacteria:			
Bacterium 5B1	methylamine, methanol, formate	dmn, tmn, tmo	Colby and Zatman, 1973
Bacterium 5H2	methylamine (not methanol	dmn, tmn, tem, tmo	Hampton and Zatman, 1973

Table 1.3 Substrates supporting growth of facultative methylotrophs (from Anthony 1975a)

Hyphomicrobium sp. grow on only those substrates listed and on ethanol, acetate and β-hydroxybutyrate. All the other facultative methylotrophs are able to grow on the following: acetate and/or ethanol; pyruvate and lactate; at least one carbohydrate; succinate and other dicarboxylic acids. The pink pseudomonads are also usually able to grow on oxalate. All the bacteria listed use the serine pathway of C_1 assimilation.

Abbreviations used: dmn, dimethylamine; tmn, trimethylamine; tem, tetramethylammonium compounds; tmo, trimethylamine-N-oxide; tms, trimethylsulphonium compounds.

*This group also includes *Vibrio extorquens*, *Pseudomonas extorquens*, *Protaminobacter ruber*, *Pseudomonas* PRL-W4.

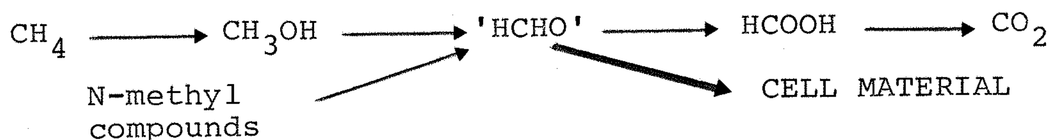
Table 1.4 Substrates supporting the growth of the restricted facultative methylotrophs (from Colby and Zatman, 1975a)

Substrate	Type M		Type L	
	W3A1	W6A	S2A1	PM6
C_1 compounds:				
tetramethylammonium	-	-	-	+
trimethylamine	+	+	+	+
trimethylamine N-oxide	-	-	+	+
dimethylamine	+	+	+	+
methylamine	+	+	+	+
methanol	+	+	-	-
Non-C_1 compounds:				
glucose	+	+	+	+
gluconate	-	-	+	+
citrate	-	-	+	+
glutamate	-	-	+	+
alanine	N.T.	N.T.	+	+
betaine	-	-	+	+
nutrient agar	-	-	+	+

Two of these isolates (W3A1 and W6A) grew only on glucose out of 56 non-C_1 compounds tested. Another two isolates (S2A1 and Bacillus PM6) grew only on betaine, D-glucose, gluconate, citrate, L-alanine and nutrient agar (Table 1.4). Such bacteria are therefore clearly distinguished from the typical facultative and obligate methylotrophs previously described. For convenience, the organisms with the more-restricted range of growth substrates (W3A1 and W6A) were designated type 'M' and the less-restricted organisms (PM6 and S2A1) designated type 'L'. The type 'L' organisms were Gram-positive while the type 'M' organisms Gram-negative. These restricted facultative methylotrophs could not utilise methane and did not possess internal membrane structures.

1.3 The oxidation of C_1-compounds

Much of the energy satisfaction of aerobic methylotrophs arises by way of the complete oxidation of the C_1 growth substrate to carbon dioxide and water. C_1 substrates are assimilated into cell material at the oxidation level of formaldehyde which can exist in the free state or bound as a tetrahydrofolate derivative. An outline of the oxidation of C_1-compounds is given below followed by a discussion of some of the individual reactions:



a) Methane oxidation

The difficulty in preparing active cell-free extracts has been a major obstacle in the study of methane oxidation. However Ferenci (1974) and Ribbons (1975) have recently demonstrated NADH and O_2-dependent methane oxidation by particulate fractions of cell-free extracts prepared from obligate methane-utilisers. With Methylococcus capsulatus (Type I membrane structure) Ribbons was able to demonstrate the simultaneous disappearance of methane and O_2 concomitant with the oxidation of NADH. The stoichiometry of the reaction however was not established as the presumed initial product of methane oxidation (methanol) was further oxidised to formate by the preparation which also contained high NADH-oxidase activity. Using similar particulate fractions of Pseudomonas methanica (Type I) and Methylosinus trichosporium (Type II) Ferenci showed that NADH oxidation and oxygen uptake formed a 1:1 ratio consistent with the involvement of a mono-oxygenase (methane hydroxylase) catalysed reaction. Carbon monoxide was also oxidised by this system and it was suggested that a common component of the mono-oxygenase system was involved in both methane and CO oxidation. That molecular oxygen is involved in methane oxidation was previously demonstrated by Higgins and Quayle (1970) who detected $CH_3{}^{18}OH$ after incubation of whole cells of P. methanica and Methanomonas methanooxidans (Methylosinus) with methane and $^{18}O_2$. By contrast, $CH_3{}^{18}OH$ was not found when these cells were incubated with methane and $H_2{}^{18}O$.

These results indicate the following mono-oxygenase

(methane hydroxylase) reaction for methane oxidation to methanol:

$$CH_4 + O_2 + NADH + H^+ \longrightarrow CH_3OH + NAD^+ + H_2O$$

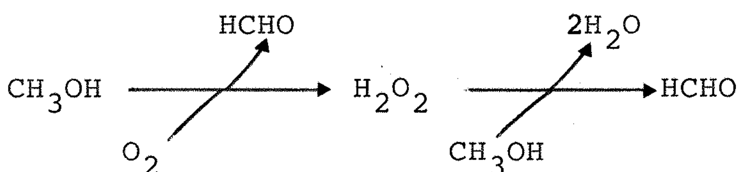
It is of interest to note that a similar mono-oxygenase appears to catalyse methane oxidation in methane-utilisers with different membrane systems and assimilation pathways. The demonstration of methane hydroxylase activity in the particulate fractions of both types of methane utiliser, and the observation that only those methylotrophs with complex internal membrane systems can oxidise methane suggests that these membranes play a specific role during methane oxidation or associated electron transport and ATP synthesis.

b) Methanol oxidation

Two enzymes are known to be responsible for methanol oxidation to formaldehyde in micro-organisms, one exclusively in yeast and the other in bacteria.

In the methylotrophic yeasts *Kloekera* sp. No 2201 (Tani, Miya and Ogata, 1972) and *Candida boidinii* (Sahm and Wagner, 1973) methanol is oxidised by an inducible FAD-dependent alcohol oxidase which has a broad substrate specificity for short chain primary alcohols. The purified enzyme from *C. boidinii* had a molecular weight of about 600,000 and consisted of eight subunits each of molecular weight 74,000. The stoichiometry of methanol oxidation by the purified enzyme was as follows:

An inducible catalase was also present in extracts of methanol-grown C. boidinii and the overall mechanism for methanol oxidation by this organism was therefore represented as follows (Roggenkamp, Sahm and Wagner, 1974):



Electron micrographs show that methanol-grown C. boidinii contain microbodies with crystalline inclusions not found during growth of the yeast on other substrates. The crystalline inclusions were also absent from a mutant lacking methanol oxidase activity even when incubated in medium containing methanol (Sahm, Roggenkamp, Wagner and Hinklemann, 1975). That the enzyme is associated with these microbodies was further demonstrated by the finding of almost all the methanol oxidase activity in the particulate fraction of cell-free extracts.

Methanol is oxidised in bacteria by an enzyme first described by Anthony and Zatman (1964) from Pseudomonas M 27. The methanol dehydrogenase is independent of NAD^+ or $NADP^+$ and extracts are absolutely dependent for activity on the artificial hydrogen acceptor phenazine methosulphate (PMS) and also on ammonia or methylamine as activator

Table 1.5 A comparison of the methanol-oxidising enzymes of bacteria and yeasts

	Bacteria	Yeast
trivial name	methanol dehydrogenase	methanol oxidase
molecular weight	120,000 - 146,000	600,000
prosthetic group	pteridine	FAD
activators	NH_3, CH_2NH_2	-
inhibitors	-	p-CMB, KCN
specificty	primary alcohols (C_1-C_{11});formaldehyde	short-chain primary alcohols (C_1-C_5)
Km for methanol	0.02mM	1-2mM
references	Anthony and Zatman, 1965, 1967	Sahm and Wagner, 1973

Abbreviations: FAD, flavin adenine dinucleotide; p CMB, p-Chloromercuribenzoate; KCN, potassium cyanide

(Anthony and Zatman, 1965). This enzyme is also responsible for ethanol oxidation to acetaldehyde during growth of several facultative methylotrophs on this compound. The molecular weight is in the region of 120,000 and the prosthetic group appears to be a pteridine. The uniformity among bacteria with respect to methanol oxidation has been demonstrated with the finding that the purified enzyme from the obligate methylotroph *M. capsulatus* is almost identical with the methanol dehydrogenase from facultative methylotrophs (e.g. *Pseudomonas* M27) (Patel, Bose, Mandy and Hoare, 1972). The physiological importance of the enzyme has also been demonstrated with the isolation of mutants which lack methanol dehydrogenase and can neither oxidise nor grow on methanol (Dunstan, Anthony and Drabble, 1972a).

The properties of these two methanol oxidising enzymes are summarised in Table 1.5.

c) Formaldehyde and formate oxidation

Formaldehyde, either in the free state or bound, is the oxidation product of methanol and N-methyl compounds, and it is at this level of oxidation that C_1-compounds are assimilated into cell-material. There is considerable variation in the enzymes capable of formaldehyde oxidation by methylotrophs:

(i) NAD^+-linked aldehyde dehydrogenase (Kung and Wagner, 1970).

(ii) Glutathione dependent, NAD^+-linked formaldehyde dehydrogenase (Johnson and Quayle, 1964).

(iii) NAD(P)-independent aldehyde dehydrogenase (Johnson and Quayle, 1964).

(iv) An NADP-linked methylenetetrahydrofolate dehydrogenase has been described which is present at a high level in Pseudomonas AM1 and oxidises 'bound formaldehyde' to the oxidation level of formate (methenyltetrohydrofolate).

(v) In methylotrophs capable of growth on methanol or methane, formaldehyde can also be oxidised by the PMS-dependent methanol dehydrogenase described above, suggesting that, in certain bacteria, methanol can be oxidised to formate by two consecutive steps catalysed by the same enzyme. An example of this is Methylococcus capsulatus in which no other formaldehyde oxidising enzyme was detected (Patel and Hoare, 1971).

In all methylotrophs so far studied, formate is oxidised to carbon dioxide by an NAD-linked formate dehydrogenase (Johnson and Quayle, 1964).

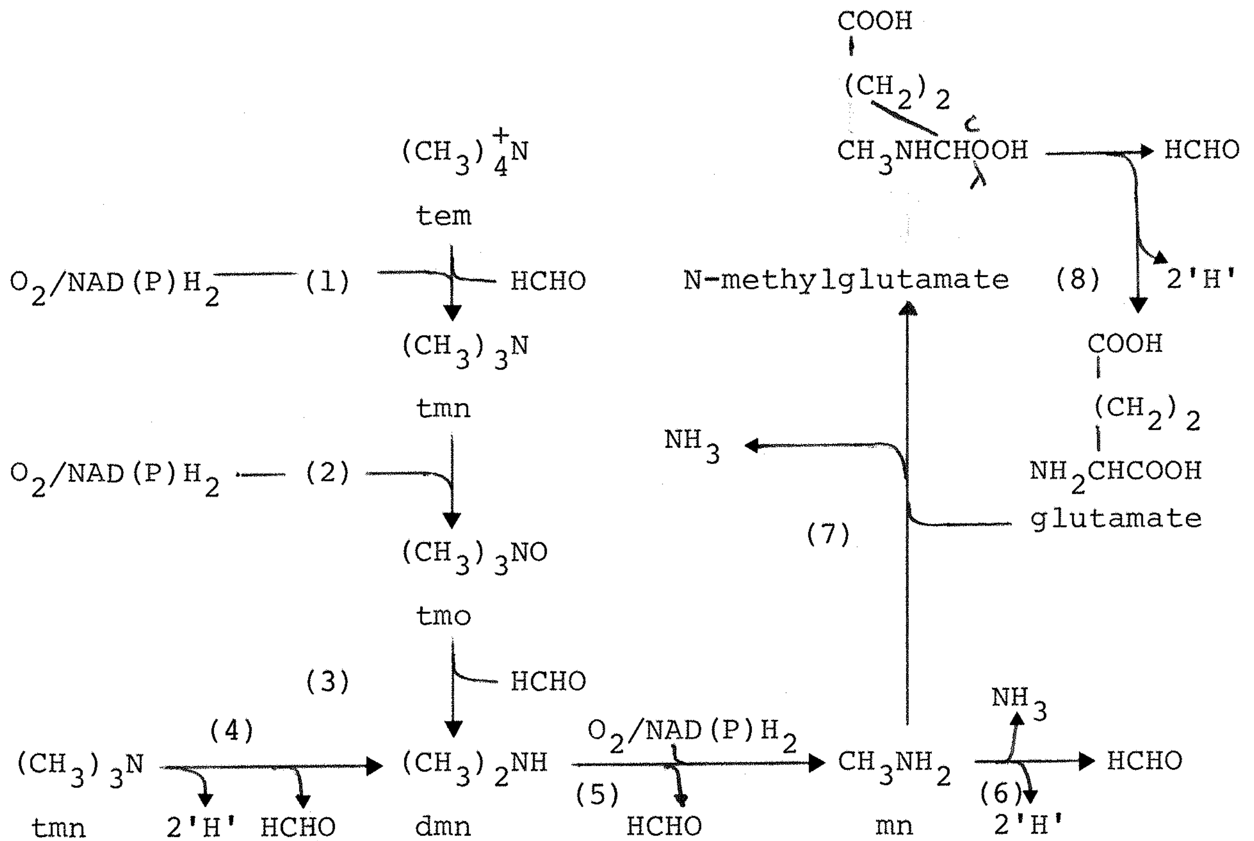
d) Oxidation of N-methyl compounds

The oxidation of these compounds has recently been reviewed by Anthony (1975a) and only the major points will be discussed here. A summary of the oxidative pathways is shown in Fig. 1.1.

(i) Tetramethylammonium oxidation

A mono-oxygenase from the facultative methylotroph Bacterium 5H2 (Hampton and Zatman, 1973) has been described

Fig. 1.1 The oxidation of N-methyl compounds (from Anthony, 1975a)



Numbers in parentheses refer to the following enzymes described in the text:

(1) tetramethylammonium (tem) mono-oxygenase

(2) trimethylamine (tmn) mono-oxygenase

(3) trimethylamine N-oxide (tmo) demethylase

(4) trimethylamine dehydrogenase

(5) dimethylamine (dmn) mono-oxygenase

(6) methylamine (mn) dehydrogenase

(7) N-methylglutamate synthase

(8) N-methylglutamate dehydrogenase

which catalyses the incorporation of molecular oxygen into one of the methyl groups. The enzyme was induced during growth on tetramethylammonium and the oxidation products were trimethylamine and formaldehyde (Reaction 1).

(ii) Trimethylamine oxidation

In the obligate methylotrophs Bacterium 4B6 and Bacterium C2A1, dimethylamine and formaldehyde are produced by anaerobic oxidative demethylation of trimethylamine catalysed by trimethylamine dehydrogenase (Reaction 4). Artificial electron acceptors must be used for assay of this enzyme and the natural electron acceptor is unknown (Colby and Zatman, 1973; 1974). By contrast, facultative methylotrophs which grow on N-methyl compounds use two enzymes to effect the same overall reaction. The product of the first enzyme, trimethylamine mono-oxygenase, (Reaction 2) is trimethylamine N-oxide which is subsequently demethylated by the second enzyme to yield formaldehyde and dimethylamine (Reaction 3). Examples of bacteria which oxidise trimethylamine via these two enzymes include Bacterium 5H2, *Pseudomonas* 3A2 (Cox and Zatman, 1973), *Hyphomicrobium vulgarae* NQ and *Pseudomonas aminovorans* (Large, Boulton and Crabbe, 1972). In addition to its role in oxidising trimethylamine, the trimethylamine mono-oxygenase from the latter organism also oxidises dimethylamine by a series of reactions involving the intermediacy of NN-dimethylhydroxylamine and N-methylhydroxylamine although the oxidation of these compounds is probably not physiologically significant (Boulton and Large, 1975).

It can be seen from Fig. 1.1 that the oxidation of trimethylamine by obligate methylotrophs produces one molecule of reduced co-factor whereas the involvement of a monooxygenase during the oxidation of this compound by facultative methylotrophs requires a molecule of reduced co-factor (NADH or NADPH).

(iii) Dimethylamine oxidation

Dimethylamine is oxidised by a mixed-function secondary amine oxidase reported in both facultative and obligate methylotrophs (Reaction 5). The enzyme was first demonstrated in *Pseudomonas aminovorans* and shown to contain a carbon monoxide-sensitive haemoprotein of the cytochrome P_{420} type (Eady, Jarman and Large, 1971; Brook and Large, 1975).

(iv) Methylamine oxidation

Two routes for methylamine oxidation have been described. The first involves the direct oxidative deamination of methylamine to formaldehyde (Reaction 6) by an inducible methylamine dehydrogenase first demonstrated in *Pseudomonas* AM1 (Eady and Large, 1968). The enzyme, which requires PMS as the electron acceptor, has been found in both facultative and obligate methylotrophs.

The second route for methylamine oxidation consists of two enzymes; a soluble N-methylglutamate synthase (Reaction 7) and a particulate N-methylglutamate dehydrogenase (Reaction 8). Both are inducible and act in concert to effect the oxidation of methylamine to formaldehyde, ammonia and water. This system is responsible for methyl-

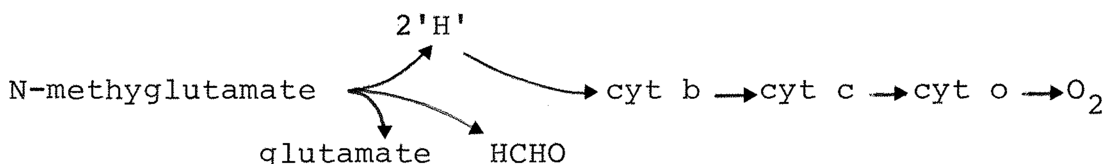
amine oxidation in *Pseudomonas* MA (Bellion and Hersh, 1972), *Pseudomonas* MS, *Pseudomonas aminovorans* (Large and Carter, 1973) and possibly in the obligate methylotroph Bacterium 4B6 (Colby and Zatman, 1973).

e) *The electron transport chain of methylotrophs*

Little is known at the moment of the energy metabolism and respiratory pathways involved during oxidation of C_1-compounds.

It has been reported that cytochromes of the a, b and c types are present in the facultative methylotrophs *Pseudomonas* MA (Hersh, Peterson and Thompson, 1971), *Hyphomicrobium* X and *Pseudomonas* AM1 (Anthony, 1975b; Widdowson and Anthony, 1976) and also in the obligate methylotrophs *Pseudomonas methanica* (Type I) and *Methylosinus trichosporium* (Type II) (Tonge, Knowles, Harrison and Higgins, 1974).

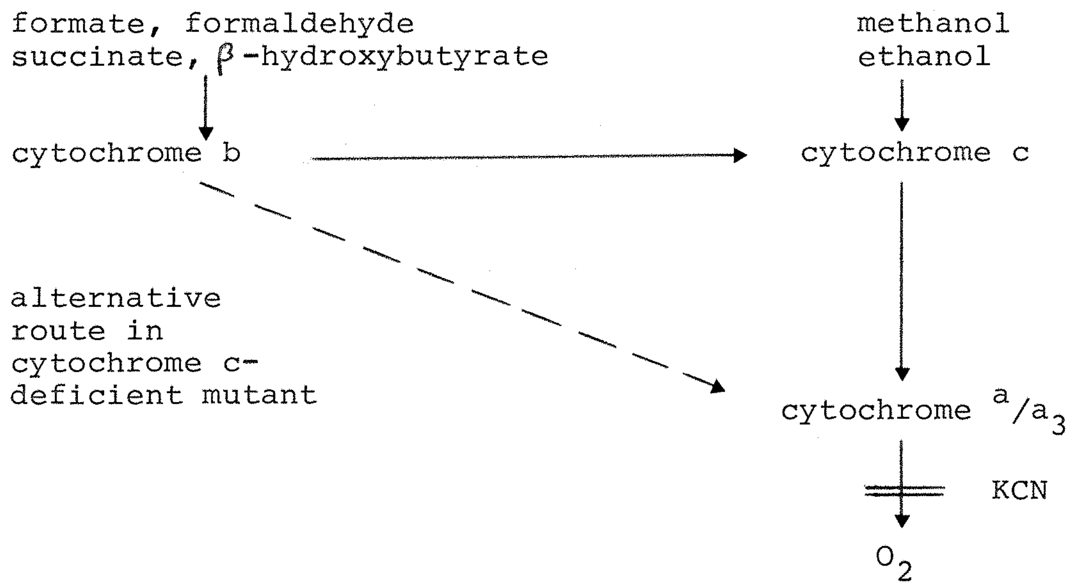
In *Pseudomonas* MA cytochromes b and c are reduced by N-methylglutamate in the presence of the membrane-bound N-methylglutamate dehydrogenase. Cytochrome o is the only CO-binding pigment in this organism and presumably acts as the terminal oxidase catalysing the reoxidation of cytochrome c (Hersh *et al.*, 1971):



In *Pseudomonas* AM1, *Hyphomicrobium* X and *Pseudomonas* MS

the $^a/a_3$ cytochromes and b-type cytochromes are able to react with carbon monoxide. In addition to this Pseudomonas AM1 and Hyphomicrobium X have CO-binding c-type cytochromes. However, these CO-binding c and b-type cytochromes do not appear to have oxygenase or oxidase functions and their ability to bind CO is probably irrelevant to the normal physiology of the organisms (Widdowson and Anthony, 1976).

The electron transport chain of Pseudomonas AM1 is thought to operate as follows (from Widdowson and Anthony, 1976)



1.4 The assimilation of C_1-compounds

Excluding the ribulose diphosphate cycle of autotrophic CO_2 fixation, two pathways are known to effect the biosynthesis of C_3- and C_4-intermediates from C_1-units (formaldehyde).

a) The ribulose monophosphate cycle

Evidence for the pathway shown in Fig. 1.2 was originally

Fig. 1.2 The ribulose monophosphate cycle of formaldehyde fixation (from Strøm et al., 1974)

Alternative routes for the cleavage of fructose 6-Phosphate are shown with dotted lines.

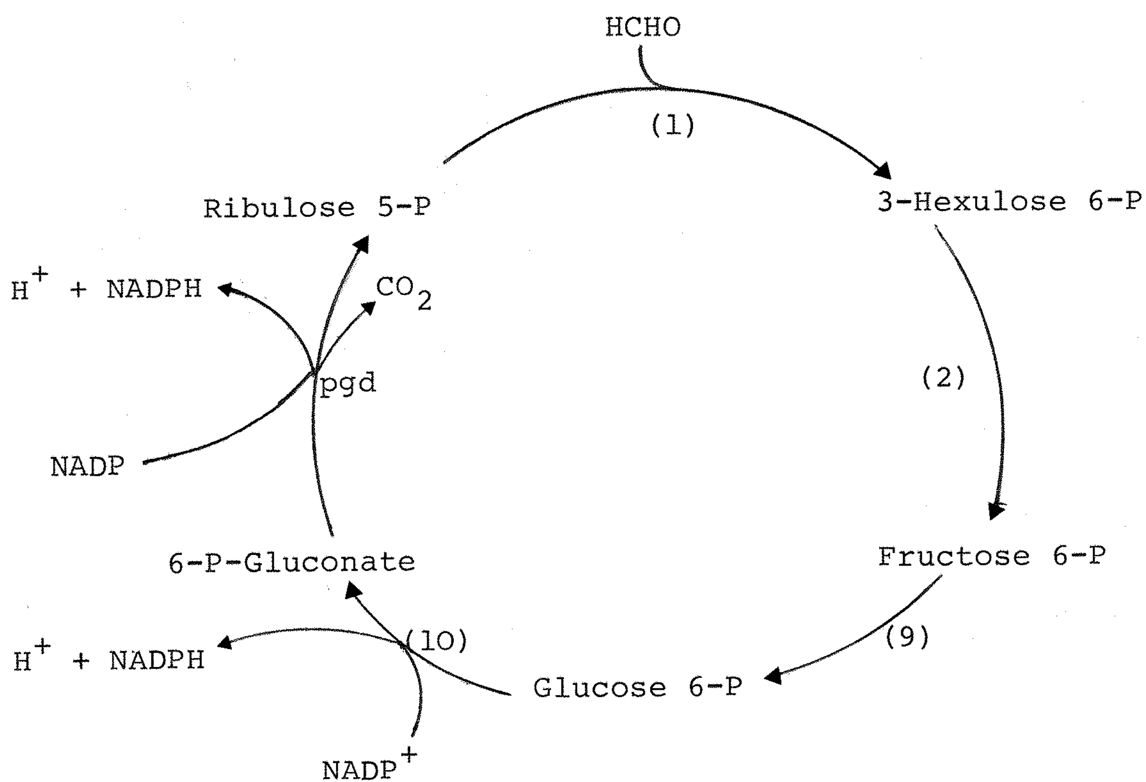
Key to the enzymes involved:

(1) hexulose phosphate synthase
(2) phospho-3-hexulose isomerase
(3) phosphofructokinase
(4) fructose diphosphate aldolase
(5) transketolase
(6) transaldolase
(7) ribulose-5-phosphate 3-epimerase
(8) phosphoriboisomerase
(9) phosphoglucoismerase
(10) glucose-6-phosphate dehydrogenase
(11) 6-phosphogluconate dehydratase
(12) phospho-2-keto-3-deoxygluconate (PKGD) aldolase

obtained by Johnson and Quayle (1965) using the obligate methane utiliser Pseudomonas methanica (Type I). The key enzyme is hexulose phosphate synthase which catalyses the initial incorporation of formaldehyde (Reaction 1). The enzyme is specific for D-ribulose 5-phosphate, the formaldehyde acceptor molecule, and the product of the condensation reaction is a hexulose phosphate, D-erythro-L-glycero-3-hexulose-6-phosphate (Ferenci, Strøm and Quayle, 1974; Kemp, 1974). The second enzyme, phospho-3-hexuloisomerase, catalyses the isomerisation of the hexulose phosphate to fructose-6-phosphate (Reaction 2). The remaining reactions of the cycle serve to regenerate the ribulose 5-phosphate acceptor and to provide either dihydroxyacetone phosphate or pyruvate as a substrate for biosynthesis of cell material. Two alternative routes for the cleavage of fructose-6-phosphate have been suggested (Strøm, Ferenci and Quayle, 1974; Colby and Zatman, 1975b). One route involves phosphofructokinase and fructose diphosphate aldolase while the other involves phosphoglucoseisomerase (Reaction 9) and phospho-2-keto-3-deoxygluconate aldolase (Reaction 12) (Fig. 1.2). Another variation of the pathway occurs in some methylotrophs which lack transaldolase activity (Reaction 6). Such organisms include the two Gram-positive, Type L restricted facultative methylotrophs (PM6 and S2A1) which use a modified cycle involving sedoheptulose 1, 7-diphosphate and sedoheptulose diphosphatase (Colby and Zatman, 1975b).

It has been suggested that certain enzymes of the ribulose monophosphate pathway are involved in the complete

Fig. 1.3 Hexulose phosphate synthase-mediated cycle of formaldehyde oxidation (from Strøm *et al.*, 1974)



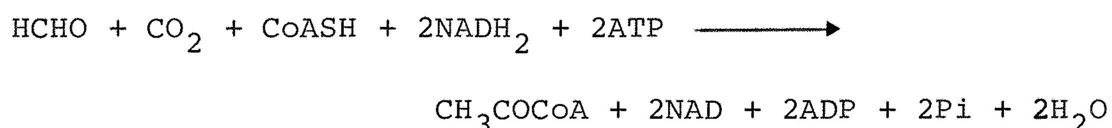
pgd, 6-phosphogluconate dehydrogenase

The key to the other enzymes is given in Fig. 1.2.

oxidation of formaldehyde shown in Fig. 1.3. This scheme for formaldehyde oxidation has been demonstrated in *P. methanica*, *M. capsulatus* (Strøm *et al.*, 1974) and Bacillus PM6 (Colby and Zatman, 1975b). The latter two organisms lack alternative enzymes for formaldehyde oxidation. The cycle may also be important in all these bacteria for the generation of NADPH for biosynthetic purposes.

b) The serine pathway

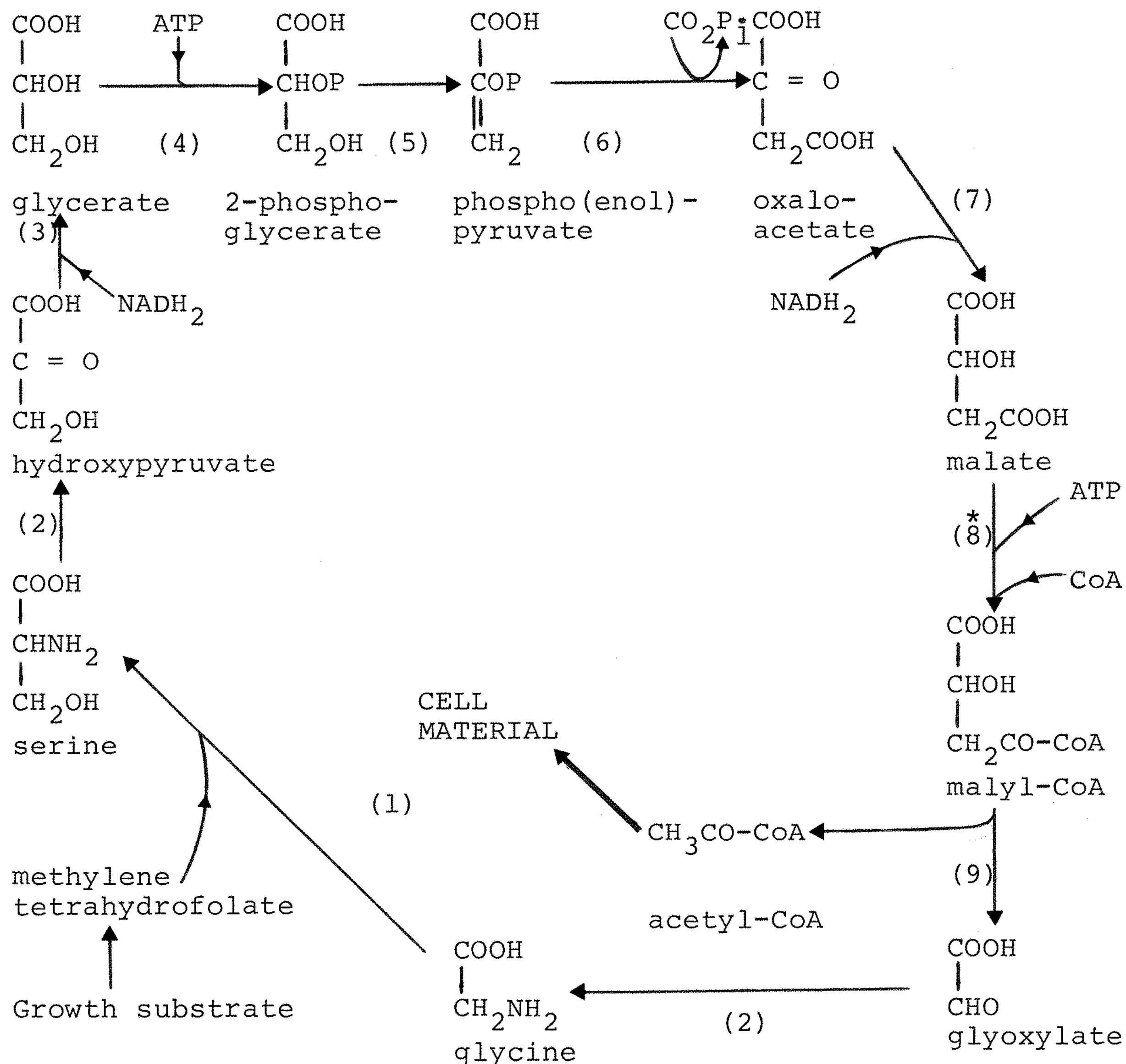
The serine pathway was first proposed by Large, Peel and Quayle (1961) who showed that serine was the earliest intermediate in the metabolism of ^{14}C-methanol and ^{14}C-formate by methanol and formate-grown *Pseudomonas* AM1. This pathway is now known to operate as a cyclic series of reactions which effect the addition of formaldehyde and CO_2 to give a molecule of acetyl-CoA (Fig. 1.4):



$$HCHO + CO_2 + CoASH + 2NADH_2 + 2ATP \longrightarrow CH_3COCoA + 2NAD + 2ADP + 2Pi + 2H_2O$$

The enzymes which probably have an exclusive function in the serine pathway are: serine-glyoxylate aminotransferase (2), hydroxypyruvate reductase (3), glycerate kinase (4) and malyl-CoA lyase (9). All are induced during methylotrophic growth and there is evidence that they are coordinately regulated in *Pseudomonas* AM1 (Dunstan *et al.*, 1972b; Salem, Hacking and Quayle, 1973b). As expected, mutants which are unable to synthesize any of the above four enzymes are unable to grow on C_1-compounds (Anthony,

Fig. 1.4 The serine pathway for methylotrophic growth (from Anthony, 1975a)



Key to enzymes:

(1) serine hydroxymethyltransferase
(2) serine-glyoxylate aminotransferase
(3) hydroxypyruvate reductase
(4) glycerate kinase
(5) enolase
(6) phospho(enol)pyruvate carboxylase
(7) malate dehydrogenase
(8) malyl-CoA synthetase
(9) malyl-CoA lyase

* No malyl-CoA synthetase (8) has been described in Pseudomonas AM1

1975a).

One outstanding problem concerning the operation of the serine pathway is the activation of malate to malyl-CoA by *Pseudomonas* AM1. Malate thiokinase (8) has not been demonstrated in this organism but it is present in *Hyphomicrobium* X, *Pseudomonas* MA, *Pseudomonas* MS (Salem *et al.*, 1973b) and Bacterium 5H2 (Cox and Zatman, 1973). No alternative enzyme (e.g. CoA-transferase) has yet been described which is capable of activating malate in extracts of *Pseudomonas* AM1.

The final problem in considering the growth of bacteria using the serine pathway is that of the conversion of acetyl-CoA, the nett product of the pathway, to C_3- and C_4-compounds required for biosynthesis. This problem is overcome by oxidation of acetyl-CoA to glyoxylate which can re-enter the pathway after transamination to glycine and thus allow C_3 and C_4 intermediates to be withdrawn.

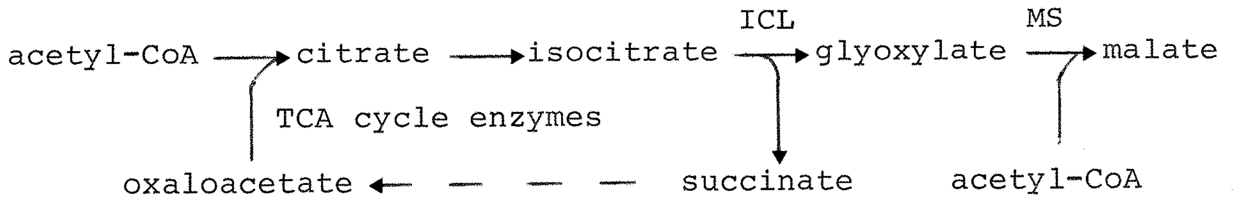
There is some diversity amongst serine-pathway methylotrophs in their mechanism of oxidation of acetyl-CoA to glyoxylate. Two routes are known, the distinction being whether or not the organism can synthesize a key enzyme of the glyoxylate cycle, isocitrate lyase.

1.5 Assimilation of acetyl-CoA produced by the serine pathway

a) Bacteria which form isocitrate lyase

In common with typical heterotrophic bacteria, the glyoxylate cycle operates during growth of many facultative methylotrophs on compounds metabolised by way of acetyl-CoA (e.g. acetate, ethanol and β-hydroxybutyrate). The two key

enzymes of the cycle, isocitrate lyase (ICL) and malate synthase (MS), together with certain TCA cycle enzymes effect the addition of two molecules of acetyl-CoA to give malate:



It might be expected that during methylotrophic assimilation of acetyl-CoA, malate synthase is not required as the activity of this enzyme coupled with malyl-CoA lyase would result in the futile cycling of malyl-CoA to malate and CoASH. This problem is overcome by certain facultative methylotrophs where malate synthase is repressed during methylotrophic growth but not during growth on compounds requiring the operation of the complete glyoxylate bypass. Such bacteria include Pseudomonas MA (Bellion and Hersh, 1972) and Hyphomicrobium X (Harder, Attwood and Quayle, 1973). Some bacteria however, (e.g. Pseudomonas MS and Bacterium 5H2, Cox and Zatman, 1973) have similar levels of malate synthase under all conditions of growth. It is possible that the malate synthase from these organisms is inhibited by certain intermediates of the serine pathway during methylotrophic growth (See Section 6.5). All these bacteria contain induced levels of isocitrate lyase during growth on C_1-compounds as well as compounds such as acetate and ethanol (Anthony, 1975a).

An overall pathway for C_1-assimilation which involves

isocitrate lyase but not malate synthase is shown in Fig. 1.5. In this scheme the intermediate withdrawn for biosynthesis is a C_3-compound; C_4-compounds required for biosynthesis may also be produced by further metabolism of 2-phosphoglycerate to oxaloacetate or malate.

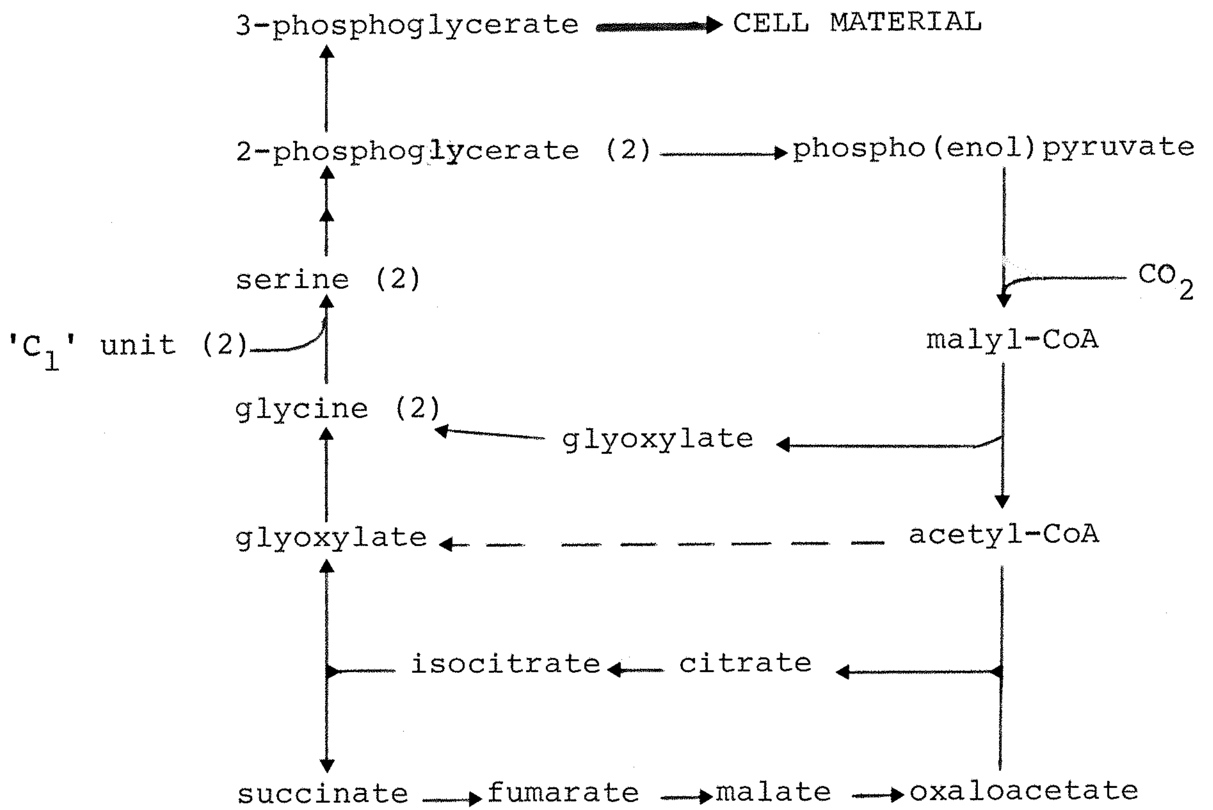
The 'unknown reactions' in Fig. 1.5 refer to the mechanism of oxidation of acetyl-CoA to glyoxylate in *Pseudomonas* AM1 and closely related bacteria which are unable to synthesize isocitrate lyase.

b) *Oxidation of acetyl-CoA to glyoxylate in Pseudomonas AM1*

Pseudomonas AM1 has no detectable isocitrate lyase activity when growing on either C_1-compounds or compounds usually requiring operation of the complete glyoxylate cycle for their assimilation (e.g. ethanol and β-hydroxybutyrate). It is now known that *Pseudomonas* AM1 possesses a novel pathway for growth on ethanol, β-hydroxybutyrate and malonate part of which is also involved in the assimilation of acetyl-CoA during methylotrophic growth. This novel pathway involves the direct oxidation of acetate or acetyl-CoA to glyoxylate during assimilation of both C_1- and C_2-compounds (Dunstan *et al.*, 1972a, 1972b; Dunstan and Anthony, 1973). During growth on C_2-compounds the glyoxylate condenses with a second molecule of acetyl-CoA in a reaction catalysed by malate synthase to give malate. The complete pathway takes its name from this enzyme and is known as the malate synthase pathway. In an analogous situation to those bacteria which do contain isocitrate lyase, it is unlikely that malate synthase is required during methylotrophic growth of *Pseudomonas*

Fig. 1.5 Scheme for C_1 assimilation in *Hyphomicrobium X* and *Pseudomonas AM1* (modified from Harder, Attwood and Quayle, 1973)

The dotted lines represent the 'unknown' reactions involved in acetyl-CoA oxidation to glyoxylate by *Pseudomonas* AM1.



AM1 despite the observation that the enzyme is present regardless of the growth substrate. This has recently been confirmed following the isolation of a mutant of *Pseudomonas* AM1 (mutant ICT 51) which lacked malate synthase but retained the ability to grow on C_1-compounds (Taylor and Anthony, 1975). Evidence for the malate synthase pathway in *Pseudomonas* AM1 is presented in Section 4.2.

The overall pathway of C_1 assimilation by *Pseudomonas* AM1 is thus similar to that for *Hyphomicrobium* (Fig. 1.5), the 'unknown reactions' being equivalent in function to the isocitrate lyase and TCA cycle enzymes which operate in bacteria such as *Hyphomicrobium*.

Similar reactions involving the oxidation of acetate or acetyl-CoA to glyoxylate may also occur in organisms closely related to *Pseudomonas* AM1 such as *Pseudomonas* M 27, *Pseudomonas* 3A2 and Bacterium 5B1. The latter organism is at present unique in that it contains high levels of isocitrate lyase during growth on acetate but the enzyme is virtually absent during growth on C_1-compounds (Colby and Zatman, 1972). It is possible that this organism uses enzymes of the glyoxylate cycle for assimilation of acetate but during methylotrophic growth acetyl-CoA is oxidised to glyoxylate by enzymes similar to those proposed for *Pseudomonas* AM1.

1.6 Distribution of the carbon assimilation pathways

Obligate methylotrophs use either of the pathways of C_1 assimilation. All the methane-utilisers with Type I membranes use the ribulose monophosphate pathway while those with Type II membranes use the serine pathway. The obligate

methylotrophs which are not capable of utilising methane: Bacterium 4B6, Bacterium C2A1 (Colby and Zatman, 1975b), Organism W1 (Dahl et al., 1972) and Methylomonas M-15 (Sahm and Wagner, 1975) all use the RMP pathway of formaldehyde fixation. It is not known if the Type II obligate methylotrophs which use the serine pathway contain isocitrate lyase for acetyl-CoA assimilation. The finding by Bellion and Woodson (1975) of two distinct isocitrate lyases in Pseudomonas MA, one elaborated during methylotrophic growth and the other during growth on acetate leads to the possibility that organisms may exist which grow on C_1-compounds using isocitrate lyase, but fail to grow on C_2-compounds.

The restricted facultative methylotrophs all use the RMP pathway of formaldehyde fixation (Colby and Zatman, 1975b).

With the exception of Pseudomonad C, all the facultative methylotrophs use the serine pathway of C_1 assimilation. However, Goldberg and Mateles (1975) have recently proposed that Pseudomonad C assimilates methanol by way of the RMP pathway while formaldehyde and formate are assimilated by way of the serine pathway. These workers demonstrated that methanol-grown Pseudomonad C contained high levels of hexulose phosphate synthase and no glycerate dehydrogenase. Conversely, extracts of bacteria grown on formate or formaldehyde contained high levels of NADPH-specific glycerate dehydrogenase but no hexulose phosphate synthase. If these results are confirmed by detailed analysis of all the enzymes of the serine and RMP pathways, this bacterium is unique in possessing both pathways of C_1 assimilation.

Methylotrophic yeasts use the ribulose monophosphate pathway of formaldehyde fixation. An inducible hexulose phosphate synthase has been demonstrated in Candida biodinii during growth on methanol and early labelled intermediates derived from ^{14}C-formaldehyde incubated with methanol-grown cells were sugar phosphates (Sahm and Wagner, 1974). Evidence has also been obtained for the operation of this pathway in Candida biodinii No 0302, Kloeckera sp. No. 2201 and Pichia pastoris although the specific activities of hexulose phosphate synthase in these yeasts were much lower than those of bacteria containing this enzyme (Diel, Held, Schlanderer and Dellweg, 1974).

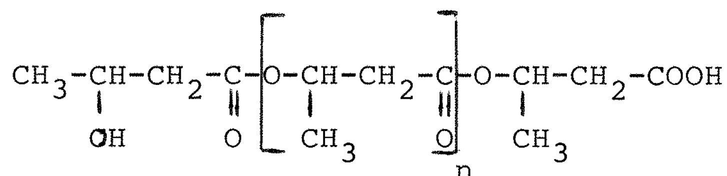
PART B

The metabolism of poly-β-hydroxybutyrate

1.7 Nature and occurrence of poly-β-hydroxybutyrate

Poly-β-hydroxybutyrate (PHB) is a carbon and energy storage compound found in a wide variety of micro-organisms including Gram-negative and Gram-positive aerobic and anaerobic species. Very large amounts of the polymer are accumulated by Azotobacter species (Stockdale, Ribbons and Dawes, 1968) and by Hydrogenomonas eutropha (Schlegel, Gottschalk and Von Bartha, 1961). In 1960, Kallio and Harrington demonstrated that 32% of the dry weight of a strain of methanol-grown Pseudomonas methanica (Iowa strain) was due to lipid material, and 92% of this lipid was poly-β-hydroxybutyrate. This endogenous storage compound was later found to form 6% of the dry weight of Pseudomonas AM1 (Peel and Quayle, 1961) and has since been observed in a number of different methylotrophs.

Poly-β-hydroxybutyrate exists in the form of a straight chain homopolymer of D(-) β-hydroxybutyrate, the formula of which is:



When extracted with neutral solvents from various bacteria, n = 600 to 2,500 representing molecular weights of between 60,000 and 250,000 (Lundgren, Alper, Schnaitmann and Marchessault, 1965). In all PHB-producing bacteria the

polymer is present within hydrophobic granules which can be stained with Sudan black. The single membrane surrounding the granule contains protein which has been associated with the enzyme system(s) concerned with polymerisation and depolymerisation of the polymer (Merrick, 1965).

1.8 The enzymes of poly-β-hydroxybutyrate biosynthesis

The most detailed study of PHB biosynthesis has come from work with *Azotobacter beijerinckii* which under certain conditions can accumulate up to 70% of its weight as PHB (Dawes and Senior, 1973). The following enzymes were found to catalyse the formation of PHB from acetyl-CoA in this bacterium: β-ketothiolase, acetoacetyl-CoA reductase and poly-β-hydroxybutyrate synthetase (Fig. 1.6). PHB synthetase is granule-bound and has been found in *H. eutropha* (Schegel, Lafferty and Krauss, 1970), *Rhodospirillum rubrum* and *Bacillus megaterium* (Greibel, Smith and Merrick, 1968) as well as *A. beijerinckii*. In the latter two organisms, the enzyme has a functional thiol group and in all cases the substrate is D(-) β -hydroxybutyryl-CoA.

β-hydroxybutyryl-CoA is produced by reduction of aceto-acetyl-CoA by an NAD(P)H-linked acetoacetyl-CoA reductase; this has been reported in several PHB-producing organisms including *H. eutropha*, *Rhodospirillum rubrum* (Stern, Del Campillo and Raw, 1956), *Rhodopseudomonas spheroides* (Carr and Lascelles, 1961), *Bacillus cereus* and *A. beijerinckii*. The product of acetoacetyl-CoA reduction has been identified in only two cases. The product of the enzyme from *A. beijer-inckii* is D(-) β -hydroxybutyryl-CoA (Ritchie, Senior and

Dawes, 1971) while the product of the enzyme from R. rubrum is L(+)β-hydroxybutyryl-CoA.

Two separate β-hydroxybutyryl-CoA dehydratases have been demonstrated in R. rubrum (Moskowitz and Merrick, 1969), one specific for the D(-) isomer, and the other for the L(+) isomer. Crotonyl-CoA was incorporated into PHB in the presence of the dehydratase specific for D(-)β-hydroxybutyryl-CoA and PHB synthetase, but substitution of the L(+) isomer dehydratase for the D(-) enzyme in this system did not lead to incorporation. It was therefore proposed that in R. rubrum, L(+)β-hydroxybutyryl-CoA from the reduction of acetoacetyl-CoA was first converted to the D(-) isomer through the intermediacy of crotonyl-CoA before being incorporated into PHB (Fig. 1.6).

The first enzyme of PHB synthesis from acetyl-CoA is β-ketothiolase which is present in all PHB-producing bacteria. The enzyme has been examined in detail from A. beijerinckii (Senior and Dawes, 1973) and H. eutropha (Oeding and Schlegel, 1973) and possesses several features of interest. The thiolysis reaction is inhibited by high concentrations of acetoacetyl-CoA (substrate inhibition) which is relieved by increasing the concentration of the second substrate, CoASH. In the direction of acetyl-CoA condensation, the reaction is inhibited by CoASH. The significance of these properties in relation to the regulation of PHB metabolism is discussed in Section 1.10.

1.9 The enzymes of poly-β-hydroxybutyrate degradation

a) Extracellular PHB degradation

It can be assumed that PHB is a common carbonaceous

Fig. 1.6 The metabolism of poly-β-hydroxybutyrate in *Azotobacter beijerinckii* (modified from Senior and Dawes, 1973)

Dotted lines indicate the scheme for reduction of acetoacetyl-CoA to D(-) β -hydroxybutyryl-CoA in *R. rubrum*.

(1) β-ketothiolase
(2) acetoacetyl-CoA reductase
(3) PHB synthetase
(4) PHB depolymerase
(5) D(-) β -hydroxybutyrate dehydrogenase
(6) acetoacetate:succinate CoA transferase
(7) L(+) β -hydroxybutyryl-CoA dehydrogenase
(8) L(+) β -hydroxybutyryl-CoA dehydratase
(9) D(-) β -hydroxybutyryl-CoA dehydratase

substance in the soil, liberated by the death and lysis of organisms such as Azotobacter and Hydrogenomonas which accumulate substantial amounts of the polymer. It is not surprising therefore to find that a number of bacteria have been isolated which are capable of utilising extracellular PHB as a sole source of carbon and energy. Several species of Hydrogenomonas and Bacillus have been reported to have this property (Dawes and Senior, 1973) as well as a number of Pseudomonads (Delafield, 1965b). The enzyme which confers this ability is an extracellular PHB depolymerase which in most cases degrades PHB to a mixture of monomeric and dimeric D(-) β -hydroxybutyrate which are transported into the cell. The dimer is then further hydrolysed to D(-) β -hydroxybutyrate by an intracellular dimer hydrolase (Delafield, 1965a).

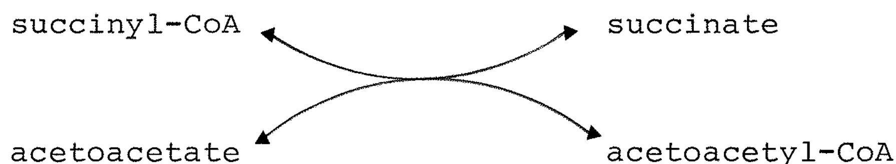
b) Intracellular PHB degradation

The catabolism of intracellular PHB reserves is probably not initiated until all available exogenous carbon and energy sources are exhausted. The first degradation step is catalysed by a soluble PHB depolymerase. The sole product of PHB hydrolysis in Hydrogenomonas was D(-) β -hydroxybutyrate (Hippe and Schlegel, 1967) whereas the products of purified PHB depolymerase from B. megaterium were identified as a mixture of dimer and monomer units. A dimer hydrolase has also been isolated from this organism which catalyses the hydrolysis of dimeric β-hydroxybutyrate to monomer units (Dawes and Senior, 1973).

In every system so far studied, D(-) β -hydroxybutyrate is further metabolised by an NAD^+-specific β -hydroxybutyrate dehydrogenase; the products being NADH and acetoacetate.

Oeding and Schlegel (1973) have demonstrated that the enzyme from H. eutropha is competitively inhibited by NADH, pyruvate and oxaloacetate. The enzyme from A. beijerinckii is also competitively inhibited by NADH and pyruvate but differs in that oxaloacetate does not inhibit whereas 2-oxoglutarate does (Senior and Dawes, 1973). The significance of these regulatory properties are discussed in Section 1.10.

Further oxidation of acetoacetate involves activation to acetoacetyl-CoA followed by cleavage of this molecule to two molecules of acetyl-CoA. In A. beijerinckii aceto-acetate is activated by transfer of CoA from succinyl-CoA in a reaction catalysed by acetoacetate:succinate CoA transferase (thiophorase):



When assayed in the direction of CoA transfer from acetoacetyl-CoA to succinate, acetoacetate was found to be a potent inhibitor of the enzyme (Senior and Dawes, 1973).

β-ketothiolase, the enzyme catalysing the first reaction of biosynthesis, catalyses the final degradation reaction of PHB to acetyl-CoA. There is no evidence to suggest that more than one enzyme is responsible for these two activities in A. beijerinckii or H. eutropha.

The overall scheme for the metabolism of PHB by A. beijerinckii is shown in Fig. 1.6. Oeding and Schlegel (1973), while not reporting the presence of an acetoacetate:

succinate CoA transferase, have concluded that PHB metabolism by *H. eutropha* proceeds by a process much the same as for *A. beijerinckii* and with almost identical regulatory controls (Fig. 1.7).

1.10 Regulation of poly-β-hydroxybutyrate metabolism

Dawes and Senior (1973) have pointed out that any regulatory mechanism of PHB metabolism must take into account the observed physiological functions of the polymer.

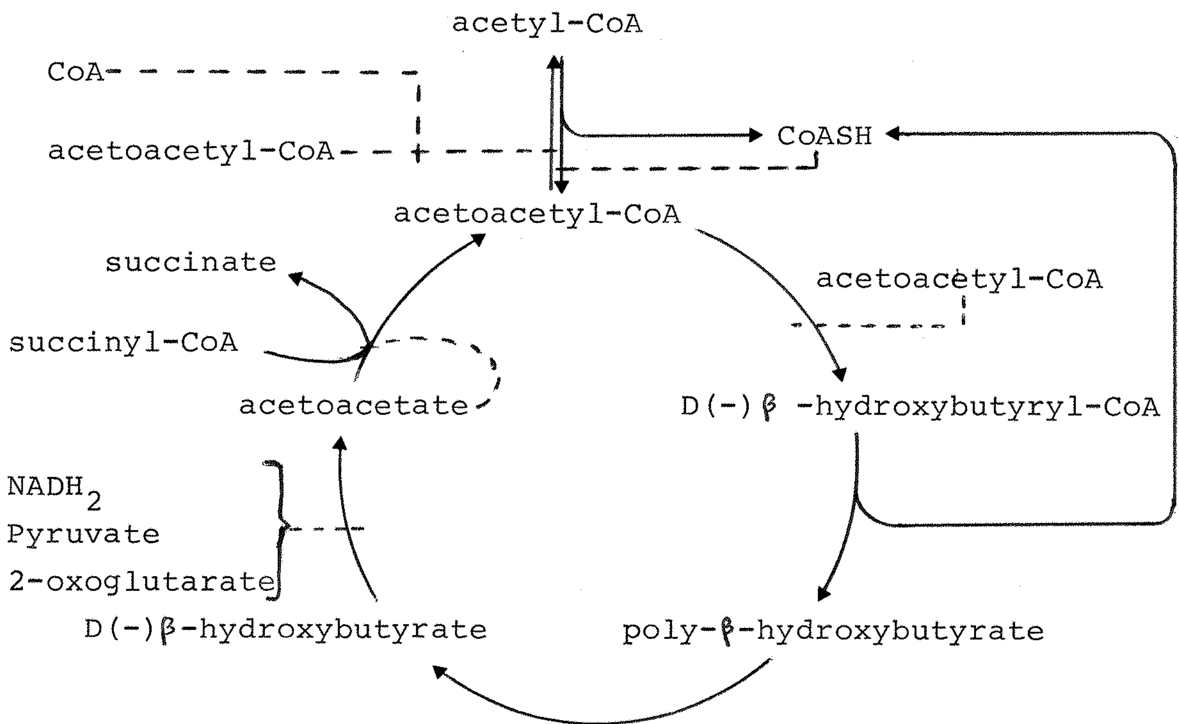
The primary function of the polymer is that of a carbon and/or energy source during starvation. This role has been demonstrated in several bacteria including *Micrococcus halodenitrificans* (Sierra and Gibbons, 1962), *Bacillus megaterium* (Macrae and Wilkinson, 1958) and *Hydrogenomonas eutropha* (Hippe, 1967). In some bacteria the polymer serves as a reserve of carbon and energy during specialised activities such as sporulation and encystement. In *Azotobacter vinelandii* PHB accumulates prior to encystement and subsequently disappears when encystement occurs (Stevenson and Socolofsky, 1966). Similiarly, in *B. cereus* the polymer accumulates at the end of exponential growth and is degraded at the onset of sporulation (Kominek and Halvorson, 1965). In the Azotobacteriaeceae it has been proposed that PHB functions as both a storage compound and as a means of regulating the oxygen environment of their natural habitat, the soil. The nitrogenase of *Azotobacter* is inhibited by oxygen concentrations in excess of about 20% air saturation. This inhibitory effect may be countered by the organism increasing its oxidative activity thus lowering the

environmental partial pressure to a more acceptable value; a process known as 'respiratory protection' (Dalton and Postgate, 1969). The possession of large amounts of PHB permits the organism to increase its oxidative activity even in the absence of an exogenous substrate. It is not surprising therefore to find that the polymer accumulates in *Azotobacter beijerinckii* during atmospheric nitrogen fixation under conditions of oxygen limitation (Senior, Beech, Ritchie and Dawes, 1972) when 'respiratory protection' is unnecessary.

The regulation of PHB metabolism has been studied in detail in two organisms: *A. beijerinckii* (Senior and Dawes, 1973) and *H. eutropha* H. 16 (Oeding and Schlegel, 1973) (Fig. 1.7). Senior and Dawes have proposed the following explanation of their findings.

Under conditions of unrestricted growth, enzymes of the TCA cycle are operating maximally and citrate synthase acts as a sink for acetyl-CoA with the simultaneous release of free CoASH. Consequently the acetyl-CoA concentration in the cell is low and the CoASH concentration high, a combination which leads to inhibition of β-ketothiolase in the direction of acetoacetyl-CoA synthesis. Oxygen limitation would lead to a build up of NADH which inhibits the citrate synthase of *Azotobacter beijerinckii* (Senior and Dawes, 1971). With few exceptions this inhibition of citrate synthase by NADH is common to Gram-negative bacteria (Section 1.13). The inhibition of this enzyme would lead to a sufficiently high acetyl-CoA concentration to saturate

Fig. 1.7 The regulation of poly-β-hydroxybutyrate metabolism in *A. beijerinckii* (from Senior and Dawes, 1973)



The dotted lines from effectors indicate inhibition.

β-ketothiolase (Km = 0.9 mM) and the simultaneous decrease in the concentration of free CoASH would relieve the inhibition by this compound on the condensation reaction of β-ketothiolase. The high NADH concentration would, in turn, inhibit degradation of the polymer at the level of β-hydroxybutyrate dehydrogenase and prevent unrestricted cycling of metabolism.

Conditions favouring a high intracellular concentration of $NAD(P)^+$ resulting from relaxation of oxygen limitation would stimulate degradation of the polymer only when the steady-state concentration of acetyl-CoA decreased and that of CoASH increased as a result of the supply of carbon becoming restricted. The increasing CoASH concentration would enable thiolysis of acetoacetyl-CoA to proceed by relieving the inhibition of β-ketothiolase by its substrate acetoacetyl-CoA.

Similar considerations apply to the regulation of PHB metabolism in organisms such as *H. eutropha* which accumulate the polymer under nitrogen limitation. Nitrogen limitation would cause a cessation of protein synthesis; pyruvate and TCA cycle intermediates would not flow into anabolic pathways resulting in high acetyl-CoA and low CoASH concentrations. Once again β-ketothiolase condensation would be uninhibited and PHB synthesis unimpaired.

In conclusion, poly-β-hydroxybutyrate is thus a highly reduced carbon and energy storage compound which, according to organism, may additionally play a role in spore or cyst formation. In the case of nitrogen-fixing organisms in the soil the possession of PHB could afford 'respiratory protection' when readily oxidisable exogenous substrates are not available.

PART C

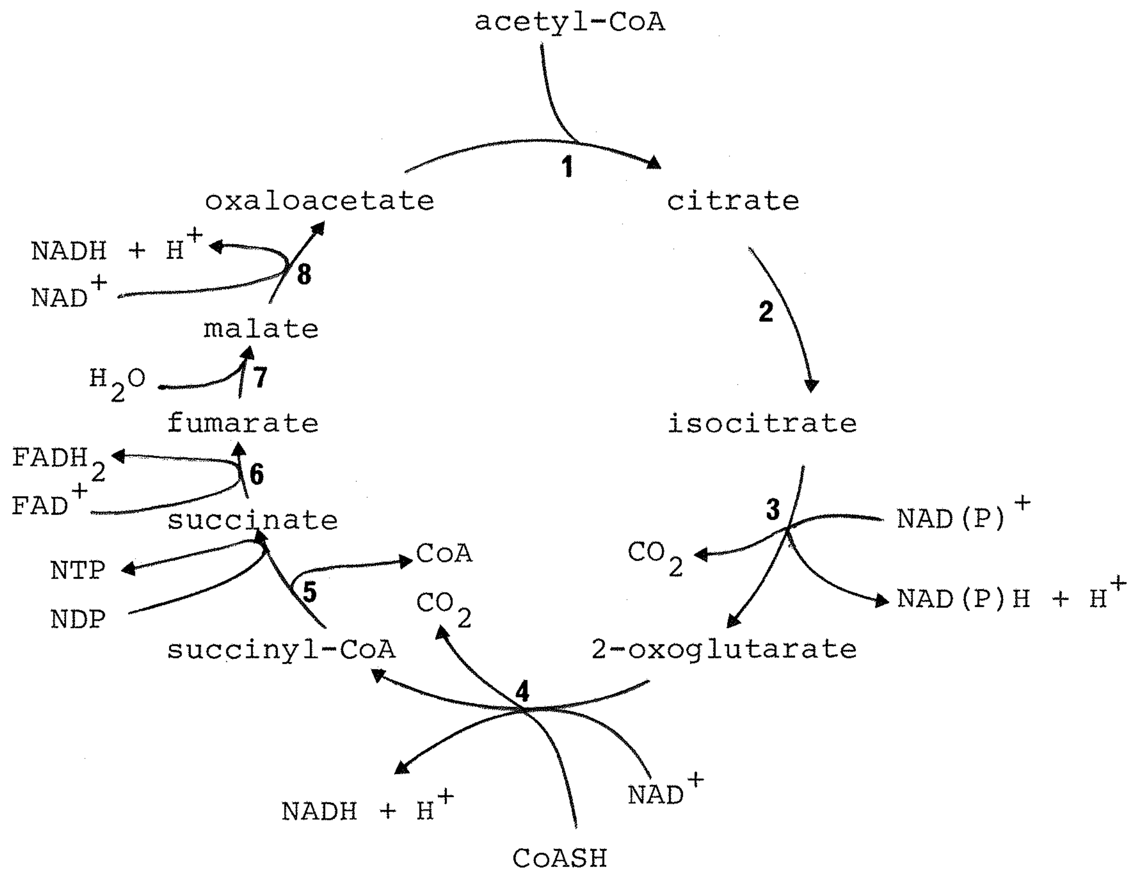
Regulation and functions of the tricarboxylic acid (TCA) cycle in bacteria

1.11 Functions of the TCA cycle

The familiar series of reactions which constitute the tricarboxylic acid cycle are shown in Fig. 1.8. The cycle can be considered to have two functions; the first is the generation of energy by the complete oxidation of acetyl-CoA to CO_2 and water and the second is the provision of carbon skeletons for biosynthesis (e.g. 2-oxoglutarate and succinate). The biosynthetic function of the cycle however will allow the removal of intermediates of the cycle only when an anaplerotic supply of C_4-dicarboxylic acids is available. Bacteria have evolved various control mechanisms to ensure the correct, co-ordinated flow of carbon either into biosynthetic channels or for energy generation depending on the environment or physiological requirements of the organism.

In aerobic bacteria growing on compounds metabolised to acetyl-CoA the TCA cycle is used for both purposes. By contrast several species of bacteria use enzymes of the cycle in a purely biosynthetic capacity and certain TCA cycle enzymes are either repressed or completely absent from these organisms. Bacteria which possess an incomplete TCA cycle include certain Gram-positive facultative anaerobes and also several obligate autotrophs and obligate methylotrophs. In addition, several Gram-negative facultative anaerobes possess the ability to switch off the oxidative TCA cycle

Fig. 1.8 The tricarboxylic acid cycle



Abbreviations: NDP, NTP, nucleotide di- and triphosphate

Enzymes:

(1) citrate synthase
(2) aconitase
(3) isocitrate dehydrogenase
(4) 2-oxoglutarate dehydrogenase
(5) succinyl-CoA synthetase
(6) succinate dehydrogenase
(7) fumarase
(8) malate dehydrogenase

under certain conditions by enzyme repression. Examples of these organisms are discussed in the following Section.

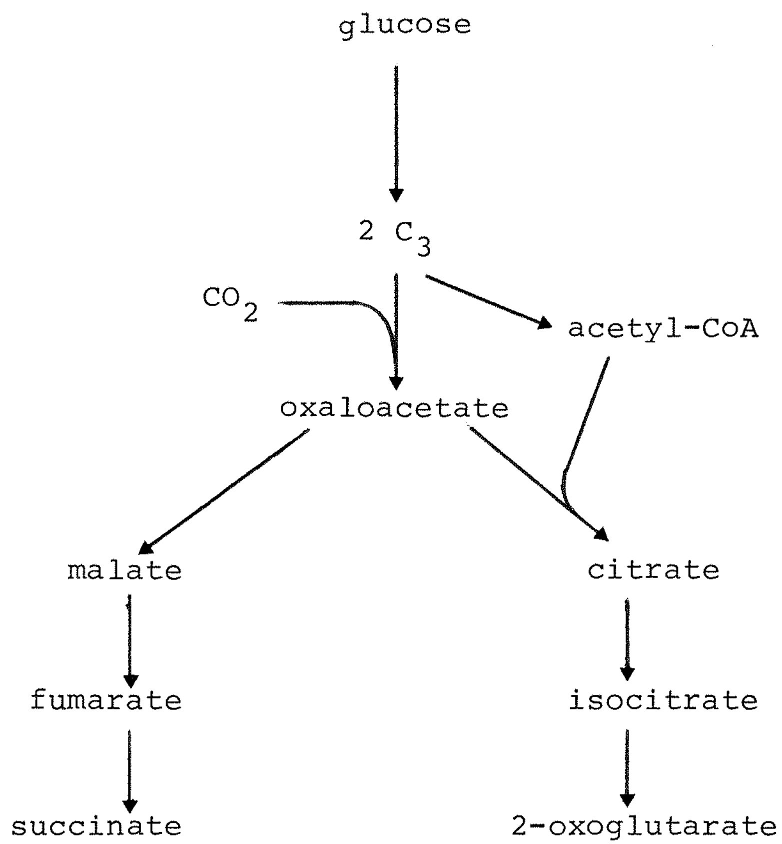
1.12 Bacteria lacking a complete TCA cycle

a) Gram-negative facultative anaerobes

During growth of Escherichia coli and related bacteria on glucose, enzymes of the TCA cycle are repressed under both anaerobic and aerobic conditions. Such enzymes include succinate dehydrogenase, succinyl-CoA synthetase and 2-oxoglutarate dehydrogenase (Amarasingham and Davis, 1965). It has also been shown that mutants of E. coli lacking succinate dehydrogenase grew well aerobically on glucose supporting the observation that the enteric bacteria live largely by anaerobic glycolysis even when growing aerobically (Hirsch, Rasininsky, Davis and Lin, 1963). An ingenious explanation of the apparently wasteful way of utilising glucose aerobically, namely by glycolysis alone, has been advanced by Amarasingham and Davis (1965). These authors suggest that the partial oxidation of glucose leads to accumulation of fermentation products which remain in the external mileu for use when the supply of glucose is exhausted. They argue that this gives a selective advantage to the enteric bacteria over other microbes which may not readily adapt to incompletely oxidised intermediates such as acetate.

Under conditions where the tricarboxylic acid cycle is incomplete, a branched, non-cyclic 'horseshoe' pathway operates to provide biosynthetic intermediates. The pathway, shown in Fig. 1.9, consists of an oxidative branch

Fig. 1.9 Biosynthetic 'horseshoe' pathway showing the end-product nature of 2-oxoglutarate (from Amarasingham and Davis, 1965)



from oxaloacetate to 2-oxoglutarate and a reductive branch to succinate (Amarasingham and Davis, 1965). In E. coli the enzymes of the 'horseshoe' pathway are the same as those of the complete cycle except that fumarate is reduced to succinate by a separate fumarate reductase rather than a reversal of succinate dehydrogenase.

b) Gram-positive facultative anaerobes

Tanaka and Hanson (1975) have recently shown that like the enteric facultative anaerobes, the Gram-positive facultative anaerobes Bacillus polymyxa Hino G, B. polymyxa Hino J and B. macerans also have incomplete tricarboxylic acid cycles. All these strains were devoid of malate dehydrogenase and only very low levels of 2-oxoglutarate dehydrogenase were detected during both anaerobic and aerobic growth on glucose. Another Gram-positive facultative anaerobe, Listeria monocytogenes has also been reported to lack a complete TCA cycle. This organism which grows on glucose but not on any of the following compounds: acetate, pyruvate, citrate, isocitrate, 2-oxoglutarate, succinate, fumarate or malate, was devoid of 2-oxoglutarate dehydrogenase, succinate dehydrogenase and the glyoxylate-bypass enzymes isocitrate lyase and malate synthase (Trivett and Meyer, 1971).

c) Autotrophic bacteria

In facultative autotrophs such as Nitrobacter, Hydrogenomonas and Micrococcus denitrificans the tricarboxylic acid cycle is complete and can provide both energy and intermediates for biosynthesis. However, with the finding that

obligate chemolithotrophs (e.g. *Thiobacillus* species) and obligate photolithotrophs (blue-green algae, *Chromatium*) lack 2-oxoglutarate dehydrogenase there is growing evidence that the TCA cycle enzymes of obligate lithotrophs serve a sole biosynthetic function (Smith, London and Stanier, 1967; Kelly, 1971). The incomplete cycle thus operates to produce 2-oxoglutarate from acetate by the oxidative branch, and succinate from oxaloacetate by the reductive branch of the biosynthetic 'horseshoe' pathway (Kelly, 1971). In general, as well as lacking 2-oxoglutarate dehydrogenase, obligate autotrophs also have low levels of succinate and malate dehydrogenases and in some cases NADH oxidase is also absent. Smith *et al*. (1967) have suggested that the absence of 2-oxoglutarate dehydrogenase may be the biochemical basis of obligate autotrophy and that obligate autotrophs have evolved from heterotrophic ancestors by the elimination of this key enzyme.

d) *Methylotrophic bacteria*

Several obligate methylotrophs which use the ribulose monophosphate pathway of formaldehyde fixation (Section 1.4) lack complete TCA cycle activity. Such bacteria include the Type I methane-utilisers (Davey, Whittenbury and Wilkinson, 1972); Bacterium 4B6 (Colby and Zatman, 1972) and organism W1 (Dahl *et al*., 1972); in each case 2-oxoglutarate dehydrogenase was not detected. Malate dehydrogenase was also absent from Bacterium 4B6 and this was associated with low specific activities of other TCA cycle enzymes, notably succinyl-CoA synthetase and succinate dehydrogenase.

In these bacteria, the absence of 2-oxoglutarate dehydrogenase has been implicated as a basis for obligate methylotrophy which is also common to obligate autotrophs. The biochemical basis of obligate methylotrophy however is still unresolved as Type II methane-utilisers which assimilate C_1-compounds by way of the serine pathway do possess all the enzymes of the TCA cycle. In addition to this, the restricted facultative methylotrophs (Section 1.2c) lack 2-oxoglutarate dehydrogenase whilst retaining the ability to grow on certain non-C_1 compounds (Colby and Zatman, 1975a). The type 'L' restricted isolates (S2A1 and PM6) had relatively high levels of the other TCA cycle enzymes whereas the type 'M' bacteria (W3A1 and W6A) had very low or undetectable levels of succinate dehydrogenase, succinyl-CoA synthetase and 2-oxoglutarate dehydrogenase.

Lack of a complete TCA cycle, particularly 2-oxoglutarate dehydrogenase is the probable reason for the limited range of substrates supporting growth of the restricted facultative methylotrophs and this situation is therefore very similar to that obtaining in *Hyphomicrobium* X (lacking pyruvate dehydrogenase) which has a complete TCA cycle but is unable to grow on any compound metabolised by way of pyruvate (Harder, Matin and Attwood, 1975). The TCA cycle-deficient restricted facultative methylotrophs are nevertheless capable of growth on a few non-C_1 compounds and this led Colby and Zatman (1975a) to conclude that a deficient TCA cycle could not be accepted as the *sole* cause of obligate methylotrophy.

1.13 Regulation of citrate synthase activity

i) Bacteria which have a complete TCA cycle

In general, the citrate synthases of aerobic bacteria with complete oxidative and biosynthetic TCA cycles are inhibited by either NADH or ATP. Inhibition by NADH is characteristic of Gram-negative bacteria and is relieved in the presence of AMP (Weitzman and Jones, 1968 (1)). The inhibition of citrate synthase by ATP is characteristic of Gram-positive bacteria with complete TCA cycles (Johnson and Hanson, 1974 (5)).

Inhibition of citrate synthase by either of these effectors can be regarded as 'end-product' inhibition where these are products of the catabolic (oxidative) function of the TCA cycle. The relief of NADH inhibition of citrate synthases by AMP is only observed with strictly aerobic Gram-negative bacteria and not with Gram-negative facultative anaerobes discussed below. This regulatory effect of AMP would ensure the highest activity of the cycle when ATP levels are low regardless of the NADH concentration.

ii) Bacteria which lack a complete TCA cycle

The citrate synthases of bacteria with incomplete TCA cycles (either lacking 2-oxoglutarate dehydrogenase or with repressed levels of key enzymes of the cycle under certain circumstances) are inhibited by 2-oxoglutarate in addition to either NADH or ATP. Examples of such bacteria include Gram-negative facultative anaerobes (Weitzman and Dunmore, 1969a (2)), Gram-positive facultative anaerobes (Tanaka and Hanson, 1975 (6)), obligate chemolithotrophs

(Taylor, 1970; Lucas and Weitzman , 1975 (7)) and certain methylotrophs (Colby and Zatman, 1975c (4)) (Table 1.6).

The inhibitory effect of 2-oxoglutarate, the product of the oxidative branch of the biosynthetic 'horseshoe' pathway can also be considered as 'end-product' inhibition (Fig. 1.9).

The citrate synthases of most Gram-negative bacteria are inhibited by NADH and this property has been proposed as an aid to establishing taxonomic relationships amongst bacteria whose reaction to the Gram-strain is not clear-cut (Weitzman and Jones, 1975). However, the citrate synthases of certain Gram-negative obligate autotrophs (Taylor, 1970 (3)), two obligate methylotrophs and one restricted facultative methylotroph (Colby and Zatman, 1975c) are insensitive to inhibition by NADH. It is possible that these bacteria lost the ability to synthesize 2-oxoglutarate dehydrogenase after being subjected to conditions where an oxidative TCA cycle became unnecessary or impossible. Regulation of citrate synthase by NADH being unnecessary, it could be lost in favour of regulation by the new 'end-product' 2-oxoglutarate.

It is thus evident that the type of regulation of citrate synthase depends on the function of the TCA cycle enzymes of the organism and its use as a taxonomic guide could be misleading.

1.14 The molecular weights of citrate synthases from different bacteria

It has been shown that the molecular weights of the

Table 1.6 The regulation of citrate synthases of Gram-positive and Gram-negative bacteria

Gram-negative	Function of TCA cycle	Inhibitors	Molecular size	Examples	Reference No.
aerobes (including facultative methylotrophs)	O/B	NADH, relieved by AMP	L	Pseudomonas sp. Acetobacter sp. Azotobacter sp.	(1)
facultative anaerobes	B	2-oxoglutarate, NADH	L	Enterobacteriaceae	(2)
obligate autotrophs	B	2-oxoglutarate, ATP	-	Thiobacillus sp.	(3)
obligate and restricted methylotrophs	B	2-oxoglutarate, ATP	-	Organisms 4B6, C2A1, W3A1	(4)
Gram-positive					
aerobes	O/B	ATP	S	Bacillus subtilis	(5)
facultative anaerobes	B	2-oxoglutarate, ATP	S	Bacillus polymyxa Hino G, J	(6)
obligate autotrophs	B	2-oxoglutarate, ATP	*L	Blue-green algae	(7)

Abbreviations: B, biosynthetic function; O/B, oxidative and biosynthetic functions; L, 'large' citrate synthase; S, 'small' citrate synthase. The reference numbers refer to references described in the text.

*The blue-green algae with 'large' citrate synthases are unique amongst Gram-positive bacteria.

All those bacteria whose citrate synthases are inhibited by 2-oxoglutarate either lack 2-oxoglutarate dehydrogenase or have repressed levels of the enzyme.

enzymes from Gram-negative bacteria susceptible to NADH inhibition are all in the region of 210,000 to 260,000. These have been designated 'large' citrate synthases (Weitzman and Dunmore, 1969b). By contrast, citrate synthases from Gram-positive bacteria inhibited by 2-oxo-glutarate and/or ATP but not NADH have molecular weights of approximately 60,000 and are called 'small' citrate synthases (Weitzmann and Dunmore, 1969b; Tanaka and Hanson, 1975). Exceptions to this pattern have, however, recently been described. Lucas and Weitzman (1975) showed that the citrate synthases of several blue-green algae (Gram-positive) were of the 'large' type and the 'large' enzyme from Acinetobacter anitratum (Gram-negative) was not regul-ated by any known modifier of citrate synthase activity including NADH, AMP, ATP and 2-oxoglutarate (Johnson and Hanson, 1974).

It would be of interest to determine the molecular weights of the enzymes from those Gram-negative bacteria which are insensitive to NADH inhibition (e.g. the methylo-trophs: 4B6, C2A1 and W3A1). If these enzymes are of the 'large' type it would seem apparent that these bacteria have evolved from bacteria with oxidative TCA cycles regulated by NADH inhibition which subsequently lost 2-oxoglutarate dehydrogenase.

Chapter 2

Materials and Methods

2.1 Materials

(a) Chemicals

All chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, except for the following provided by the firms listed below.

The Radiochemical Centre, Amersham, Bucks.

U-^{14}C-acetate sodium salt; specific activity 56 μCi/m mole

Calbiochem Ltd., London

Diothiothreitol (Clelands reagent)

The Boehringer Corporation (London) Ltd., Ealing.

Glycerate-3-phosphate, crystalline; monobarium salt.

Nicotinamide adenine dinucleotide (NAD^+)

Nicotinamide adenine dinucleotide, reduced form (NADH)

Glaxo Laboratories Ltd., Greenford, Middlesex.

Sodium benzylpenicillin

Streptomycin sulphate

Koch Light Laboratories Ltd., Colnbrook, Bucks.

DL-glyceric acid

Glyoxylic acid monohydrate

N-methyl-N-nitroso-N'-nitro-guanidine

Phenylhydrazine hydrochloride

L-2-hydroxyglutaric acid

Nuclear Enterprises Ltd., Edinburgh

NE 250 liquid scintillator

Nutritional Biochemical Corporation, Cleveland, Ohio.

Hydroxypyruvic acid, lithium salt

Oxoid Ltd., London

Nutrient agar

Nutrient broth No. 2

Purified agar

Sigma (London) Chemical Co. Ltd., London.

Nicotinamide adenine dinucleotide phosphate ($NADP^+$)

Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)

3-acetyl-NAD^+, grade I

DL-6, 8-thioctic acid amide (DL-Lipoamide)

Pyridoxal-5-phosphate

Diketene (acetoacetic anhydride)

Dowex 50W, hydrogen form

Ovalbumin

Cytochrome c, horse heart

Urease, type VII from jack beans

Phospho(enol)pyruvate, tricyclohexylamine salt

Lactate dehydrogenase, crystalline suspension from pig heart, type IV

Pyruvate kinase, crystalline suspension from rabbit skeletal muscle, type I

β-Hydroxyacyl-CoA dehydrogenase, crystalline suspension from pig heart, grade III

Malic dehydrogenase, crystalline suspension from beef heart

Citrate synthase, crystalline suspension from pig heart, type III

Pharmacia, Uppsala, Sweden.

Sephadex G - 200

Blue dextran

P-L Biochemicals Inc., Milwaukee.

Coenzyme A, lithium salt

(b) Preparation of CoA derivatives

Acetyl- CoA, succinyl-CoA and acetoacetyl-CoA were prepared by reaction of CoASH with the respective anhydrides of acetate, succinate and acetoacetate according to the methods of Srere (1969) (acetyl-CoA) and Simon and Shemin (1953) (succinyl-CoA and aceto-acetyl-CoA). Solutions of acetyl-CoA were assayed in the presence of citrate synthase, oxaloacetate and DTNB according to the method of Tubbs and Garland (1969).

(c) Bacterial Strains

Pseudomonas AM1 (NClB 9133) was obtained from The National Collection of Industrial Bacteria. *Hyphomicrobium* X was a gift from Dr. M.M. Attwood, Department of Microbiology, University of Sheffield and *Escherichia coli* B was from the Microbiological Research Establishment, Porton, Wilts.

2.2 Purification of U-^{14}C-acetate

The U-^{14}C-acetate obtained from Amersham contained small but significant amounts of radioactive impurities; it was purified before use by gas liquid chromatography, using a 25% diethylene glycol adipate, phosphoric acid

treated AW-DMCS Chromosorb W 100-120 mesh column at a temperature of 140° and with a nitrogen flow rate of 100 ml/minute. The column tended to deteriorate rapidly, and a precolumn was therefore used which could be refilled before each set of purifications.

The radioactive sodium acetate was converted to acetic acid by the addition of 2 μl concentrated hydrochloric acid, then injected into the column. Pure acetic acid was collected in a trap cooled in liquid nitrogen, dissolved in dilute sodium hydroxide and adjusted to pH 7.0 before use; the yield was about 40%. This treatment separates acetate from all radioactive impurities, as demonstrated by chromatography of 10 μCi purified acetate in the solvent systems described in Section 2.18.

2.3 Media and maintenance of cultures

The basal medium (referred to as 'salts medium') contained (g/100ml solution): $(NH_4)_2SO_4$, 0.2: NaCl, 0.05; $MgSO_4.7H_2O$, 0.0025; $FeSO_4.7H_2O$, 0.0001; and KH_2PO_4, 0.2; the phosphate was dissolved separately, adjusted to pH 7.0, and added last. The medium was sterilised by autoclaving at 121° 15lb/in^2 for twenty minutes.

Carbon sources were added after sterilization to a final concentration of 0.2%(w/v), except methanol and methylamine, which were used at a final concentration of 0.4%(w/v) and β-hydroxybutyrate and malonate used at a final concentration of 0.1%(w/v).

Stock solutions were made up 100 times the final

concentration, and the pH was adjusted to 7.0 when necessary. The carbon sources were sterilized by filtration through ultrafine sintered glass (porosity 5) and added to the salts medium just before use.

Solid media were prepared by adding Purified agar (1% w/v) to the salts medium. The mixture was autoclaved, then cooled to 45° before adding the carbon source and pouring the plates.

Stock cultures were maintained on methylamine- or succinate-agar slopes stored at 2° and subcultured every two months. Cultures were also stored as suspensions in 30% (v/v) glycerol at -20°.

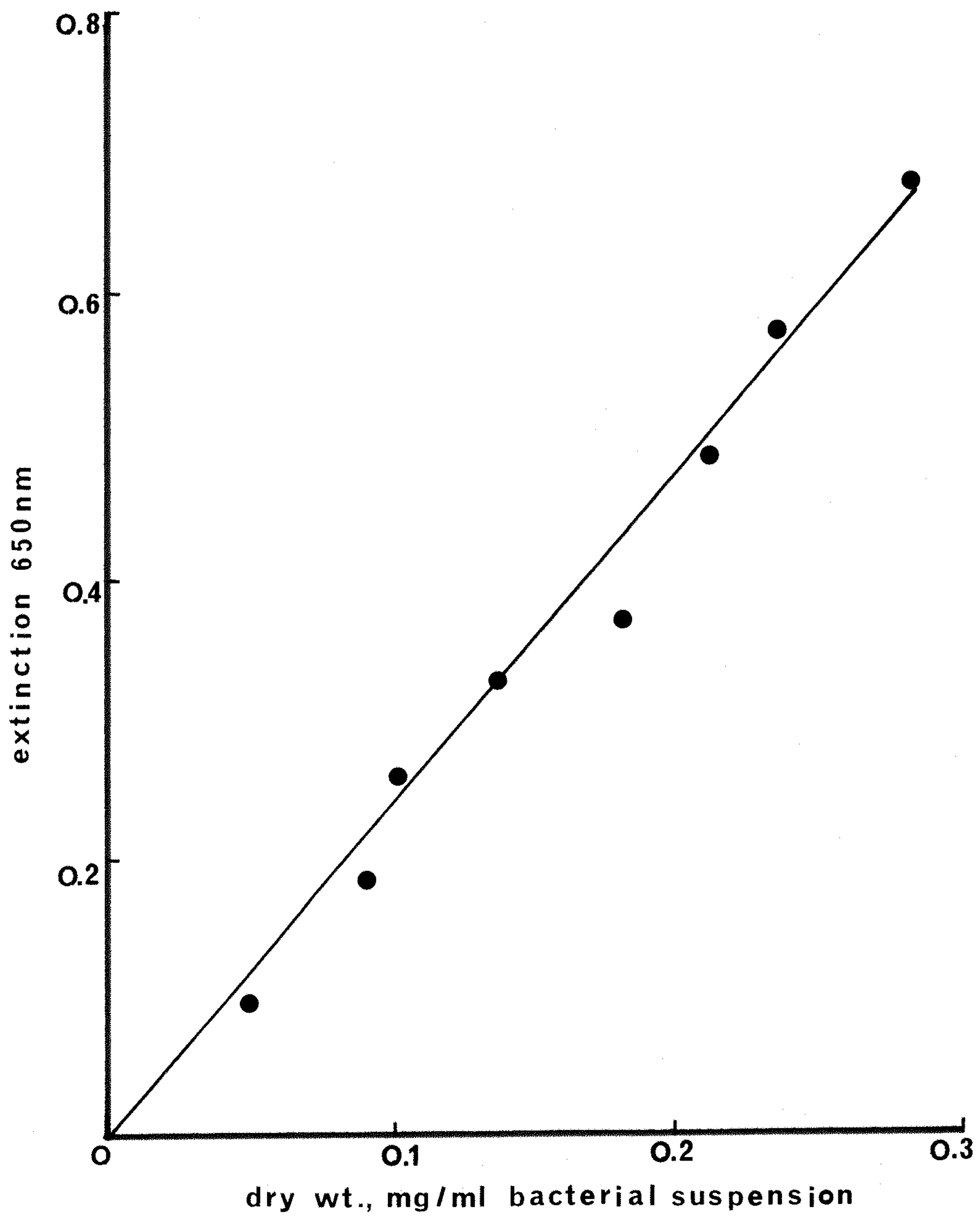
2.4 Growth and harvesting of cultures

Cultures were grown in shake flasks at 30° from a 5% inoculum of cells grown on the same carbon source. When the cells were in the mid-logarithmic phase of growth they were harvested by centrifugation at 10,000g for 15 minutes at 4°. The harvested cells were washed twice in 0.02M phosphate buffer, pH 7.0, then resuspended in buffer and stored if necessary, either at 2° (for whole cell experiments) or frozen at -15° (for preparation of cell-free extracts).

2.5 Estimation of the dry weight of cells in suspension

The dry weight of cells in suspension was estimated by measuring the extinction at 650nm in a Unicam SP 600 spectrophotometer. Extinction is proportional to dry weight of cells, as shown in Figure 2.1. This curve was obtained by suspending washed cells in buffer

Fig. 2.1 Graph relating extinction at 650 nm and dry weight of a suspension of whole cells of Pseudomonas AM1



and measuring the extinction at various cell dilutions; dry weight was determined by evaporating samples of the original suspension to dryness at 104°.

2.6 Isolation of mutants of Pseudomonas AM1

Mutants were isolated by a method based on that of Heptinstall and Quayle (1970). Wild-type cells were inoculated with the mutagen N-methyl-N-nitroso-N'-nitro-guanidine (NG) and then treated with penicillin under conditions designed to select for the required mutants. The example below was used to isolate mutants capable of growth on malate, but not on a combination of acetate plus glyoxylate.

A 50 ml overnight culture of Pseudomonas AM1 grown on malate was harvested aseptically, washed in sterile salts medium and resuspended in sterile 50mM-tris-maleate buffer, pH 6.0, to give an extinction at 650 nm of 1.3. 0.5 ml of this suspension was incubated with 0.2 ml of NG solution (0.6 mg/ml) for 30 minutes at 30°. The cells were centrifuged, washed twice in sterile salts medium, resuspended in 5 ml of salts medium containing malate, and incubated aerobically at 30° for 18 hours to allow expression of the required mutation. The cells were then washed twice and resuspended in 5 ml of salts medium containing acetate and glyoxylate. After 6 - 10 hours incubation at 30° penicillin was added (final concentration of 1000 units/ml) and the culture was incubated for a further 15 hours. Under these conditions all cells should grow except those unable to utilize

acetate plus glyoxylate as a carbon source; the growing cells are killed by the penicillin but the non-growing mutant cells remain viable and are therefore enriched. The penicillin enrichment treatment was repeated twice. Dilutions were then made in salts medium and sufficient suspension to yield approximately 200 colonies per plate were spread on to malate-agar plates. The plates were incubated at 30° for 4 days before replication on to acetate plus glyoxylate- and malate-agar plates using a pad of sterile velvet. Colonies of putative mutants able to grow on malate but not on acetate plus glyoxylate were picked off and simultaneously re-tested on the above carbon sources using a 27 - pronged metal replicator. Mutants were purified by three successive single colony isolations on malate-agar plates before they were stored on succinate slopes and as suspensions in 30% (v/v) glycerol.

2.7 Characterisation of the growth response of mutant strains

Growth characteristics of mutant strains were determined by observation of growth in liquid media (3 ml salts medium with appropriate carbon sources incubated in sloped test tubes at 30°). When growth occurred the mutant was subcultured at least twice into the same medium using a wire loop. After the second subculture the growth properties were confirmed on solid media to ensure that contamination by wild-type bacteria had not occurred.

In cases where growth properties were difficult

to interpret growth was monitored by measuring extinction at 650 nm using Erlenmeyer flasks (100 ml) containing the medium under test (25 ml).

When large scale cultures of mutant cells were used for experiments the growth responses of these cultures to a number of different carbon sources were tested on solid media to ensure that wild-type contaminants or spontaneous revertants were not present.

2.8 Isolation of revertants

Revertant strains were isolated by plating approximately 10^8 bacteria on to agar plates containing the carbon source the mutant was unable to utilize in the initial selection procedure. A small crystal of NG was placed in the centre of each plate and revertant colonies which appeared outside the zone of growth inhibition were picked off and purified by single colony isolation.

In some cases the original mutant was first made resistant to streptomycin by plating approximately 10^8 bacteria on to succinate- or methanol-agar plates containing 1 mg/ml streptomycin. Streptomycin resistant colonies were tested to ensure that they retained the growth properties of the original mutant and streptomycin resistant revertants were obtained after treatment with NG as described above. These revertants were useful in being easily distinguishable from wild-type contaminants.

Revertants were tested for their growth properties as described in Section 2.7, and, when the site of the

lesion of the original mutant was known, for reappearance of activity of the defective enzyme.

2.9 Measurement of oxygen uptake by bacterial suspensions

Oxygen uptake by bacterial suspensions was measured using a Rank oxygen electrode. The incubation vessel contained in a final volume of 2 ml, 50 μmoles Hepes buffer, pH 7.0 and 0.05 - 2 mg dry weight equivalent of washed cells. After measuring the endogenous rate of oxygen uptake substrate was injected and oxygen uptake was measured for 5-10 minutes. Sufficient substrate was used to give maximum rate with that substrate (this was 40 μmoles, but for formaldehyde, ethanol and methanol 20 μmoles was used and for formate 80 μmoles was necessary). The QO_2 was calculated by assuming that 0.45 μg atoms oxygen are dissolved in 1 ml buffer at 30°.

2.10 Preparation of sonic extracts

Unless otherwise stated, washed cells were suspended in 2-3 ml of 0.02 M-phosphate buffer, pH 7.0, to a concentration of about 0.1 g wet weight/ml, and sonicated for periods of 2 - 3 minutes in a 100w MSE ultrasonic disintegrator at 20 kH_z using a probe tip of diameter 0.9 cm. The total exposure time was 10 minutes and throughout treatment the cells were cooled in an ice bath.

The sonicate was centrifuged at 40,000g for 1 hour at 2°. The supernatant was decanted and stored in ice. When required, particulate fractions were prepared by

first centrifuging the crude sonicate at 4,000g for 10 minutes to remove unbroken bacteria before centrifugation at 40,000g. The pellet was resuspended in buffer equal to the original volume of the extract. Sonic extracts were assayed immediately for enzyme activity but often stored frozen at -20° before being assayed for protein concentration.

2.11 Protein assay

Protein was assayed by the method of Lowry, Rosebrough, Farr and Randall (1951); crystalline bovine serum albumin (fraction V) was used as standard.

2.12 Enzyme and cytochrome assays

Unless otherwise stated, cell-free extracts were prepared as described in Section 2.10. Most enzymes were assayed spectrophotometrically at 25° using Unicam SP 8000 or SP 1800 ultraviolet spectrophotometers fitted with Unicam AR 25 linear recorders. Where necessary published spectrophotometric assay methods were modified for 1 ml cuvettes. Enzyme activities are expressed as specific activities (nmoles substrate used or product formed/mg protein/minute) determined in the region of proportionality between initial reaction velocity and protein concentration. In all cases correction was made for endogenous activity in the absence of substrate.

Solutions of the substrates and cofactors used in enzyme assays were made up fresh, either in buffer or in distilled water; in the latter case solutions

were adjusted to the required pH where necessary.

(a) Enzymes of β-hydroxybutyrate metabolism

The following enzymes of β-hydroxybutyrate metabolism were assayed by published methods: β-hydroxybutyrate dehydrogenase, EC 1.1.1.30. (Schuster and Doudoroff, 1962); acetoacetate-succinate CoA transferase, EC 2.8.3.5. (Stern, Coon, del Campillo and Schneider, 1956); β-ketothiolase, EC 2.3.1.9, cleavage reaction (Senior and Dawes, 1973); condensation reaction (Oeding and Schlegel, 1973); acetoacetyl-CoA reductase, EC 1.1.1.36 (Ritchie, Senior and Dawes, 1971).

(b) Enzymes of the TCA cycle

The following enzymes were assayed by published procedures: citrate synthase, EC 4.1.3.7. (Srere, 1969); isocitrate dehydrogenase (NAD^+) EC 1.1.1.41 (Cox, 1969); Isocitrate dehydrogenase ($NADP^+$) EC 1.1.1.42 (Ochoa, 1955a); fumarase EC 4.2.12 (Racker, 1950) and malate dehydrogenase EC 1.1.3.7. (Ochoa, 1955b). Membrane bound succinate dehydrogenase was assayed using the particulate fraction by the method of Veeger, Der Vartanian and Zeylemaker (1969). Overall 2-oxoglutarate and pyruvate dehydrogenase activities were assayed by the method of Guest and Creaghan (1973). The decarboxylase (E_1) components of the 2-oxoacid dehydrogenase complexes were assayed spectrophotometrically at 420 nm with ferricyanide as the electron acceptor (modified from Hager and Kornberg, 1961). The reaction mixture contained (μmoles in 1 ml final volume); potassium phosphate, pH 6.3, 100; thiamine

pyrophosphate, 0.5; potassium ferricyanide, 1.5; sodium 2-oxoglutarate or sodium pyruvate, 20 and extract up to 2 mg protein. After incubation for 2-3 minutes the reaction was started by adding ketoacid. Lipoamide dehydrogenase (lpdh) was assayed by recording the lipoamide-dependent oxidation of NADH at pH 6.5 according to a modified method of Massey (1966). Succinyl-CoA synthetase, EC 6.2.1.1. was assayed by the hydroxamate method of Kaufmann (1955).

(c) Enzymes of C_1 metabolism

The phenazine methosulphate-dependent methanol (alcohol) dehydrogenase from *Pseudomonas* AM1 was assayed by the spectrophotometric method of Anthony (1971). Methylamine dehydrogenase was also assayed in the presence of phenazine methosulphate according to the method of Eady and Large (1968). Hydroxypyruvate reductase, EC 1.1.1.29 was measured by a method based on that of Large and Quayle (1963). The reaction mixture in a final volume of 1 ml contained; sodium acetate - acetic acid buffer pH 4.5, 100 μmoles, NADH, 0.1 μmoles; lithium hydroxypyruvate, 2 μmoles (added to start reaction) and extract up to 1 mg protein. Glyoxylate reductase, EC 1.1.1.26. was measured by the same method, except that 6 μmoles sodium glyoxylate were substituted for lithium hydroxypyruvate. Malyl-CoA lyase was assayed by the method of Salem, Hacking and Quayle (1973).

(d) Enzymes of C_2 metabolism

Acetyl-CoA synthetase (acetate thiokinase), EC 6.2.1.1. was assayed by the method of Jones and Lipmann

(1955) except that the temperature of incubation was 40° instead of 30°. Acetokinase, EC 2.7.2.1 and aldehyde dehydrogenase (NAD^{+}, CoA dependent) were assayed by the methods of Rose (1955) and Dawes and Foster (1956) respectively.

(e) Enzymes of the glyoxylate bypass

Isocitrate lyase, EC 4.1.3.1. was assayed by the continuous method of Dixon and Kornberg (1959). Malate synthase, EC 4.1.3.2. was initially assayed by the method of Dixon and Kornberg (1959) except that phosphate buffer pH 8.0 was used instead of Tris-HCl. A more reliable and sensitive assay of this enzyme was used in later experiments which involved the use of the chromogen, 5,5'-dithiobis-(2-nitrobenzoate)(DTNB) to measure CoASH production. The reaction mixture contained (μmoles in 1 ml final volume); potassium phosphate buffer, pH 8.0, 80; magnesium chloride, 5; DTNB 0.1; acetyl-CoA, 0.05, and extract up to 0.2 mg protein. Sodium glyoxylate (0.2μmoles) was added to start the reaction. The extinction at 412 nm was monitored and correction was made for endogenous CoASH production.

(f) Cytochrome assays

Cytochromes were determined as described by Widdowson and Anthony (1976).

2.13 Molecular weight and kinetic studies of citrate synthase

The molecular weight of citrate synthase was determined by gel-filtration on Sephadex G-200 by the procedure of Andrews (1965). A sonic extract prepared

from methanol-grown Pseudomonas AM1 as described in Section 2.10 was partially purified by the following procedure:

(a) Protamine sulphate precipitation

To 6.0 ml of the sonic extract, previously adjusted to pH 6.0 with 0.1M HCl, a 1.8% solution of neutralised protamine sulphate was added slowly with continuous stirring at room temperature until no further precipitate was observed. The precipitate was discarded after centrifugation at 10,000g for 10 min.

(b) Ammonium sulphate fractionation

The remaining supernatant was adjusted to pH 7.0 and solid ammonium sulphate was added at room temperature over a period of 15 min. to give 50% saturation. The precipitate was removed by centrifugation and discarded; further ammonium sulphate was added to the supernatant to give 70% saturation. Before centrifugation was commenced a further 15 min. was allowed for completion of precipitation. The 50-70% ammonium sulphate precipitate was dissolved in a small volume of buffer (pH 7.0) to a protein concentration of about 7mg/ml.

A sample (3ml) of the partially purified enzyme was applied to a column of Sephadex G-200 (dimensions 2.5 x 51cm) previously equilibrated with 0.05M tris-HCl buffer, pH 7.4. Proteins were eluted with the same buffer and collected in 1.9ml fractions at 0°. Citrate synthase was assayed by the method described

in Section 2.12b.

The column used was calibrated with the following standard proteins: horse heart cytochrome c (M.wt. 12,400), ovalbumin (45,000), pig heart lactate dehydrogenase (135,000), rabbit muscle pyruvate kinase (237,000) and urease (490,000). Blue Dextran (Pharmacia) was used to measure the void volume (Vo) of the column. The calibration curve of the column is shown in Fig. 2.2.

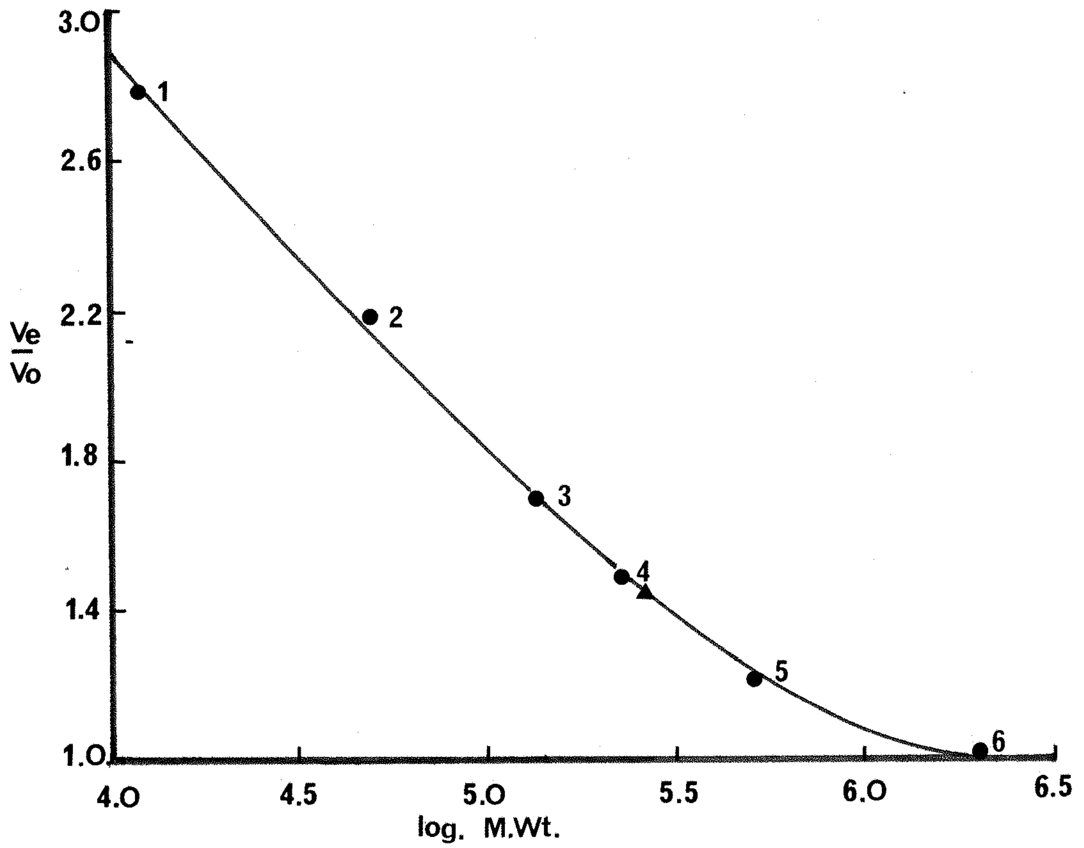
The assay used in kinetic studies of citrate synthase was modified from that of Srere (1969). The standard reaction mixture contained (μmoles in 1 ml); tris-HCl buffer, pH 8.0, 100; EDTA, 1.0; DTNB, 0.1; acetyl-CoA, 0.1; oxaloacetate, 0.5 and enzyme 90 μg. The reaction was started by the addition of oxaloacetate. The enzyme had been slightly purified by heating the sonic extract at 60° for 5 minutes to remove malate dehydrogenase activity. Precipitated protein was removed by centrifugation. For the determination of the K_m for oxaloacetate 0.8 μmoles of acetyl-CoA was used. Potential inhibitors were incubated with the enzyme for 1 min. before addition of oxaloacetate.

The kinetic data were processed using the methods of Wilkinson (1961) and Atkins (1973) on a Hewlett-Packard Model 10 desk top computer.

2.14 Partial purification of acetyl-CoA synthetase from Pseudomonas AM1

A typical purification procedure involved the

Fig. 2.2 Calibration of Sephadex G-200 column



Standards: (1) cyctochrome C (12,4000)
(2) ovalbumin (45,000)
(3) lactate dehydrogenase (135,000)
(4) pyruvate kinase (237,000)
(5) urease (490,000)
(6) blue dextran

▲ indicates citrate synthase of Pseudomonas AM1

following steps: a sonic extract of β-hydroxybutyrate-grown bacteria was dialysed at 2° against 20 vols of 0.02M phosphate buffer, pH 7.0 for 15 hours. The dialysate was treated with protamine sulphate as described in Section 2.13a and precipitated nucleic acid and protein were removed by centrifugation. The supernatant was heated at 60° for 15 min. and solid ammonium sulphate was added at room temperature to the supernatant after centrifugation to remove precipitated protein. After addition of salt, periods of 15 min. were allowed for the completion of precipitation. The fraction precipitating between 52% and 75% saturation was dissolved in 0.02M phosphate buffer to give a protein concentration of approximately 10mg/ml. The percentage saturation values recorded are relative to full saturation at room temperature.

The partially purified acetyl-CoA synthetase obtained from the above procedure was separated from adenylate kinase by gel-filtration on a column of Sephadex G-150. Approximately 2.0ml of the acetyl-CoA synthetase fraction was applied to the column (dimensions 1.5 x 23cm) previously equilibrated with 0.02M phosphate buffer, pH 7.0. Proteins were eluted with the same buffer at room temperature and collected in 1.0ml fractions. Blue Dextran (Pharmacia) was used to measure the void volume (Vo) of the column. Acetyl-CoA synthetase and adenylate kinase were separated as shown in Fig. 3.4.

2.15 Assay of ADP production and adenylate kinase

ADP formation was coupled to NADH oxidation by the use of pyruvate kinase and lactate dehydrogenase in the presence of phospho(enol)pyruvate and NADH (Fig. 3.3).

The following assay mixture (μmoles in a total volume of 3.0ml) was used in an attempt to assay ADP-producing acetyl-CoA synthetase: CoASH, 4.0; ATP, 2.0; sodium acetate, 20; potassium phosphate buffer, pH 7.4, 100; $MgCl_2$, 10; reduced glutathione, 10; NADH, 2.5; phospho(enol)pyruvate, 5.0; pyruvate kinase and lactate dehydrogenase, 10 units each and acetyl-CoA synthetase fraction, 50-200μg protein (added to start reaction). Endogenous NADH oxidation was monitored at 340nm for 1 min. before the addition of enzyme.

Adenylate kinase was assayed by the above method except that acetate and CoASH were replaced with 1.0 μmole of AMP.

2.16 Amino acid analysis and poly-β-hydroxybutyrate estimation

(a) Poly-β-hydroxybutyrate was estimated by the method of Law and Slepeky (1961).

(b) Amino acid analysis

Amino acids present in the growth medium of bacterial cultures were analysed on a JEOL model JLC 6AH amino acid analyser with the help of Dr. M. Gore and Mr P. Vincent. Bacteria were harvested by centrifugation at 10,000g for 10 minutes and portions of the supernatant were filtered to remove any remaining cells.

Samples of 2.5 ml were analysed after the pH had been adjusted to 2.0. Norleucine was used as standard.

Lysine could not be detected by the amino acid analyser because of the high concentration of ammonia present in the samples. Attempts to remove ammonia before analysis by heating at 60°, pH 12 were unsuccessful and it was necessary to assay lysine separately by the spectrophotometric method of Vogel and Shimura (1971).

2.17 Bacterial incorporation of ^{14}C-acetate

Bacterial cultures were harvested in the mid-logarithmic phase of growth as described in Section 2.4, washed and suspended in salts medium to a concentration of 2-3 mg dry weight/ml. A suitable volume of this suspension was incubated at 30° for 10 minutes in the presence of 2.5 μmoles/ml of non-radioactive acetate. To each sample 5 μCi/ml of U-^{14}C-acetate was added; 1 ml aliquots were taken after 17 and 60 seconds incubation, transferred immediately to 3.0 ml of absolute ethanol (boiling) and left for 10 minutes. The ethanolic suspensions were then transferred to a bath at 50° for a further 15 minutes. Insoluble material was removed by centrifugation and resuspended in 1.0 ml of 20% aqueous ethanol before being centrifuged again. The supernatants were combined and evaporated to dryness under reduced pressure at 35°.

2.18 Chromatographic analysis

The residues obtained from the above procedure

were suspended in 0.5 ml of 0.01M HCl and applied to small Dowex columns (10 x 0.6 cm, H^+ form). Neutral and acidic compounds were eluted with 10 ml water and basic compounds with 4.0 ml 2N NH_4OH. These fractions were evaporated to dryness, dissolved in 0.3 ml 20% ethanol and spotted on to Whatman No. 1 chromatography paper (dimensions 20 x 57 cm). The acidic fractions were run in ethanol:ammonia:water (16:1:3) with a mixture of standard carboxylic acids (30 µg each of citric, malic, succinate, fumaric and glycollic acids) either side of the samples to be analysed. Basic compounds were separated in phenol:ammonia:water (200 ml water satd. phenol+1 ml NH_3) with a mixture of the following amino acids (6 µg each): alanine, glycine, serine, aspartic and glutamic acids.

2.19 Detection of radioactive compounds

After drying the chromatograms, strips either side of the unknown samples containing the standard amino or carboxylic acids were cut off and stained as follows:

Carboxylic acids; acridine (0.1% w/v) in 99.5% ethanol. Carboxylic acids appeared as yellow spots which fluoresced under U.V. light.

Amino acids; ninhydrin (0.5% w/v) in acetone. After drying the colour was developed for a few minutes at 110°. 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 and placed in 2.0 ml water and shaken for 5 minutes. The paper was removed and a sample of the eluant counted in NE250 scintillant. All counting was carried out in a Phillips liquid scintillation analyser programmed for automatic quench correction.

2.20 Co-chromatography of radioactive compounds

The position of the radioactive peaks compared with the positions of the standards gave the possible identity of the labelled compounds. The identity of these compounds was confirmed by co-chromatography with authentic standards in the original solvent and a second solvent. The second solvent was butanol: acetic acid:water (12:3:5 by vol.) for both amino and carboxylic acids.

A suitable volume of the radioactive sample was evaporated to dryness together with authentic sample. The residue was dissolved in 0.05 ml of 20% aq. ethanol and spotted on to the chromatography paper. After drying, chromatograms were stained as described previously. The paper was cut into small strips and counted in scintillation fluid. When radioactivity was detected in the strips corresponding to the stained area in both solvents the radioactive sample was taken to be identical with the standard.

2.21 Incorporation of radioactivity from ^{14}C-acetate into growing cultures of bacteria

Cultures (200 ml) were shaken at 30° in Erlenmeyer

flasks (500 ml) fitted with side arms for assessment of growth at 650 nm. The medium contained β-hydroxybutyrate (0.1%) to which 2 μCi of U-^{14}C-acetate together with non-radioactive acetate (final conc. 2 mM) were added once the cells had started to grow exponentially. Samples containing 0.2 - 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 x 5 ml, 0.1M) and water (2 x 5 ml), transferred on the filters to scintillation vials and dissolved in 10 ml of NE 250 scintillant for estimation of radioactivity. Incorporation of radioactivity is expressed as specific activities (d.p.m/μg dry weight of bacteria).

Chapter 3

The formation of acetyl-CoA during growth of Pseudomonas AM1 on β-hydroxybutyrate, ethanol and C_3-compounds

3.1 Introduction

In addition to the central role of acetyl-CoA in intermediary metabolism, acetyl-CoA or acetate have been shown to be of vital importance during the assimilation of a number of growth substrates by Pseudomonas AM1. Such substrates include C_1-compounds and the multicarbon compounds β-hydroxybutyrate, ethanol and malonate (Dunstan and Anthony, 1973); lactate and pyruvate (Salem, Wagner, Hacking and Quayle, 1973a).

The function of this chapter is to describe some of the enzymes involved in the metabolism of these multicarbon growth compounds to acetyl - CoA by Pseudomonas AM1. Probable pathways operating during growth of Pseudomonas AM1 on β-hydroxybutyrate, ethanol and malonate are proposed as a result of enzymic analyses of bacterial extracts prepared from cells grown on a variety of carbon sources and the properties of a mutant lacking a key enzyme of C_2 metabolism (acetyl-CoA synthetase).

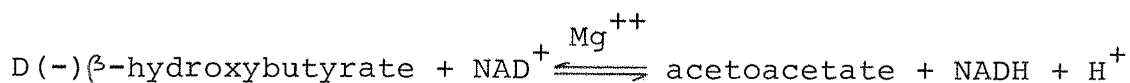
3.2 The enzymology of β-hydroxybutyrate oxidation to acetyl-CoA

Pseudomonas AM1 grows on β-hydroxybutyrate with a mean generation time of between 6 and 7 hours. Furthermore, an insoluble polymer of β-hydroxybutyrate (poly-β-hydroxybutyrate, PHB) is present in granules within the organism under all conditions of growth and it is likely that the metabolism of this endogenous carbon source is similar to the metabolism

of exogenous β-hydroxybutyrate.

The following enzymes have been assayed in the soluble fraction of extracts of Pseudomonas AM1 grown on a variety of carbon sources; β-hydroxybutyrate dehydrogenase, acetoacetate : succinate CoA transferase, β-ketothiolase and acetoacetyl-CoA reductase (Table 3.1).

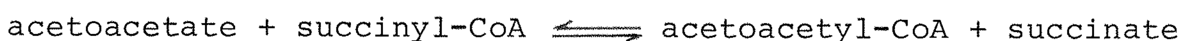
(a) β-Hydroxybutyrate dehydrogenase



$$D(-)\beta\text{-hydroxybutyrate} + NAD^{+} \overset{Mg^{++}}{\rightleftharpoons} \text{acetoacetate} + NADH + H^{+}$$

This is the first of three enzymes involved in the oxidation of β-hydroxybutyrate to acetyl-CoA. The enzyme was NAD^{+}-specific, no activity being detected with $NADP^{+}$ as the hydrogen acceptor. Magnesium ions (10 mM - $MgCl_2$) were required for maximum activity although 30% of full activity was observed in their absence. β-hydroxybutyrate dehydrogenase was induced (2.5 - 10 fold) during growth of Pseudomonas AM1 on β-hydroxybutyrate compared with the other substrates (Table 3.1).

(b) Acetoacetate:succinate CoA transferase



$$\text{acetoacetate} + \text{succinyl-CoA} \rightleftharpoons \text{acetoacetyl-CoA} + \text{succinate}$$

The specific activities of acetoacetate:succinate CoA transferase showed much less variation with carbon source than β-hydroxybutyrate dehydrogenase. The lowest value was 36.0 for extracts of ethanol-grown bacteria compared with 95 nmoles/min/mg protein for lactate-grown bacteria. It is

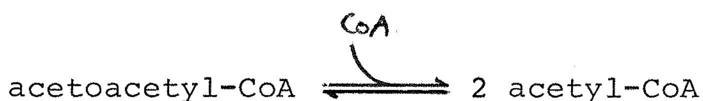
Table 3.1 Specific activites of enzymes of β-hydroxybutyrate metabolism in extracts of Pseudomonas AM1 grown on different carbon sources

Cells were harvested in the mid-logarithmic phase of growth and enzymes were assayed as described in Section 2.12. Enzyme levels are expressed as nmoles/mg protein/min, and are the average of those obtained with at least two separate extracts.

Enzyme	Growth substrate				
	β-hydroxy-butyrate	succinate	methanol	ethanol	lactate
β-hydroxy-butyrate 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

therefore unlikely that the enzyme is induced during growth on β-hydroxybutyrate. The enzyme was routinely assayed by following the disappearance of acetoacetyl-CoA (which absorbs at 303 nm) on addition of succinate to the reaction mixture. The following compounds were inactive as replacements for succinate in extracts prepared from both β-hydroxybutyrate- and methanol-grown bacteria: malate, acetate, propionate, glycollate and formate. As these compounds were not activated by transfer of CoA from acetoacetyl-CoA it can be assumed that in the direction of acetoacetate activation the enzyme is specific for succinyl-CoA.

(c) β-ketothiolase



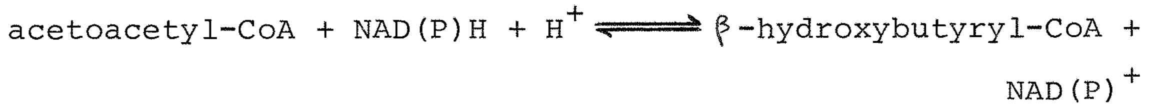
The final enzyme of β-hydroxybutyrate metabolism to acetyl-CoA is β-ketothiolase. This enzyme like β-hydroxybutyrate dehydrogenase was induced during growth of Pseudomonas AM1 on β-hydroxybutyrate (Table 3.1). β-ketothiolase was assayed in the directions of both acetoacetyl-CoA cleavage and acetyl-CoA condensation. The ratio of specific activities was constant; the rate of the cleavage reaction being about four times that of condensation (Table 3.2). The constant ratio demonstrates that one enzyme is involved in both acetyl-CoA condensation and acetoacetyl-CoA cleavage and indicates that β-ketothiolase is also involved in poly-β-hydroxybutyrate biosynthesis.

Table 3.2 Activities of β-ketothiolase cleavage and condensation reactions in extracts of Pseudomonas AM1 grown on various carbon sources

Extracts were prepared and assayed as described in Sections 2.10 and 2.12 respectively. Activities are expressed as nmoles acetoacetyl-CoA used or produced /mg protein/min.

	β-ketothiolase activity		
Growth substrate	cleavage reaction	condensation reaction	ratio of activities
β-hydroxybutyrate	1260	310	4.15
malate	-	50	-
malate + β-hydroxybutyrate	1370	312	4.40
methanol	279	75	3.70
succinate	330	87	3.90

(d) Acetoacetyl-CoA reductase (β-hydroxybutyryl-CoA dehydrogenase)

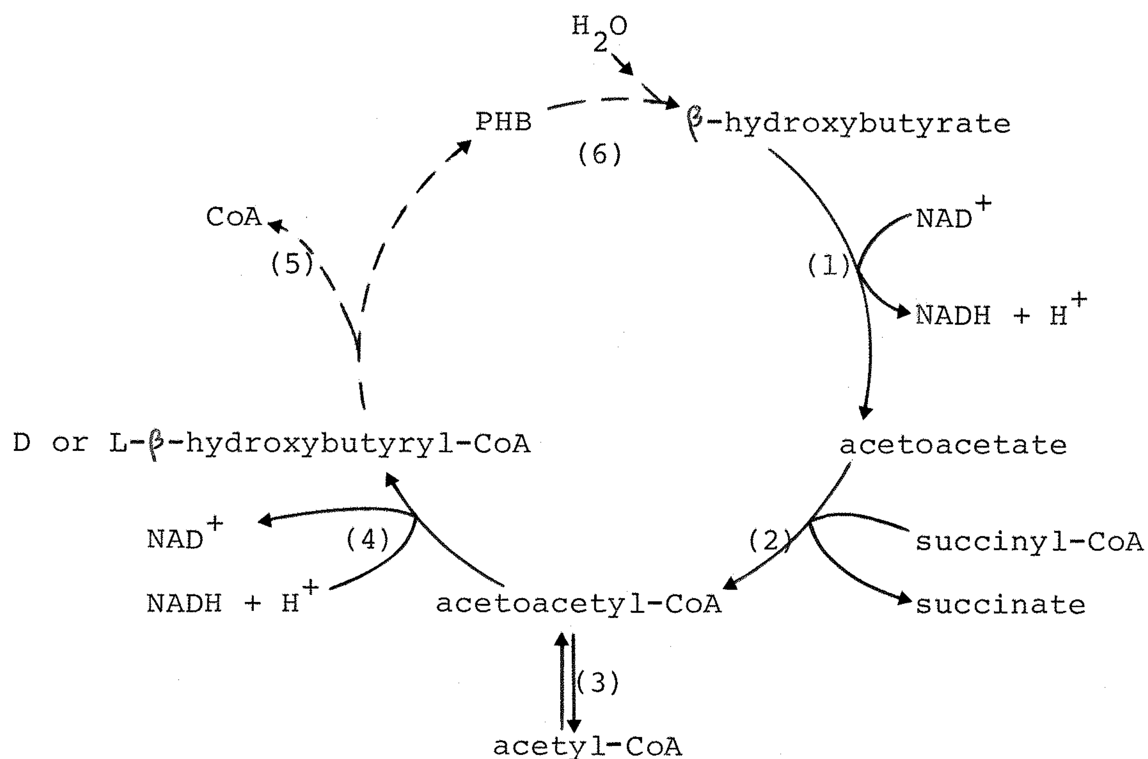


$$\text{acetoacetyl-CoA} + \text{NAD(P)H} + \text{H}^{+} \rightleftharpoons \beta\text{-hydroxybutyryl-CoA} + \text{NAD(P)}^{+}$$

Acetoacetyl-CoA reductase is the second enzyme of poly-β-hydroxybutyrate biosynthesis. The enzyme from *Pseudomonas* AM1 was present with similar activities in extracts of bacteria grown on all the carbon sources used. In the presence of NADPH approximately 10% of the activities measured with NADH was detected.

As discussed in Section 1.8 nicotinamide nucleotide-linked acetoacetyl-CoA reductases have been reported in several PHB-producing organisms. Whilst the enzymes from *Hydrogenomonas* and *Bacillus* cereus favoured NADH as the coenzyme, the enzyme from *Azotobacter beijerinkii* preferred NADPH, NADH giving 20% of maximum activity (Dawes and Senior, 1973). The product of acetoacetyl-CoA reduction in *A. beijerinkii* was identified as D(-)β-hydroxybutyryl-CoA which is directly incorporated into PHB (Ritchie, Senior and Dawes, 1971); conversely the product of acetoacetyl-CoA reduction in *Rhodospirillum rubrum* was identified as L(+)β-hydroxybutyryl-CoA and it was proposed that this isomer is first converted into the D(-) form through the intermediacy of crotonyl-CoA before incorporation into PHB (Moskowitz and Merrick, 1969). It would be of interest to identify the product of acetoacetyl-CoA reduction in *Pseudomonas* AM1 to distinguish between the two possible routes

Fig. 3.1 β-hydroxybutyrate and poly-β-hydroxybutyrate metabolism in Pseudomonas AM1



(1) NAD^+ specific β-hydroxybutyrate dehydrogenase

(2) acetoacetate:succinate CoA transferase

(3) β-ketothiolase

(4) acetoacetyl-CoA reductase (NAD-specific)

Reactions shown as dotted lines have not been investigated in Pseudomonas AM1.

(5) D(-)β-hydroxybutyryl-CoA polymerase (PHB synthetase)

(6) PHB depolymerase

of β-hydroxybutyrate incorporation into PHB.

The presence of the enzymes described above suggests the scheme shown in Fig. 3.1 for β-hydroxybutyrate (and PHB) metabolism in Pseudomonas AM1. Although no attempt has been made to assay PHB polymerase and depolymerase activities in the present study, it is likely that these enzymes are similar to those described in other systems (Sections 1.8,9; Senior and Dawes, 1973).

3.3 Factors affecting the poly-β-hydroxybutyrate (PHB) content of Pseudomonas AM1

In an attempt to demonstrate the physiological significance of PHB accumulation in Pseudomonas AM1, the PHB content was measured in bacteria subjected to varying conditions of growth. A number of factors which may affect levels of PHB accumulation were examined; these include carbon source, nitrogen or oxygen limitation and growth phase.

(a) Carbon source

Bacteria were grown on various carbon sources, harvested at the end of exponential growth and analysed for poly-β-hydroxybutyrate as described in Section 2.14. The results in Table 3.3 show that the carbon source has relatively little effect on the level of PHB accumulation. Values ranged from 3% of the organisms dry weight during growth on β-hydroxybutyrate to 8.0% during growth on methanol. The mean value was 6% PHB. It is of interest to note that the lowest value of PHB accumulation occurred when bacteria were grown on β-hydroxybutyrate; the reason for this may be related to the higher activities of enzymes involved in

PHB degradation during growth of Pseudomonas AM1 on β-hydroxybutyrate.

(b) Nitrogen concentration and oxygen limitation

The preliminary experiments described below indicated that neither the nitrogen concentration nor oxygen limitation had any significant effect on the levels of PHB accumulation in Pseudomonas AM1 growing on succinate.

In an experiment designed to measure the effect of nitrogen concentration, bacteria were grown in batch cultures on salts medium containing ammonium sulphate varied from 0.1 g (limiting) to 2.0 g/litre. The levels of PHB in cells harvested at the end of exponential growth were constant and formed approximately 5% of the dry weight.

Similarly, a 10 litre batch culture of bacteria growing on succinate and limited by oxygen during the exponential phase of growth showed no change in the level of PHB in samples taken throughout the period of oxygen limitation (5 hours). However, once the culture had reached the stationary phase of growth there was a steady decrease in the level of PHB from 6.5% to 3.0% of the dry weight of the bacteria. It is reasonable to assume that this was due to the degradation of this endogenous storage compound in the absence of an exogenous carbon source.

To obtain a better understanding of the significance and regulation of PHB accumulation in this (and other) methylotrophic bacteria it would be necessary to extend the present study with the use of more reliable methods involving continuous culture techniques. Bacteria could then be held in a steady state of carbon, nitrogen or oxygen

Table 3.3 PHB content of Pseudomonas AM1 grown on various carbon sources

Bacteria were harvested at the end of exponential growth, washed and analysed for PHB as described in Section 2.16. Values are given as a percentage of the dry weight of the sample.

Growth substrate	PHB content
methanol	8.0
succinate	4.1
lactate	5.6
malonate	7.6
ethanol	7.5
β-hydroxybutyrate	2.8

limitation for long periods, and other factors such as the effect of the growth rate of the culture on PHB accumulation could be assessed.

3.4 The enzymology of ethanol metabolism to acetyl-CoA

(a) Alcohol dehydrogenase

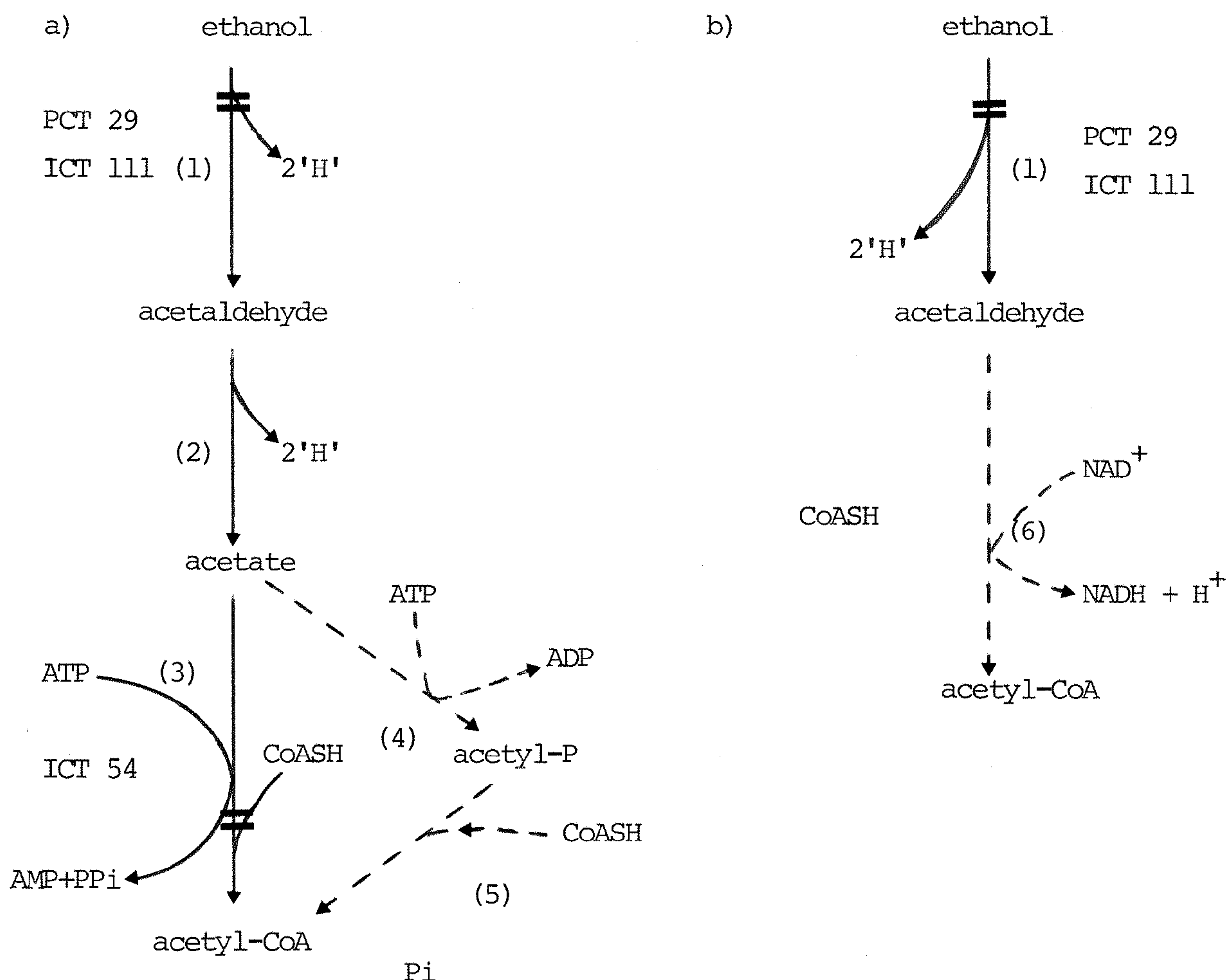
In *Pseudomonas* AM1 ethanol is oxidised to acetaldehyde by the alcohol dehydrogenase responsible for the oxidation of methanol by this organism. The enzyme is independent of NAD^+ or $NADP^+$ and extracts are absolutely dependent on the artificial hydrogen acceptor phenazine methosulphate for activity, and also ammonia or methylamine as activator (Anthony and Zatman, 1965). Mutants deficient in alcohol dehydrogenase activity do not oxidise ethanol and cannot utilise ethanol as a carbon source; such mutants include PCT 29 (Dunstan *et al.*, 1972a) and ICT 111 (Section 7.3).

(b) Oxidation of acetaldehyde

There are two potential routes for the metabolism of acetaldehyde produced during ethanol oxidation. The first of these involves the direct oxidation and activation to acetyl-CoA by an NAD^+, CoA-dependent aldehyde dehydrogenase (Burton and Stadtman, 1953). The second route requires oxidation to free acetate which would then be activated to acetyl-CoA in a reaction catalysed by acetyl-CoA synthetase, or by the sequential action of acetate kinase and phosphotransacetylase (Fig. 3.2).

Ethanol-grown *Pseudomonas* AM1 contained no detectable NAD^+, CoA-dependent acetaldehyde dehydrogenase although extracts of ethanol-grown *Hyphomicrobium* X contained significant

Fig. 3.2 Possible routes of ethanol oxidation by Pseudomonas AM1



The reactions shown as dotted lines indicate enzymes which were not detected in extracts of ethanol-grown Pseudomonas AM1. Metabolic lesions in the designated mutants are shown with parallel bars.

(1) methanol dehydrogenase
(2) NAD(P)-independent aldehyde dehydrogenase
(3) acetyl-CoA synthetase
(4) acetate kinase
(5) phosphotransacetylase
(6) NAD^+, CoA-dependent aldehyde dehydrogenase

activity of this enzyme (specific activity 40 nmoles/min/mg. protein). Acetaldehyde is therefore presumably oxidised to the level of free acetate by the NAD(P)-independent aldehyde dehydrogenase described by Johnson and Quayle (1964). However, the activity of this enzyme (assayed with DCPIP as electron acceptor) was very low and this would account for acetaldehyde accumulation which is observed during growth of *Pseudomonas* AM1 on ethanol. It should be noted that no nicotinamide nucleotide-linked aldehyde dehydrogenase has been measured in extracts of *Pseudomonas* AM1.

(c) Activation of acetate to acetyl-CoA

No attempt was made to assay phosphotransacetylase activity in *Pseudomonas* AM1 as acetate kinase could not be detected in extracts of bacteria grown on a number of different carbon sources. Acetyl-CoA synthetase was present in extracts of *Pseudomonas* AM1 and showed induced levels in bacteria grown on β-hydroxybutyrate and malonate (Table 3.4). The relatively low specific activity of this enzyme in extracts of ethanol-grown bacteria (20 nmoles/min/mg. protein) is nonetheless sufficient to account for the growth rate of *Pseudomonas* AM1 on this compound. Further evidence for the requirement of acetyl-CoA synthetase during growth of *Pseudomonas* AM1 on ethanol is presented in Section 4.7.

These results suggest that ethanol is oxidised to acetyl-CoA by the following enzymes: methanol dehydrogenase, NAD(P)-independent aldehyde dehydrogenase and acetyl-CoA synthetase (enzymes (1) - (3), Fig. 3.2a). This contrasts

Table 3.4 Specific activites of acetyl-CoA synthetase in extracts of Pseudomonas AM1 grown on various carbon sources

Cell-free extracts of bacteria were prepared as described in Section 2.10. Acetyl-CoA synthetase was assayed as described in Section 2.12. Enzyme activities are expressed as nmoles product formed/min/mg protein. Figures in parentheses indicate the number of separate extracts assayed.

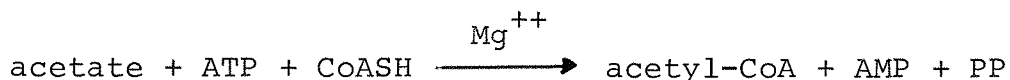
Growth substrate	Specific activity
methanol	13.4 (2)
methylamine	15.2 (2)
ethanol	20.0 (2)
malonate	84.5 (2)
β-hydroxybutyrate	94.6 (3)
lactate	39.6 (2)
malate	12.1 (2)
succinate	17.2 (3)

with the mechanism of ethanol oxidation in *Hyphomicrobium* X. Ethanol-grown *Hyphomicrobium* X contained NAD^+-specific ethanol and acetaldehyde dehydrogenases and acetyl-CoA was produced by an NAD^+, CoA-dependent acetaldehyde dehydrogenase as well as acetyl-CoA synthetase (Attwood and Harder, 1974).

3.5 The significance of acetyl-CoA synthetase induction during growth of Pseudomonas AM1 on β-hydroxybutyrate

The presence of induced levels of acetyl-CoA synthetase during growth of *Pseudomonas* AM1 on β-hydroxybutyrate was unexpected. If acetyl-CoA is the product of β-hydroxybutyrate oxidation (Section 3.2) then an inducible enzyme, β-keto-thiolase, is responsible for acetyl-CoA formation and it might be more logical to find repressed levels of acetyl-CoA synthetase in this situation. The possibility thus arose that acetyl-CoA synthetase from *Pseudomonas* AM1 might function under conditions of high acetyl-CoA concentrations in the direction of acetate and ATP formation. This, or a similar system would be required if acetate was the substrate for oxidation to glyoxylate during operation of the malate synthase pathway (Section 4.2).

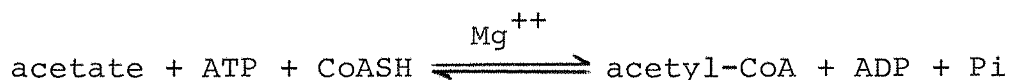
The classical acetyl-CoA synthetase activates acetate in the presence of CoASH and Mg^{++} using energy derived from the hydrolytic cleavage of ATP to AMP and pyrophosphate:



$$\text{acetate} + \text{ATP} + \text{CoASH} \xrightarrow{Mg^{++}} \text{acetyl-CoA} + \text{AMP} + \text{PP}$$

The pyrophosphate is subsequently hydrolysed by pyrophosphatase and its removal renders the synthetase reaction virtually

irreversible. However, an enzyme which could activate acetate using the energy of ATP hydrolysis to ADP and Pi would be readily reversible and during acetyl-CoA formation from β-hydroxybutyrate could be important in both energy and acetate production:



$$\text{acetate + ATP + CoASH} \xrightleftharpoons{Mg^{++}} \text{acetyl-CoA + ADP + Pi}$$

(Such an enzyme would be similar to succinyl-CoA synthetase).

3.6 Identification of the products of acetyl-CoA synthetase

(a) Measurement of ADP production

In an attempt to detect a novel ADP-producing acetyl-CoA synthetase a partially purified preparation was used which was substantially free of succinyl-CoA synthetase activity. The partially pure enzyme was obtained from an extract of β-hydroxybutyrate-grown bacteria after, dialysis, protamine sulphate treatment, heating at 60° for 15 min. and ammonium sulphate fractionation (Section 2.14; Table 3.5).

ADP formation by acetyl-CoA synthetase was coupled to NADH oxidation by lactate dehydrogenase and pyruvate kinase in the presence of phospho(enol)pyruvate and NADH (Fig. 3.3) as described in Section 2.15.

No NADH oxidation was observed when acetate was replaced by either succinate or malate or when any of the following constituents were omitted from the assay: CoASH, ATP, acetate, pyruvate kinase and lactate dehydrogenase. NADH was oxidised by the complete system at a rate proportional to the amount of protein added, and equivalent to the production of 260

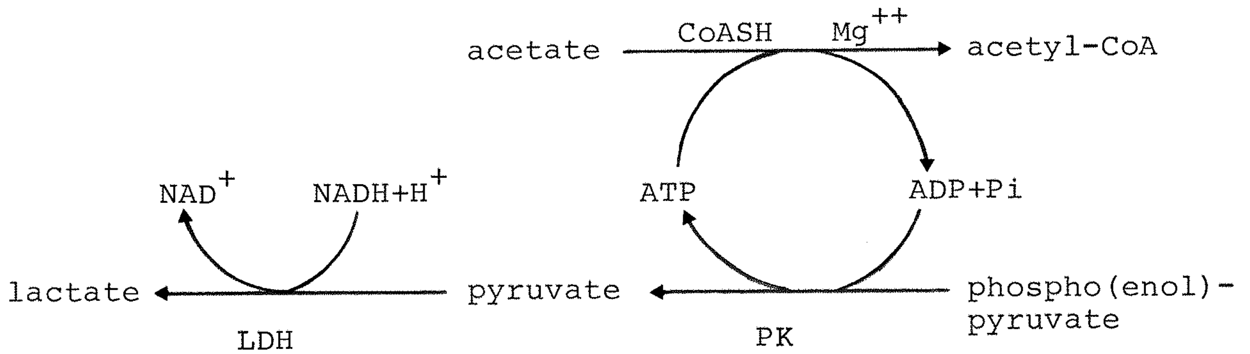
Table 3.5 Purification of acetyl-CoA synthetase

	Step	vol. (ml)	prot/ml (mg)	Munits/ml (nmol)	spec. act. (units/mg protein	total Munits	yield (%)	purification factor
1.	Crude ext.	66	6.8	250	36.4	16,400	-	-
2.	Dialysis	67	5.9	250	42.0	16,700	101	1.15
3.	Prot. Sulph. (SN)	69	3.87	232	58.5	15,820	97	1.60
4.	Heated @60° for 15 mins	65	2.65	200	75.1	12,950	79	2.05
5.	AS 52-75% satd. ppt.	4.2	11.0	1290	118.0	5,310	36	3.2
6.	Active fractions from G150 column (9-16)	8.0	1.73	370	221	2,990	18.4	6.1

Acetyl-CoA synthetase activities were determined by the hydroxylamine method of Jones and Lipmann (1955). The purification procedure was as described in Section 2.14.

Fig. 3.3 Coupled assay for ADP measurement

NADH oxidation (ΔE_{340}) was measured in the presence of acetate, CoASH, Mg^{++}, ATP, NADH, pyruvate kinase (PK) and lactate dehydrogenase (LDH) as described in Section 2.15. Fractions obtained from step 5 of the purification procedure (Table 3.5) were added to start the reaction.



nmoles ADP/min/mg. protein. This rate of ADP production was approximately twice the specific activity of the enzyme when assayed by the standard acetohydroxamate method (120 nmoles/min/mg. protein) and it was assumed that 2 moles of ADP were being produced for every mole of acetyl-CoA. This stoichiometry would be possible if the acetyl-CoA synthetase preparation contained adenylate kinase (myokinase) activity. Adenylate kinase catalyses the interconversion of adenine nucleotides according to the equation:

AMP + ATP $\rightleftharpoons$ 2ADP

Any AMP produced by a 'normal' acetyl-CoA synthetase would therefore be converted to 2ADP in the presence of ATP provided in the reaction mixture. Adenylate kinase was therefore assayed in the acetyl-CoA synthetase preparation (5) by the method described in Section 2.15 and was present with a specific activity of 530 nmoles/min/mg. protein.

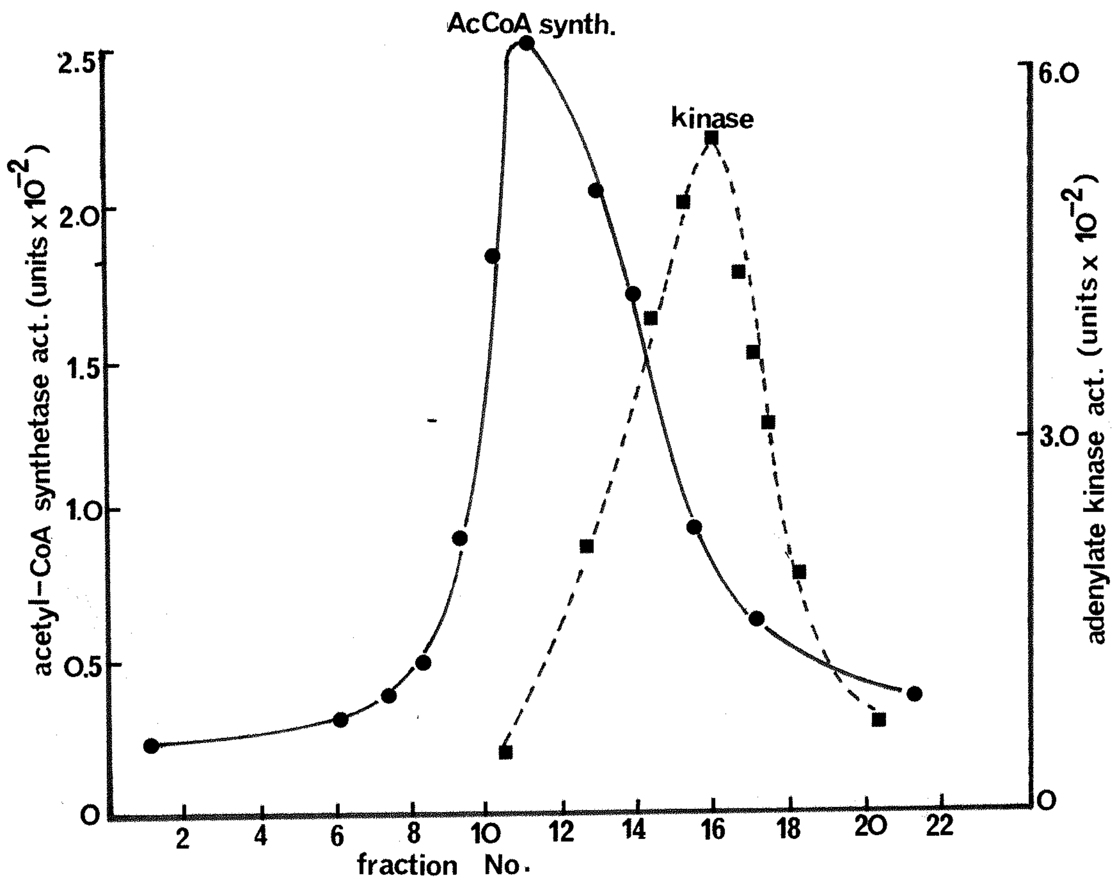
(b) Separation of adenylate kinase and acetyl-CoA synthetase

These enzymes were separated by gel-filtration on a column of Sephadex G-150 as described in Section 2.14, (Fig. 3.4). On repeating the ADP/acetyl-CoA synthetase assay using a fraction with high acetyl-CoA synthetase activity free of adenylate kinase (fraction 10) no ADP formation was detected except when adenylate kinase (fraction 17) was included in the reaction mixture.

The requirement for adenylate kinase for ADP production conclusively demonstrates that AMP and not ADP is the product of ATP hydrolysis by acetyl-CoA synthetase. The enzyme from

Fig. 3.4 Separation of acetyl-CoA synthetase and adenylate kinase activities by Sephadex G150 column chromatography

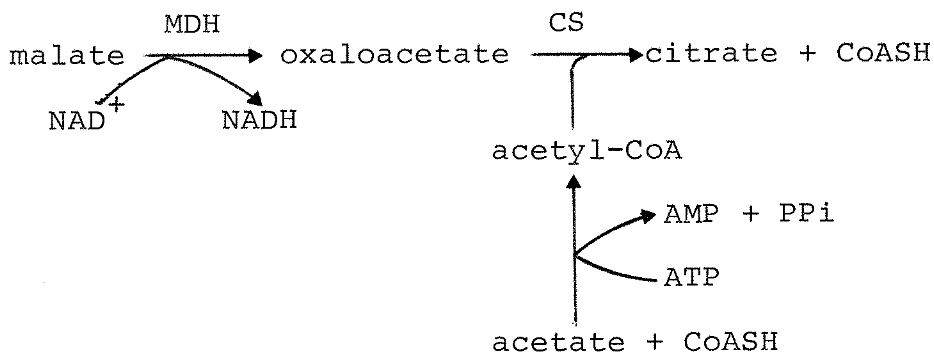
Enzymes were assayed as described in Sections 2.12 and 2.15. Activities are expressed in units (nmoles/min/ml.).



Pseudomonas AM1 therefore resembles that from other sources with respect to the products of ATP hydrolysis and irreversibility in the presence of pyrophosphatase (Jencks, 1962). The results in Section 3.7 with an acetyl-CoA synthetase-deficient mutant of Pseudomonas AM1 show that this enzyme is not required for growth on β-hydroxybutyrate and the high levels acetyl-CoA synthetase in extracts of bacteria grown on this compound are not physiologically significant.

(c) Identification of acetyl-CoA

Acetyl-CoA was shown to be the 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 (μmoles in 2.0 ml): tris-HCl buffer, pH 7.4, 200; sodium malate, 20; sodium acetate, 20; CoASH, 0.1; ATP, 10; $MgCl_2$, 10; NAD^+, 1 and acetyl-CoA synthetase fraction, 50 μg. This fraction was obtained by pooling the most active fractions (9 - 16) from the Sephadex G-150 column. Malate dehydrogenase (20 units) was added and the OD_{340} monitored for 2 - 3 min. before the addition of citrate synthase (5 units). The rate of NADH production

in the absence of citrate synthase was subtracted from the final rate and was equivalent to approximately 120 nmoles acetyl-CoA produced/min/mg. protein.

3.7 Characterisation of mutant ICT 54

Mutant ICT 54 is one of a group of mutants selected for their ability to grow on malate, but not on a combination of acetate + glyoxylate. This selection was designed for the isolation of mutants lacking either acetyl-CoA synthetase or malate synthase activity. The growth responses of mutant ICT 54 to a number of carbon compounds (Table 3.6) show that this mutant cannot grow on either ethanol or malonate, although growth on C_1-compounds, β-hydroxybutyrate, lactate, pyruvate and C_4-acids is unaffected. Malate synthase was present in extracts of ICT 54 with activities comparable to extracts of wild-type bacteria grown on the same carbon source (Table 3.7). Acetyl-CoA synthetase was present with specific activities ranging from 0 - 10% of the activities of the enzyme from wild-type bacteria. These low levels are presumably not sufficient to allow growth of the mutant on ethanol and malonate. Acetyl-CoA synthetase is therefore not required for growth of *Pseudomonas* AM1 on C_1-compounds, lactate, pyruvate and β-hydroxybutyrate despite the observation that the enzyme is induced during growth on the latter. That acetyl-CoA synthetase is not required during growth of *Pseudomonas* AM1 on C_1-compounds is expected because acetyl-CoA would be generated by the malyl-CoA lyase induced during methylotrophic growth (Salem *et al.*, 1973b). Likewise, acetyl-CoA is produced by pyruvate dehydrogenase during growth

Table 3.6 Growth responses of mutants ICT 54, ICT 51 and wild-type Pseudomonas AM1

Growth responses were determined in liquid media as described in Section 2.7. 0 indicates that no growth was observed; 1-5 represents the relative amount of growth after 2 subcultures.

Growth substrate	Strain		
	wild-type	ICT 54	ICT 51
succinate	5	5	5
malate	5	5	5
methanol	5	4	4
ethanol	3	0	0
β-hydroxybutyrate	5	4	0
malonate	4	0	0
lactate	5	4	5
pyruvate	4	4	4
acetate + glyoxylate	3	0	0

Table 3.7 Specific activities of acetyl-CoA synthetase and malate synthase in extracts of wild-type Pseudomonas AM1 and mutant ICT 54

Extracts were prepared as described in Section 2.10. Enzymes were assayed as described in Section 2.12 using the method of Dixon and Kornberg (1959) for malate synthase. Activities are expressed as nmoles substrate used or product formed/min/mg protein.

n.d., not detected; -, not assayed.

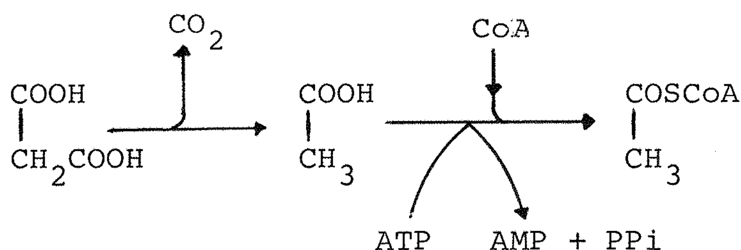
Growth substrate	acetyl-CoA synthetase		malate synthase	
	wild-type	ICT 54	wild-type	ICT 54
methanol	13.4	1.50	45.0	44.5
β-hydroxybutyrate	74.0	n.d.	35.0	30.2
lactate	50.0	0.70	45.6	-
malate	15.0	1.35	24.2	32.0

of Pseudomonas AM1 on lactate and pyruvate, and by β-keto-thiolase during β-hydroxybutyrate assimilation (Section 3.2).

The inability of ICT 54 to grow on ethanol or malonate confirms that acetyl-CoA synthetase is required during growth of wild-type organisms on ethanol (Scheme proposed in Fig. 3.2) and indicates that free acetate is also a product of malonate metabolism by Pseudomonas AM1.

3.8 The metabolism of malonate to acetyl-CoA

Pseudomonas AM1 grows on malonate with a mean generation time of between 7 and 10 hours and whole cells oxidise this compound with a QO_2 of approximately 20 μℓ/hr/mg dry weight. The failure of mutant ICT 54 to grow on malonate indicates that malonate is either directly decarboxylated to free acetate without prior activation to malonyl-CoA or malonate itself is a substrate for acetyl-CoA synthetase which activates it to malonyl-CoA before decarboxylation to acetyl-CoA. Evidence has been obtained for the activation of malonate to malonyl-CoA by extracts of Pseudomonas fluorescens and by mammalian and plant mitochondria (Wolfe, Ivler and Rittenberg, 1954; Giovanelli and Stumpf, 1957). However the very low levels of hydroxamate formed in an activating system containing malonate, CoASH, ATP and Mg^{++} demonstrate that malonate is neither a substrate for acetyl-CoA synthetase, nor is there a separate malonyl-CoA synthetase in Pseudomonas AM1 (Table 3.8). The results suggest that malonate is directly decarboxylated to free acetate which is subsequently activated to acetyl-CoA:



3.9 Oxidation of lactate and pyruvate to acetyl-CoA

It has recently been shown that pyruvate and lactate are metabolised exclusively by way of acetyl-CoA in Pseudomonas AM1 (Salem et al., 1973a). The activity of the pyruvate dehydrogenase complex is induced during growth of Pseudomonas AM1 on lactate and pyruvate (Section 5.3) and the properties of mutant ICT 54 (Table 3.6) show that as expected acetyl-CoA synthetase is not required for growth on these compounds.

3.10 Acyl-CoA synthetase activities in crude extracts of Pseudomonas AM1

The results in Table 3.8 demonstrate that in addition to the absence of a direct activating enzyme for malonate there is no system for the direct activation of β-hydroxybutyrate, malate and glycollate in β-hydroxybutyrate-grown bacteria. The products of acetate and succinate activation in the presence of hydroxylamine were identified as aceto- and succinohydroxamate respectively by paper chromatography with authentic hydroxamates in the solvent system butan-2-ol: formic acid:water (75:15:10). In this system the Rfs for aceto- and succinohydroxamate were 0.60 and 0.41 respectively. Propionate was also activated by this extract at 50% of the rate of acetate activation and it is reasonable to assume

Table 3.8 Acyl-CoA-synthetase activities in β-hydroxybutyrate-grown Pseudomonas AM1

Cell-free extracts of β-hydroxybutyrate-grown bacteria were prepared as described in Section 2.10. Activities (expressed as nmoles hydroxamate produced/min/mg protein) were assayed by the method of Jones and Lipmann (1955) for acetyl-CoA synthetase except that the temperature of incubation was 30° instead of 40°. Correction has been made for hydroxamate formation in the absence of substrate.

n.d., not detected.

Substrate	Activity
acetate	70.0
β-hydroxybutyrate	n.d.
malate	5.6
glycollate	5.6
malonate	5.9
succinate	76.0
propionate	38.0

that this compound is a substrate for acetyl-CoA synthetase as shown with other systems (Jencks, 1962).

3.11 Summary

The results presented in this Chapter show that β-hydroxybutyrate, ethanol, malonate, lactate and pyruvate are all metabolised to acetyl-CoA by Pseudomonas AM1. β-hydroxybutyrate is oxidised by β-hydroxybutyrate dehydrogenase, acetoacetate:succinate CoA transferase and β-ketothiolase. Degradation of poly-β-hydroxybutyrate also involves these enzymes, and β-ketothiolase and acetoacetyl-CoA reductase are used for synthesis of the polymer indicating a scheme for PHB metabolism similar to that in other PHB-producing bacteria (Senior and Dawes, 1973; Oeding and Schlegel, 1973; Fig. 3.1).

The characteristics of a mutant (ICT 54) lacking acetyl-CoA synthetase demonstrate that free acetate is the precursor of acetyl-CoA during assimilation of ethanol and malonate and that acetyl-CoA synthetase is not required for growth of Pseudomonas AM1 on C_1-compounds, lactate, pyruvate and β-hydroxybutyrate despite the observation that the enzyme is induced during growth on the latter compound. Activation of acetate by acetyl-CoA synthetase requires the AMP-PP hydrolysis of ATP and presumably the formation of an acetyl-AMP intermediate.

Chapter 4

The assimilation of acetyl-CoA during growth of Pseudomonas AM1 on C_1-compounds, β-hydroxybutyrate, malonate, ethanol, lactate and pyruvate

4.1 Introduction

In the previous Chapter the metabolism of a number of multicarbon growth substrates to acetyl-CoA by *Pseudomonas* AM1 was described. The subject of this Chapter is the subsequent assimilation of acetyl-CoA to C_4-dicarboxylic acids required for biosynthesis. Two of the pathways used by bacteria for assimilation of C_2 units into cell material have been mentioned in Section 1.5. The glyoxylate cycle does not operate in *Pseudomonas* AM1 and evidence is summarised below for the existence of a novel pathway (the malate synthase pathway) of acetyl-CoA assimilation by this bacterium.

4.2 The malate synthase pathway for growth of Pseudomonas AM1 on ethanol, β-hydroxybutyrate, malonate, lactate and pyruvate

This pathway is essentially similar to the glyoxylate cycle, one molecule of acetyl-CoA (or acetate) is oxidised to glyoxylate and this condenses with a second molecule of acetyl-CoA, in a reaction catalysed by malate synthase, to give malate. It is the route for oxidation of acetyl-CoA (or acetate) to glyoxylate which differs from the glyoxylate cycle and this part of the pathway is involved in methylotrophic growth as well as growth on the above multicarbon coupounds (Dunstan *et al*. 1972a,b; Dunstan and Anthony, 1973). Glycollate may be an intermediate in the oxidation process but the two versions of the pathway shown in Fig. 4.1

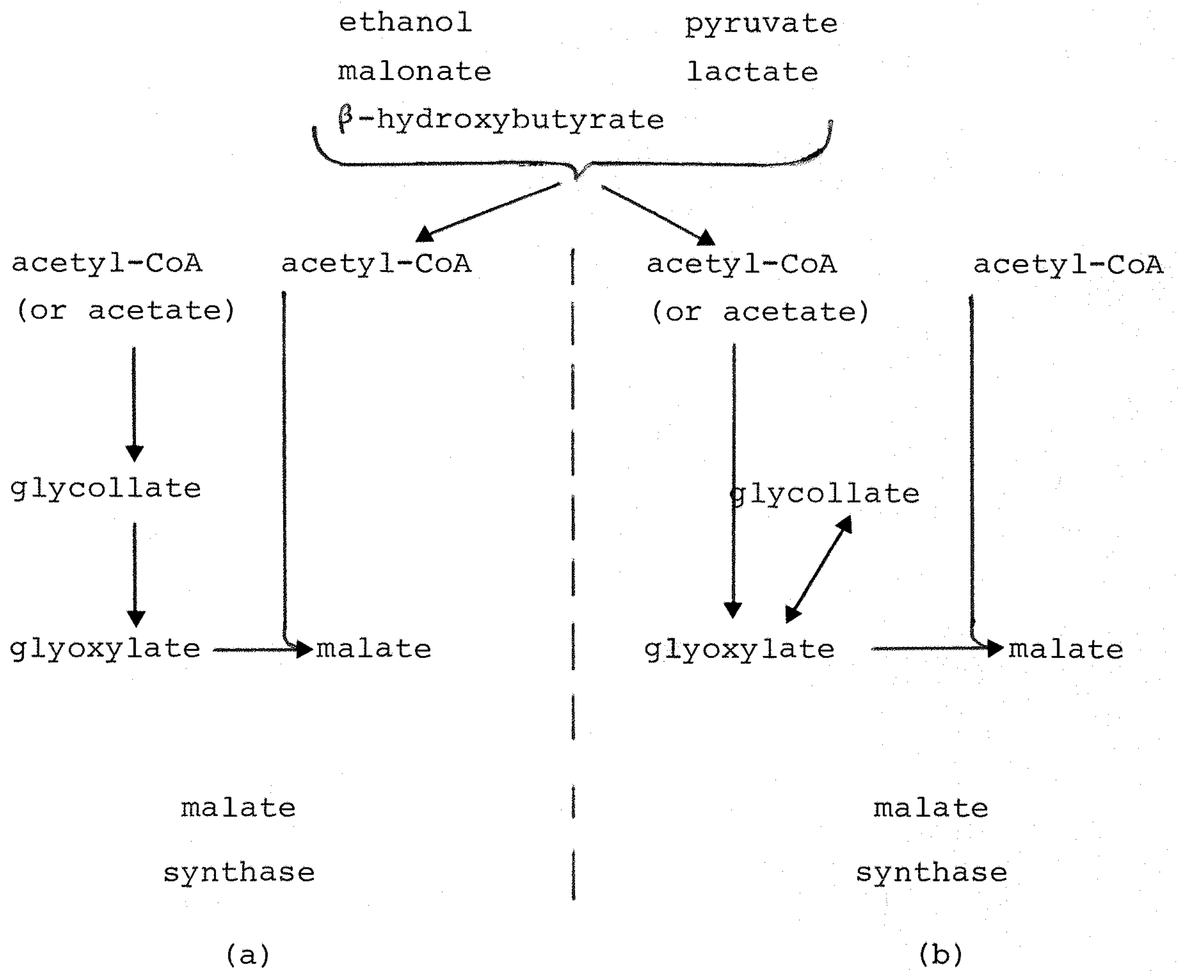
have not been distinguished.

That glyoxylate does not arise from acetyl-CoA by way of the glyoxylate cycle in *Pseudomonas* AM1 is indicated by the lack of isocitrate lyase and labelling patterns found after short-term incubation experiments with ^{14}C-acetate (Dunstan *et al.*, 1972a; 1972b). Results with labelling experiments led to the conclusion that malate is an early intermediate during the assimilation of acetate; glycollate was also an early intermediate but it is possible that this compound was detected because of an equilibrium with glyoxylate mediated by hydroxypyruvate reductase. The labelling patterns differed from those observed in bacteria using the glyoxylate cycle for acetate assimilation; in these bacteria glycollate is not labelled but citrate is labelled in a similar fashion to malate (Kornberg, 1966).

Further evidence for the existence of enzymes able to oxidise acetyl-CoA (or acetate) to glyoxylate during methylotrophic growth as well as growth on β-hydroxybutyrate, ethanol and malonate has come from results with mutants of *Pseudomonas* AM1 (Dunstan *et al.*, 1972b). One mutant, PCT 48, is unable to oxidise acetate to glyoxylate and, as a result, will only grow on C_1-compounds, ethanol, β-hydroxybutyrate or malonate when provided with supplements of glyoxylate or glycollate. Another mutant (PCT 57) lacks malyl-CoA lyase and therefore cannot regenerate glyoxylate in the serine pathway. Growth of this mutant on C_1-compounds occurs only when an alternative source of glyoxylate is provided; this source can be glyoxylate itself or glycollate

Fig. 4.1 The malate synthase pathways of C_2 assimilation

(from Anthony, 1975a)



or acetate. Because glyoxylate formation from acetate is unimpaired in mutant PCT 57, it grows normally on ethanol, β-hydroxybutyrate or malonate (Dunstan and Anthony, 1973).

Evidence for the operation of the malate synthase pathway during growth of Pseudomonas AM1 on lactate and pyruvate has been provided by Salem et al. (1973a). These workers showed that Pseudomonas AM1 contained no enzyme(s) capable of catalysing the phosphorylation of pyruvate to phospho-(enol)pyruvate and demonstrated that the carboxyl carbon atom (C-1) of 1-^{14}C-lactate is lost by decarboxylation of pyruvate to acetyl-CoA during lactate assimilation. It was concluded that lactate and pyruvate are assimilated to C_4-compounds exclusively by way of acetyl-CoA and the malate synthase pathway. Phospho(enol)pyruvate required for carbohydrate biosynthesis is produced by decarboxylation of oxaloacetate derived from acetyl-CoA metabolism via the malate synthase pathway.

Despite the considerable amount of evidence that acetyl-CoA (or acetate) is readily oxidised to glyoxylate by Pseudomonas AM1, doubt about the actual route of oxidation will remain until the enzymes responsible are assayed in cell-free extracts. The function of this Chapter is to confirm the proposed role for malate synthase during assimilation of certain multicarbon compounds, to distinguish between acetate and acetyl-CoA as precursors of glyoxylate and to investigate the possible intermediacy of glycollate in the oxidation of the C_2 unit.

4.3 Characterisation of mutant ICT 5

Mutant ICT 5, isolated by a selection procedure designed to allow growth of mutants on succinate but not on β-hydroxybutyrate, is one of a group of mutants isolated in this way with the same nutritional properties. Mutant ICT 5 grows on succinate, lactate, pyruvate and oxalate but is unable to grow on C_1-compounds, ethanol, malonate or β-hydroxybutyrate unless provided with either glyoxylate or glycollate (5 mM) in the medium (Table 4.1). Extracts of mutant ICT 5 contained all the enzymes of β-hydroxybutyrate oxidation to acetyl-CoA and also enzymes possibly involved in C_2 assimilation (Table 4.2). The growth properties of ICT 5 are the same as those described for mutant PCT 48 (Dunstan and Anthony, 1973) and are very similar to the properties of mutant C_5 reported by Salem et al. (1973a).

Mutant PCT 48 was isolated by selection for growth on succinate but not on methanol. This mutant was shown to possess enzymes of the serine pathway for methylotrophic growth and also enzymes responsible for ethanol (and methanol) oxidation (Dunstan, 1972). The characteristics of mutant PCT 48 led to the conclusion that the mutant was unable to form glycollate and glyoxylate from acetate, necessary for growth on C_1- and C_2-compounds. This conclusion was confirmed by the demonstration that ^{14}C-acetate was not metabolised to glycollate by mutant PCT 48.

Mutant C_5 was isolated by a screening procedure designed to select mutants unable to grow on lactate unless provided with a supplement of succinate (2 mM). Mutant C_5 was

Table 4.1 Growth properties of mutants ICT 5, ICT 51, 20 BL and wild-type Pseudomonas AM1

The growth properties were investigated in liquid media as described in Section 2.7. 0 indicates that no growth occurred; 1-5 represent relative amounts of growth based on visual estimates after two subcultures in the same medium; -, not tested.

Growth substrate	Strain			
	wild-type	ICT 5	ICT 51	20 BL
methanol	5	0	5	0
methylamine	5	0	5	0
ethanol	3	0	0	1
malonate	3	0	0	1
lactate	5	5	4	4
pyruvate	4	4	4	-
β-hydroxybutyrate	5	0	0	4
succinate	5	5	5	5
methanol + glycollate	5	2	-	0
methanol + glyoxylate	5	4	-	0
ethanol + glycollate	5	2	0	0
ethanol + glyoxylate	5	4	0	4
β-hydroxybutyrate + succinate (1mM)	5	-	4	-

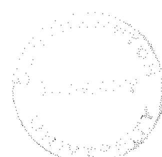


Table 4.2 Specific activities of enzymes of β-hydroxybutyrate oxidation and assimilation in extracts of wild-type Pseudomonas AM1 and mutant ICT 5

Extracts were prepared as described in Section 2.10. Enzyme activities are expressed as nmoles product formed or substrate used/min/mg.prot. Malate synthase was assayed by the method of Dixon and Kornberg (1959).

Enzyme	Specific activity		Growth substrate(s)
	wild-type	ICT 5	
β-hydroxybutyrate dehydrogenase	126	143	succinate
acetoacetate: succinate CoA transferase	70	41	succinate
β-ketothiolase (cleavage reaction)	1360	370	malate + β-hydroxybutyrate
acetyl-CoA synthetase	51	20	lactate
hydroxypyruvate reductase	170	139	malate + β-hydroxybutyrate
malate synthase	41	32	malate + β-hydroxybutyrate

unable to grow on methanol or ethanol even when supplemented with succinate but growth on these compounds was possible when glycollate or glyoxylate was present at a concentration of 10 mM. Growth of C_5 occurred on lactate and pyruvate supplemented with succinate although even in the absence of succinate growth on these compounds was observed after an initial lag of 25 hours (lactate) and 45 hours (pyruvate). Apart from this initial delay before growth of C_5 occurred on these C_3-compounds, this mutant appears very similar to mutants ICT 5 and PCT 48 which grow on lactate and pyruvate at the same rate as wild-type Pseudomonas AM1.

Despite their similarity mutants ICT 5, PCT 48 and C_5 were all isolated from different screening procedures. The preponderance of this type of mutant of Pseudomonas AM1 suggests that the phenotype has not arisen by double mutation. It is also possible that a number of enzymic reactions are involved in the oxidation of acetate or acetyl-CoA to glyoxylate or glycollate.

The growth properties of mutant ICT 5 support the results obtained with mutant PCT 48 which indicate that the reaction(s) involved in the oxidation of acetate (or acetyl-CoA) to glyoxylate are required for growth of Pseudomonas AM1 on C_1-compounds, ethanol, β-hydroxybutyrate and malonate. However, the ability of mutants ICT 5 and PCT 48 to grow on lactate and pyruvate at the same rate as wild-type bacteria, and also the observed growth of mutant C_5 on these compounds (after an initial delay) is inconsistent with the suggestion that similar C_2 oxidation reactions are involved during

growth of *Pseudomonas* AM1 on these C_3-compounds (Salem *et al.*, 1973a).

4.4 Characterisation of mutant ICT 51

Mutant ICT 51 was isolated in an attempt to obtain mutants lacking malate synthase activity. The selection procedure was the same as that used to isolate the acetyl-CoA synthetase deficient mutant, ICT 54 (Section 3.7). The results in Table 4.3 show that mutant ICT 51 lacks malate synthase activity while acetyl-CoA synthetase is present with activities similar to those found in wild-type bacteria. Mutant ICT 51 does not grow on ethanol, malonate or β-hydroxybutyrate (Table 4.1), demonstrating the essential function of malate synthase during growth of *Pseudomonas* AM1 on these substrates. Growth of the mutant occurred on all other substrates (including C_1-compounds) but only trace amounts of malate synthase were detected. The virtual absence of the enzyme from methanol-grown ICT 51 confirms the expectation that malate synthase is not required for methylotrophic growth despite its presence in wild-type *Pseudomonas* AM1 regardless of growth compound (Table 4.3).

The ability of the malate synthase-deficient mutant to grow on lactate and pyruvate suggests that alternative enzymes to those of the malate synthase pathway may exist for the assimilation of these C_3-compounds by *Pseudomonas* AM1. This would provide an explanation for the growth of mutants ICT 5 and PCT 48 on lactate and pyruvate despite their failure to oxidise acetate to glyoxylate.

Table 4.3 Specific activities of malate synthase and acetyl-CoA synthetase in extracts of wild-type Pseudomonas AM1 and mutant ICT 51

Bacterial extracts were prepared as described in Section 2.10. Malate synthase was assayed by the method of Dixon and Kornberg (1959) and acetyl-CoA synthetase by the method of Jones and Lipmann (1955).

Enzyme activities are expressed as nmoles acetyl-CoA used/min/mg. protein. -, not assayed.

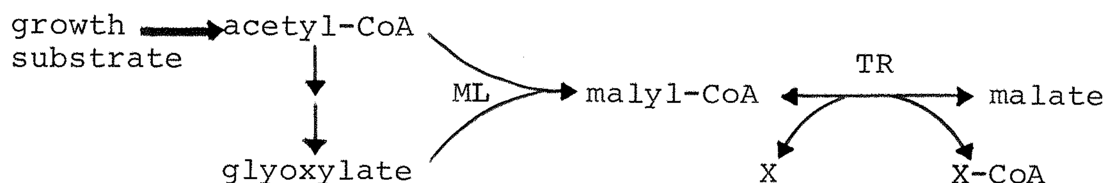
Growth substrate	malate synthase		acetyl-CoA synthetase	
	wild-type	ICT 51	wild-type	ICT 51
methanol	44	1.9	13.4	16.3
β-hydroxybutyrate	35	-	75.0	-
malonate	26	-	84.5	-
ethanol	42	-	20.6	-
malate	25	2.0	13.3	15.0
lactate	45	3.4	54.0	-

4.5 Properties of mutant ICT 51R

To confirm the properties of mutant ICT 51 an attempt was made to isolate a revertant of this mutant which regained malate synthase activity and simultaneously, the ability to utilise β-hydroxybutyrate, ethanol and malonate.

A streptomycin-resistant revertant of mutant ICT 51 was obtained which could grow on a mixture of acetate + glyoxylate, ethanol, β-hydroxybutyrate and malonate. However, extracts of the revertant (ICT 51R) grown on these compounds contained no detectable malate synthase activity (Table 4.4). Attempts to detect malate synthase activity using the method of Dixon and Kornberg (1959) and the DTNB method at pH values ranging from 6.4 to 8.5 were unsuccessful.

Mutant ICT 51R was similar to mutant ICT 51 and wild-type Pseudomonas AM1 in containing significant levels of malyl-CoA lyase even when grown on non-C_1 compounds (Table 4.5). The presence of this enzyme permits the following explanation for the growth of mutant ICT 51R on ethanol, β-hydroxybutyrate and malonate in the absence of malate synthase:



In the above scheme, malate synthase activity is effected by a reversal of the 'normal' malyl-CoA lyase reaction (ML) followed by transfer of CoA from malyl-CoA to an acceptor molecule by a CoA-transferase (TR) The

Table 4.4 Specific activities of malate synthase in extracts of wild-type Pseudomonas AM1 and mutants PCT 57 and ICT 51R

Extracts were prepared from bacteria grown on different carbon sources as described in Section 2.10. Malate synthase was assayed by the method of Dixon and Kornberg (1959).
-, not assayed; n.d., not detected.

Growth Substrate	Strain		
	wild-type	PCT 57	ICT 51R
succinate	31.4	5.2	-
ethanol	52.0	6.2	n.d.
β-hydroxybutyrate	45.0	5.2	n.d.
malonate	26.0	-	n.d.

Table 4.5 Specific activities of malyl-CoA lyase in extracts of wild-type Pseudomonas AM1 and mutants ICT 51 and ICT 51R

Extracts were prepared as described in Section 2.10. Malyl-CoA lyase was assayed by the method of Salem *et al.*, (1973b) and enzyme activities are expressed as nmoles glyoxylate produced/min/mg protein. -, not tested.

Growth substrate	Specific activity		
	wild-type	ICT 51	ICT 51R
methanol	840	920	1320
succinate	200	160	120
lactate	130	160	240
β-hydroxybutyrate	210	-	170

combined activities of malyl-CoA lyase and the hypothetical CoA-transferase would not be detected by either assay of malate synthase as free CoASH is not released, and the thio-ester bond is conserved.

Although the CoA-transferase is purely speculative, such an enzyme could be physiologically important for activation of malate to malyl-CoA during methylotrophic growth of Pseudomonas AM1. It is possible that mutant ICT 51R is a double mutant with a mutation allowing the constitutive production of the CoA-transferase in addition to the original mutation resulting in the loss of malate synthase activity.

Clearly, the factors contributing to the unusual properties of mutant ICT 51R require further investigation.

4.6 Properties of mutant PCT 57

Mutant PCT 57 was isolated by P.M. Dunstan from a screening procedure designed to allow growth of mutants on succinate but not on C_1-compounds. The mutant grew on succinate, pyruvate, ethanol, β-hydroxybutyrate and malonate. No growth was observed on methanol or methylamine unless supplemented with glycollate (5 mM). From these properties Dunstan et al. (1972b) proposed that PCT 57 lacked an enzyme capable of cleaving a C_4-compound to two C_2-compounds necessary for methylotrophic growth of Pseudomonas AM1. This was later confirmed by Salem et al. (1974) who demonstrated that this mutant lacked malyl-CoA lyase activity.

This mutant has now been shown to have very low levels of malate synthase as well as malyl-CoA lyase (Table 4.4). The actual specific activities of malate synthase in extracts

of this strain were approximately 5.0 nmoles/min/mg. protein, corresponding to about 10% of the activity of the enzyme from wild-type bacteria. The level of malyl-CoA lyase in mutant extracts was also about 5.0 nmoles/min/mg. protein (Salem *et al.*, 1974) and neither the individual nor the combined activities of the two enzymes are high enough to account for acetyl-CoA assimilation by the malate synthase pathways during growth of *Pseudomonas* AM1 on compounds such as β-hydroxybutyrate and ethanol.

The absence of both malate synthase and malyl-CoA lyase from PCT 57 is difficult to interpret and further investigations are necessary to determine the route for assimilation of acetyl-CoA in this mutant. These observations lead to another consideration; that malate synthase and malyl-CoA lyase may be the same enzyme in *Pseudomonas* AM1. However this is unlikely because (a) the ratio of malyl-CoA lyase to malate synthase varies in wild-type *Pseudomonas* AM1 grown on different carbon sources (Salem *et al.*, 1973a) and (b) although mutants ICT 51 and ICT 51R contained levels of malyl-CoA lyase similar to wild-type bacteria, no malate synthase was formed. This problem however, will not be unequivocally solved until either the two purified activities are separated or a mutant lacking malyl-CoA lyase while retaining malate synthase is isolated.

4.7 The involvement of glycollate during acetyl-CoA assimilation by the malate synthase pathway

The identification of early labelled glycollate after short-term incubation of ^{14}C-acetate with whole cells of

ethanol-grown Pseudomonas AM1 led Dunstan et al. (1972b) to suggest that the reactions involved in the oxidation of acetate or acetyl-CoA to glyoxylate probably involve glycollate as an intermediate (Fig. 4.1a).

Additional evidence for this was obtained using a mutant of Pseudomonas AM1 lacking hydroxypyruvate reductase. Mutant 20 BL was isolated by Heptinstall and Quayle (1970) who showed that it was unable to grow on C_1-compounds thus demonstrating the essential function of hydroxypyruvate reductase in the serine pathway. It was later shown that this mutant could not grow on ethanol unless supplemented with glyoxylate (but not glycollate) indicating a second function for hydroxypyruvate reductase during growth of Pseudomonas AM1 on ethanol (Dunstan et al., 1972b). Large and Quayle (1963) had previously shown that the glyoxylate reductase and the hydroxypyruvate reductase of Pseudomonas AM1 were the same enzyme.

In the present study, the growth response of mutant 20 BL to an extended range of carbon sources has been investigated. As previously reported, no growth was observed on C_1-compounds and very poor growth resulted on ethanol. Mutant 20 BL also failed to grow significantly on malonate but growth occurred on β-hydroxybutyrate at a similar rate to wild-type bacteria. No hydroxypyruvate (glyoxylate) reductase activity could be detected in extracts of mutant 20 BL grown on β-hydroxybutyrate when assayed at pH 4.6 or 7.0 (Table 4.6). These results suggest that glycollate is not an intermediate during operation of the malate synthase pathway for growth on β-hydroxy-

Table 4.6 Specific activities of hydroxypyruvate reductase with hydroxypyruvate and glyoxylate as substrates in extracts of wild-type Pseudomonas AM1 and mutant 20 BL

Extracts were prepared and assayed for enzyme activity as described in Sections 2.10 and 2.12. Specific activities are expressed as nmoles/min/mg. protein.

-, not assayed; n.d., not detected.

Growth Substrate	Strain				Ratio of activities
	20 BL		wild-type		
	hydroxy-pyruvate	glyoxylate	hydroxy-pyruvate	glyoxylate	
methanol	-	-	1520*	232	6.5
succinate	n.d.	n.d.	264*	30	8.8
ethanol	-	-	500*	66	7.5
β-hydroxy-butyrate	n.d.	n.d.	630	93	6.8

*Values of Dunstan *et al.* (1972b)

butyrate. It is therefore unlikely that glycollate is involved in the oxidation of acetate to glyoxylate during growth of *Pseudomonas* AM1 on ethanol and malonate despite the poor growth of mutant 20 BL on these compounds.

The involvement of glycollate during acetyl-CoA assimilation by the malate synthase pathway was further investigated by tracing the distribution of radioactivity from ^{14}C-acetate incubated with whole cells of β-hydroxybutyrate-grown mutant 20 BL.

4.8 Metabolism of U-^{14}C-acetate by β-hydroxybutyrate-grown wild-type Pseudomonas AM1 and mutant 20 BL

If glycollate is not an intermediate in acetate oxidation it can be expected that the amount of labelled glycollate derived from U-^{14}C-acetate would be much lower in mutant 20 BL than in wild-type *Pseudomonas* AM1 where hydroxypyruvate reductase is present to catalyse an equilibrium between glyoxylate and glycollate. In this situation the ratio of labelled glycollate to labelled malate would be low in whole cells of 20 BL incubated with U-^{14}C-acetate. Conversely, if glycollate is an intermediate during acetate oxidation the intracellular glycollate pool of mutant 20 BL would be relatively high and high levels of radioactive glycollate would be detected. This may possibly be accompanied with a lower level of radioactive malate in 20 BL compared with wild-type bacteria. In this case the ratio of labelled glycollate to labelled malate would be expected to be ~~low~~ high in whole cells of 20 BL incubated with ^{14}C-acetate.

Whole cells of β-hydroxybutyrate-grown *Pseudomonas* AM1

and mutant 20 BL were incubated with purified U-^{14}C-acetate as described in Section 2.17; after 1 minute the ethanol-soluble radioactive intermediates were extracted, counted and identified, (Sections 2.17 to 2.20).

The experiment, unlike the labelling experiments described by Dunstan et al., (1972a) was not primarily designed to identify early labelled intermediates in the pathway of acetate assimilation. Instead, an indication of the pool sizes of different components in wild-type bacteria and two mutants of known metabolic deficiencies was required. These pool sizes are estimated by comparing the relative proportion of the total label in the compounds when the distribution of radioactivity among them has reached equilibrium. Equilibrium was previously shown to occur after about 1 minute of incubation with ^{14}C-acetate (Dunstan, 1972).

The results presented in Table 4.7 show the distribution of label from ^{14}C-acetate into ethanol-soluble components of wild-type and mutant 20 BL, (the results obtained with mutant ICT 54 are discussed in Section 4.9). In both cases, label was detected after 1 minute in the carboxylic acids: malate, succinate, fumarate glycollate and citrate, and the amino acids: aspartate, alanine and glutamate. Glutamate formed over 80% of the total label incorporated into ethanol-soluble constituents of both wild-type and mutant 20 BL. It is assumed that glutamate was formed by transamination of the TCA cycle intermediate 2-oxoglutarate. A significant difference between the two strains was the ratio of label incorporated into glycollate and malate. The glycollate/

Table 4.7 Distribution of radioactivity among components of the ethanol-soluble fraction of β-hydroxybutyrate grown Pseudomonas AM1, mutant ICT 54 and mutant 20 BL incubated with U-^{14}C-acetate

Washed organisms were incubated with 2.5 μmoles acetate/ml as described in Section 2.17. 5 μCi U-^{14}C-acetate/ml was added at zero time and after 1 min incubation the ethanol-soluble radioactive compounds were isolated, counted and identified as described in Sections 2.17 to 2.20. Correction has been made for quenching and for the background counts of the vials. Results are expressed as d.p.m./min. n.d., no radioactive peaks were detected by the chromatogram scanner; the lowest detectable limit under the conditions used was approximately 300 d.p.m.

Radioactivity in:	Strain		
	wild-type	ICT 54	20 BL
malate	9,750	1,300	10,044
succinate	6,670	760 (succinate + fumarate)	6,740
fumarate	13,290		9,010
glycollate	6,350	n.d.	2,810
citrate	7,400	n.d.	6,500
aspartate	16,810	n.d.	12,920
glutamate	404,380	7,680	260,390
alanine	44,770	n.d.	20,630
others	-	1,230	-
total	502,920	10,970	322,600
%wt incorporation	100	2.1	62.0
glycollate/malate	0.65	-	0.28

malate ratio was 0.65 with wild-type bacteria and 0.28 with β-hydroxybutyrate-grown 20 BL. This is a reflection on the relative pool sizes of these compounds. Labelled glycollate forms 0.85% of the total incorporation from ^{14}C-acetate into components of 20 BL compared to 1.3% of the total incorporation into wild-type components. In addition to the lower glycollate pool of mutant 20 BL, the relative size of the malate pool in this mutant is higher than that of wild-type <u>Pseudomonas</u> AM1. These two factors when combined are represented by a significant difference in the ratios of these compounds in the two strains.

That radioactive glycollate was detected in the ethanol-soluble components of both wild-type and mutant 20 BL suggests that acetate is assimilated by the malate synthase pathway during growth of both strains on β-hydroxybutyrate. The low glycollate/malate ratio found with mutant 20 BL compared to wild-type bacteria is consistent with the direct oxidation of acetate (or acetyl-CoA) to glyoxylate without the intermediacy of glycollate. Despite the absence of glyoxylate reduction in vitro with extracts of β-hydroxybutyrate-grown 20 BL it is possible that the labelled glycollate formed in this mutant was derived from glyoxylate reduction in vivo by an enzyme other than hydroxypyruvate reductase.

4.9 Metabolism of U-^{14}C-acetate by β-hydroxybutyrate-grown ICT 54

As described in Section 3.7 mutant ICT 54 lacks acetyl-CoA synthetase and consequently the ability to grow on ethanol and malonate. Acetyl-CoA synthetase is not required for growth on β-hydroxybutyrate and mutant ICT 54 grows on

this compound at the same rate as wild-type bacteria. The results shown in Table 4.7 demonstrate that radioactive acetate is not incorporated into whole cells of β-hydroxybutyrate-grown mutant ICT 54. Only 2% of the label incorporated into wild-type ethanol-soluble components was incorporated into these components of mutant ICT 54. This is a strong indication that acetate must first be activated to acetyl-CoA before being incorporated into cellular material and rules out the possibility that free acetate is oxidised to glyoxylate during operation of the malate synthase pathway.

4.10 Incorporation of radioactivity from U-^{14}C-acetate into cultures of Pseudomonas AM1 and mutant ICT 54 growing on β-hydroxybutyrate

The results presented above indicate that free acetate cannot be rapidly incorporated into cell constituents of *Pseudomonas* AM1 without prior activation to acetyl-CoA. This was confirmed by investigating the incorporation of U-^{14}C-acetate into growing cultures of mutant ICT 54 and wild-type bacteria. Bacteria were grown on β-hydroxybutyrate and 2 μCi of U-^{14}C-acetate together with non-radioactive acetate (final concentration of 2mM) were added to the medium when the cells had reached exponential growth. Specific radioactivities of samples removed at suitable time intervals were measured as described in Section 2.21.

The low specific radioactivities recorded in samples of ICT 54 after 8 hours of incubation did not significantly increase with incubation time (Table 4.8). By contrast, the specific radioactivities measured in samples of wild-type bacteria were initially much higher than mutant ICT 54 and

Table 4.8 Incorporation of radioactivity from ^{14}C-acetate into growing cultures of wild-type Pseudomonas AM1 and mutant ICT 54

Bacteria growing on β-hydroxybutyrate were incubated with 2 μCi U-^{14}C-acetate (specific activity, 1 μCi/mmole) as described in Section 2.21. Samples were taken at suitable time intervals for estimation of specific radioactivities. Results are expressed as d.p.m./μg dry weight of bacteria.

Strain	Incubation time with ^{14}C-acetate (hours)	Specific activity
wild-type	8	0.60
	24	1.6
mutant ICT 54	8	0.15
	24	0.25

increased with incubation time.

4.11 Summary and Discussion

The aim of the work presented in this Chapter was to provide a logical extension of the study made by Dunstan et al. (1972a, 1972b, 1973) into the assimilation of acetyl-CoA into cell material by Pseudomonas AM1. Attempts have been made to confirm the proposed malate synthase pathway during assimilation of ethanol, β-hydroxybutyrate, malonate and possibly lactate and pyruvate through the isolation of mutants lacking malate synthase activity and mutants similar to PCT 48 which lack the ability to oxidise acetate or acetyl-CoA to glyoxylate. The intermediacy of glycollate during acetate oxidation has also been investigated and experiments designed to distinguish between acetate and acetyl-CoA as substrates for oxidation have been described.

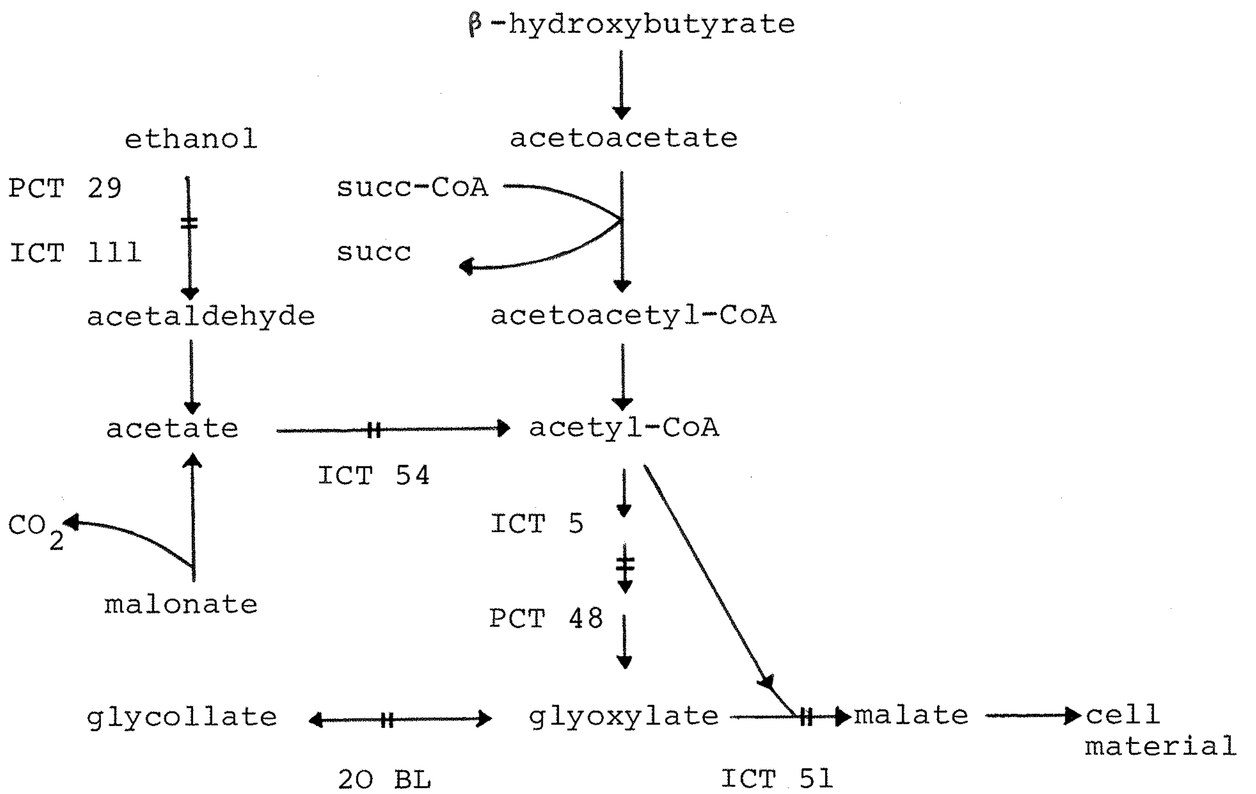
The isolation and properties of the malate synthase-deficient mutant (ICT 51) indicate that this enzyme is required for growth of Pseudomonas AM1 on β-hydroxybutyrate, ethanol and malonate, but not for growth on C_1-compounds, lactate or pyruvate (Taylor and Anthony, 1975). The properties of mutant ICT 5 provide further evidence for the oxidation of acetate or acetyl-CoA to glyoxylate during assimilation of C_1-compounds, β-hydroxybutyrate, ethanol and malonate, but this mutant, like mutants PCT 48 (Dunstan et al., 1972b) and C_5 (Salem et al., 1973a) grows on lactate and pyruvate. These results suggest that these C_3-compounds may be assimilated by a route other than the malate synthase pathway, despite the elegant demonstration by Salem et al.

(1973a) who showed that the C_1-atom of lactate is lost during its assimilation. A result more difficult to interpret was the observation that mutant ICT 51R, a streptomycin-resistant revertant of mutant ICT 51, regained the capacity to utilise β-hydroxybutyrate, malonate and ethanol while apparently failing to synthesise malate synthase. A possible explanation for this is provided whereby malyl-CoA lyase, present in mutant ICT 51R, catalyses the condensation of acetyl-CoA and glyoxylate to give malyl-CoA. In the presence of a CoA-transferase, the CoA is donated to an acceptor molecule to give free malate. If such CoA-transferase exists, it is possible that malate is activated by the same enzyme during growth of *Pseudomonas* AM1 on C_1-compounds. No malyl-CoA synthetase (thiokinase) activity has been demonstrated in extracts of *Pseudomonas* AM1 grown on C_1-compounds (Salem *et al.*, 1973b) and to date no transferase capable of activating malate has been described. The above explanation for growth of mutant ICT 51R on β-hydroxybutyrate, malonate and ethanol in the absence of malate synthase is not, however, valid for mutant PCT 57 which grows on these compounds despite the lack of both malate synthase and malyl-CoA lyase.

The results with mutant ICT 54 which lacks acetyl-CoA synthetase demonstrate that free acetate is not directly incorporated into cellular material unless it is first activated to acetyl-CoA. This precludes the possibility that acetate itself is oxidised (hydroxylated) to either glyoxylate or glycollate.

The demonstration that mutant 20 BL grows on β-hydroxy-

Fig. 4.2 Proposed scheme for the assimilation of ethanol, β-hydroxybutyrate and malonate by Pseudomonas AM1



Broken lines indicate the reaction(s) involved in the oxidation of acetyl-CoA to glyoxylate. Metabolic lesions in the designated mutants are indicated by parallel bars.

butyrate without the capacity to reduce glyoxylate (and presumably oxidise glycollate) indicates that glycollate is not an intermediate of acetyl-CoA oxidation during growth of *Pseudomonas* AM1 on β-hydroxybutyrate and possibly other compounds assimilated by this pathway. The glycollate/malate ratio, estimated by measuring the radioactivity in these compounds after incubation of mutant 20 BL and wild-type bacteria with ^{14}C-acetate was higher by a factor of 2.3 in wild-type bacteria compared to mutant 20 BL. This is in agreement with the direct oxidation of acetyl-CoA to glyoxylate.

Some of the results described in this and the previous Chapter are summarised in Fig. 4.2, which shows the proposed scheme for the assimilation of ethanol, malonate and β-hydroxybutyrate by *Pseudomonas* AM1. Pyruvate and lactate are not included in the scheme and further investigations into the metabolism of these compounds is necessary. It would be of interest to trace the path of radioactivity from ^{14}C-lactate or pyruvate incubated with whole cells of wild-type *Pseudomonas* AM1 and mutants ICT 51 and ICT 5 grown on these C_3-compounds.

CHAPTER 5

Properties of a mutant of Pseudomonas AM1 lacking 2-oxoglutarate dehydrogenase: a biochemical basis for obligate methylotrophy

5.1 Introduction

The absence of 2-oxoglutarate dehydrogenase has been suggested as an explanation of the failure of obligate autotrophs to grow heterotrophically (Kelly, 1971) and its absence from some obligate methylotrophs suggests a common basis for obligate autotrophy and methylotrophy. Obligate methylotrophs which lack a complete oxidative tricarboxylic acid cycle through the absence of 2-oxoglutarate dehydrogenase have been discussed in Section 1.12 and include methane-utilisers with Type I membrane systems, Bacterium 4B6 and Organism W1. The restricted facultative methylotrophs also lack 2-oxoglutarate dehydrogenase (Colby and Zatman, 1975a). It has been concluded that although the lack of 2-oxoglutarate dehydrogenase may be important in conferring the characteristic of obligate methylotrophy in some bacteria, other mechanisms may be involved particularly as the obligate Type II methane-utilisers contain all the enzymes of the TCA cycle. Furthermore, doubt has been expressed that a single lesion could be expected to account for the inability of a large number of potential growth substrates to support growth (Ribbons, Harrison and Wadzinski, 1970).

The isolation of a 2-oxoglutarate dehydrogenase-deficient mutant of a facultative methylotroph may provide an indication of the contribution the lack of this enzyme has in conferring the characteristic of obligate methylotrophy. Such a mutant

has been isolated from Pseudomonas AM1 (mutant ICT 41) and its properties are presented in this Chapter.

5.2 Growth and oxidative properties of mutant ICT 41

Mutant ICT 41 was isolated by its ability to grow on methanol, but not on β-hydroxybutyrate. The mutant grew only on C_1-compounds and oxalate; no growth was observed on β-hydroxybutyrate, malonate, ethanol, lactate, pyruvate, nutrient broth or C_4 dicarboxylic acids (Table 5.1). Supplements (1 mM) of succinate did not promote growth of mutant ICT 41 on malate, β-hydroxybutyrate or lactate nor was growth observed on β-hydroxybutyrate or ethanol supplemented with succinate and acetate. Although growth of mutant ICT 41 did occur on oxalate this compound is assimilated by way of the serine pathway in Pseudomonas AM1 after ~~oxidation~~ reduction to glyoxylate and decarboxylation to formate, and can thus be classed as a substrate for methylotrophic growth (Blackmore and Quayle, 1970). These growth properties demonstrate that mutant ICT 41 is an obligate methylotroph. Growth of mutant ICT 41 on compounds such as glucose, citrate, glutamate and alanine was not tested as wild-type Pseudomonas AM1 grows poorly, or not at all on these substrates which support growth of the restricted facultative methylotrophs lacking 2-oxoglutarate dehydrogenase (Section 1.2c).

The results in Table 5.2 show that C_1-compounds, ethanol, succinate, malate and malonate were oxidised at similar rates to those measured in wild-type bacteria. By contrast, pyruvate, lactate, β-hydroxybutyrate acetoacetate and

Table 5.1 Growth responses of mutant ICT 41 and revertants ICT 41-R_1 and -R_2

Growth responses were determined in liquid media as described in Section 2.7. The relative amounts of growth are represented by values 0-5 (based on visual estimates). Supplements of acetate and succinate were at a conc. of 1mM. -, not tested.

Growth substrate	Strain			
	wild-type	ICT 41	ICT 41-R_1	ICT 41-R_2
methanol	5	5	4	5
methylamine	5	5	-	-
formate	3	3	-	-
oxalate	3	3	-	-
ethanol	3	0	3	3
β-hydroxybutyrate	5	0	5	5
malonate	3	0	3	3
pyruvate	3	0	-	-
lactate	5	0	5	5
fumarate	5	0	-	-
succinate	5	0	5	5
malate	5	0	4	5
malate+succinate	5	0	-	-
β-hydroxybutyrate+succinate	5	0	-	-
β-hydroxybutyrate+succinate+acetate	5	0	-	-
ethanol+succinate+acetate	4	0	-	-
lactate+succinate	5	0	-	-
nutrient broth	3	0	3	3

Table 5.2 Comparison of rates of oxygen uptake by cell suspension of methanol-grown mutant ICT 41 and wild-type bacteria

Rates of oxygen uptakes measured as described in Section 2.9, are expressed as percentages of the rate with wild-type bacteria. Figures in parentheses indicate absolute values of oxygen-uptake ($\mu\ell$ O_2/mg. dry wt./hr). Results given are the average of a number of determinations with different cell suspensions. The endogenous rate of oxidation was subtracted. -, not measured.

Substrate for oxidation	O_2 uptake			
	wild-type	ICT 41	ICT 41-R_1	ICT 41-R_2
methanol	100 (120)	70	75	90
formate	100 (120)	97	-	-
formaldehyde	100 (110)	103	-	-
ethanol	100 (84.6)	80	-	-
succinate	100 (21.0)	82	75	62
malate	100 (35.0)	93	-	-
malonate	100 (17.7)	70	-	-
pyruvate	100 (14.0)	9	-	-
lactate	100 (19.5)	20	120	100
β-hydroxy-butyrate	100 (15.0)	13	65	90
acetoacetate	100 (20.0)	10	-	-
2-oxoglutarate	100 (21.0)	13	75	87
endogenous substrate	100 (11.0)	10	130	70

Table 5.3 Specific activities of TCA-cycle enzymes in extracts of methanol grown wild-type Pseudomonas AM1 and ICT 41

Extracts were prepared as described in Section 2.10 and enzymes were assayed by the methods described in Section 2.12. Specific activities are expressed as nmoles substrate used or product formed/min/mg. protein. n.d., not detected.

Enzyme	Specific activity	
	wild-type	ICT 41
citrate synthase	41.7	38.2
isocitrate dehydrogenase ($NADP^+$)	142	163
isocitrate dehydrogenase (NAD^+)	n.d.	n.d.
2-oxoglutarate dehydrogenase	25	n.d.
succinyl-CoA synthetase	80	72
succinate dehydrogenase	38.3	46.8
fumarase	28.2	28.2
malate dehydrogenase	1080	2650
NADH dehydrogenase	8.3	16.3

2-oxoglutarate were oxidised at only 9-20% of the wild-type rates. The endogenous respiration rate was 10% of that measured with wild-type bacteria.

These growth and oxidative properties of mutant ICT 41 suggested that the mutant was deficient in one or more of the tricarboxylic acid cycle enzymes.

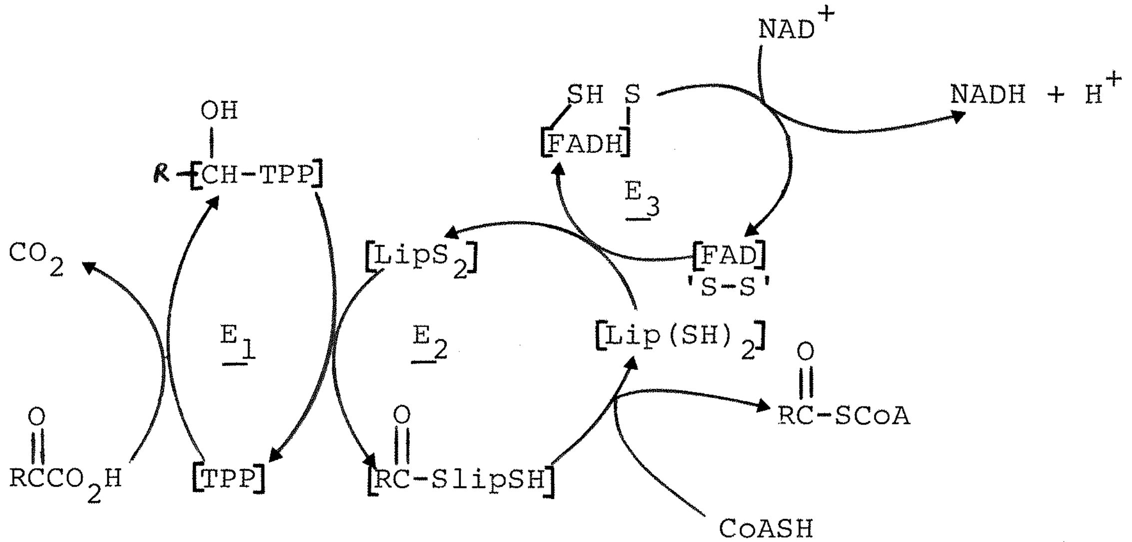
5.3 Activities of tricarboxylic acid cycle enzymes in mutant ICT 41

The results in Table 5.3 confirm the expectation that mutant ICT 41 has a deficient TCA cycle. No 2-oxoglutarate dehydrogenase activity could be detected in any extract of mutant ICT 41 using either NAD^+ or 3-acetyl NAD^+ as the electron acceptor. The specific activities of all the other TCA cycle enzymes were similar to the wild-type levels (aconitase was not measured). The levels of cytochromes a, b and c, and of carbon monoxide-binding cytochromes (potential oxidases) were also similar to those of wild-type Pseudomonas AM1.

2-oxoglutarate dehydrogenase is usually a multienzyme complex consisting of three enzymatic components (Fig. 5.1; Reed and Cox, 1970). The three components are the 2-oxoglutarate decarboxylase (E_1), the dihydrolipoamide transsuccinylase (E_2) and the lipoamide dehydrogenase (E_3) which act in concert to catalyse the oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA. Pyruvate is converted to acetyl-CoA by an analogous series of reactions.

The results in Table 5.4 show the activities of the overall pyruvate and 2-oxoglutarate dehydrogenase complexes together with their component enzymes. Mutant ICT 41 has no detectable 2-oxoglutarate dehydrogenase activity when grown on methanol or methanol plus succinate. The E_1 component

Fig. 5.1 Reaction sequence in pyruvate and 2-oxoglutarate oxidation (From Reed and Cox, 1970)



The abbreviations used are; TPP, thiamine pyrophosphate; $LipS_2$ and $Lip(SH)_2$ lipoyl moiety and its reduced form; CoASH, coenzyme A; FAD, flavin adenine dinucleotide; E_1, 2-oxoacid decarboxylase; E_2, dihydrolipoamide trans-acylase; E_3, lipoamide dehydrogenase.

(2-oxoglutarate decarboxylase) was present with 26-50% of the activity measured in wild-type bacteria although the K_m value for 2-oxoglutarate of this component of mutant ICT 41 was the same as that for the E_1 component of wild-type _Pseudomonas_ AM1 (60 μM) (Fig. 5.2). Lipoamide dehydrogenase (E_3) activity was present in extracts of mutant ICT 41 with higher levels than in wild-type bacteria. This may account for the high pyruvate dehydrogenase activity measured in the mutant as pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase share a common E_3 component (Guest and Creaghan, 1973).

It was not possible to measure the transsuccinylase (E_2) component in crude extracts of mutant ICT 41 or wild-type _Pseudomonas_ AM1. Purified preparations are required for the assay of this enzymatic component in _Escherichia coli_ (Dr. J.R. Guest, personal communication) and _Acinetobacter lwoffi_ (Dr. E. Hall, personal communication).

A comparison of the levels of 2-oxoglutarate dehydrogenase, pyruvate dehydrogenase and their components in extracts of wild-type _Pseudomonas_ AM1 grown on various carbon sources indicates that these enzymes are relatively unimportant during growth of the organism on methanol compared with growth on β-hydroxybutyrate, lactate or succinate (Table 5.4). Both E_1 and E_3 components, as well as the overall dehydrogenase activities were low during growth on methanol. The lack of 2-oxoglutarate dehydrogenase activity in mutant ICT 41 is unlikely to be due to the lower activity of the decarbooxylase (E_1) component. During growth on methanol wild-type

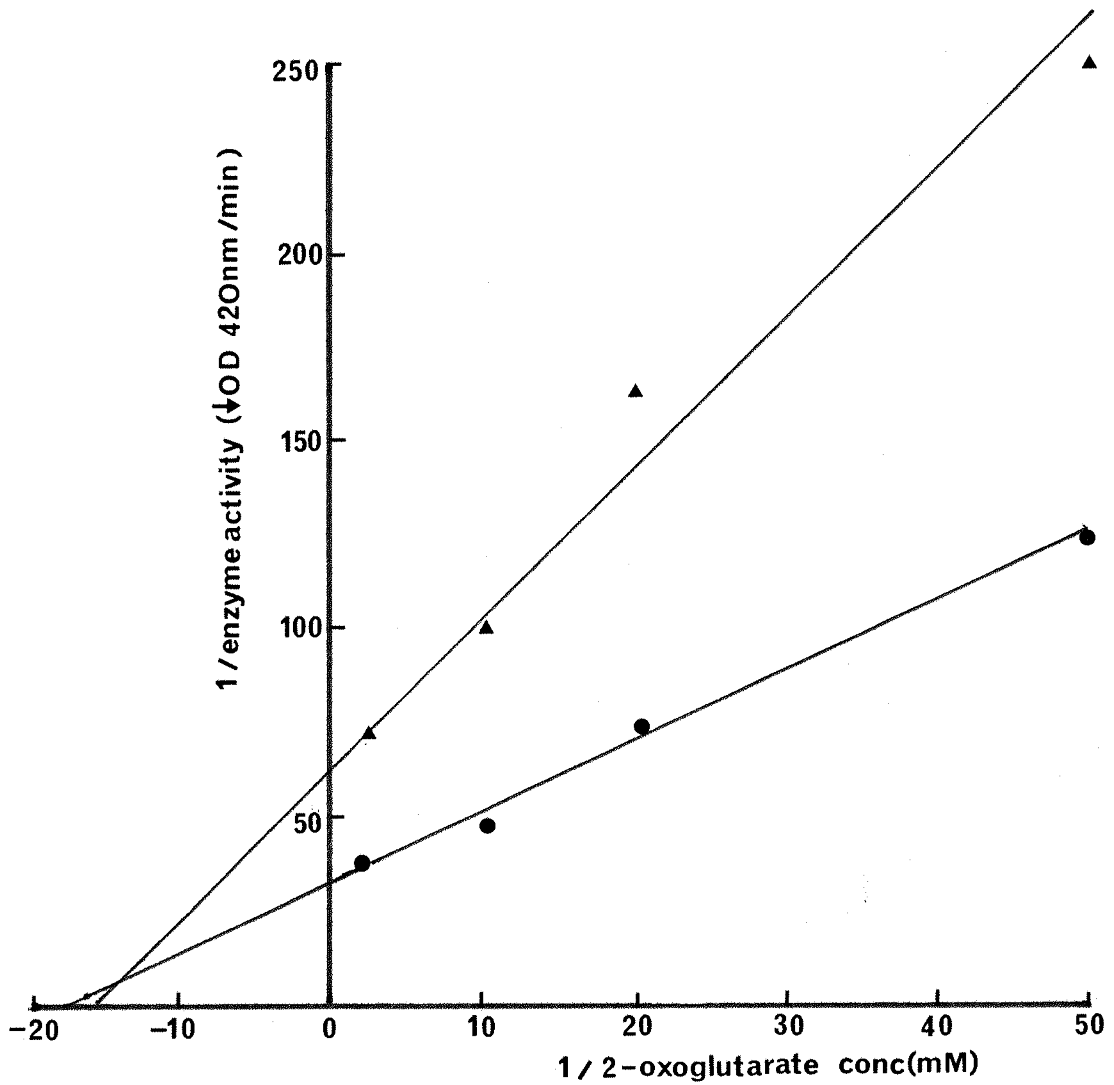
Table 5.4 Specific activities of the 2-oxoglutarate and pyruvate dehydrogenase complexes and component enzymes in wild-type Pseudomonas AM1 and derived mutants

Enzymes were assayed in ultrasonic extracts as described in Section 2.12. The average activities for determinations with several different extracts are quoted (nmoles/min/mg. protein); all values were within 10% of the average value quoted. 2-ogdh and pyrdh = activity of the overall 2-oxoglutarate and pyruvate dehydrogenase complexes; E_1(2-og) and E_1(pyr) = 2-oxoglutarate and pyruvate decarboxylase respectively; lpdh(E_3) = lipoamide dehydrogenase. -, not assayed; n.d., not detected.

		Specific activity				
Strain	growth substrate	2-ogdh	E_1(2-og)	lpdh(E_3)	pyrdh	E_1(pyr)
Wildtype	methanol	24.6	6.7	25	2.3	2.0
	methanol + succinate	43.4	16.6	58.5	11.2	3.3
	succinate	138	21.7	143	21.7	4.7
	lactate	93.5	21.7	139	40.0	7.0
	β-hydroxy-butyrate	58.5	10.0	108	17.1	4.7
ICT 41	methanol	n.d.	3.3	42	7.7	1.8
	methanol + succinate	n.d.	4.3	190	14.2	2.7
ICT 41-R_1	methanol	11.7	6.7	18.3	2.3	-
	succinate	48.4	23.4	58.5	10.8	-
ICT 41-R_2	methanol	10.0	15.0	10.8	4.0	-
	succinate	26.8	33.2	108	33.2	-

Fig. 5.2 Effect of 2-oxoglutarate concentration on the activity of the E_1-components of mutant ICT 41 and wild-type Pseudomonas AM1

The assay was as described in Section 2.12b using 1.4 mg. protein and 1.26 mg. protein in extracts of methanol-grown mutant ICT 41 (●) and wild-type (▲) respectively.



bacteria have only twice as much E_1 activity as the lowest value recorded in the mutant and yet they have significant overall 2-oxoglutarate dehydrogenase activity. It is more probable that mutant ICT 41 has an altered (inactive) dihydrolipoamide transsuccinylase (E_2) component and that this also has some effect on decreasing the activity of the E_1 component and increasing the activity of the E_3 component of the multienzyme complex of the mutant.

5.4 Properties of revertant strains derived from mutant ICT 41

Two revertants of mutant ICT 41 were obtained as described in Section 2.8. Both revertants (ICT 41-R_1 and ICT 41-R_2) had regained overall 2-oxoglutarate dehydrogenase activity (Table 5.4) and had concomitantly regained the ability to oxidise and grow on the same substrates as wild-type *Pseudomonas* AM1 (Tables 5.1 and 5.2). These results suggest that the characteristics of mutant ICT 41 are not the result of multiple lesions and emphasise the importance of a complete TCA cycle for growth of *Pseudomonas* AM1 on multicarbon compounds.

5.5 Amino acid accumulation by mutant ICT 41

Table 5.5 shows the results of analysis of amino acids present in the growth medium of cultures of mutant ICT 41 and wild-type *Pseudomonas* AM1 grown on methanol. The growth rate and extent of growth were similar (within 10%) for both cultures which were harvested as they entered the stationary phase and analysed as described in Section 2.16. The greatest differences are the high levels of glutamate

Table 5.5 Amino acid accumulation by cultures of mutant ICT 41 and wild-type Pseudomonas AM1 grown on methanol

Cultures were harvested at the end of exponential growth and amino acids in samples of the growth supernatants were analysed by a JEOL amino acid analyser as described in Section 2.16. Lysine was assayed separately by the spectrophotometric method of Vogel & Shimura (1971). Amino acid concentrations (expressed as nmoles/ml culture medium) were obtained by averaging the results of analysis of three separate cultures. All the values were within 20% of the average values quoted. Amino acids present with concentrations less than 1.0 nmole/ml have not been recorded.

Amino acid	Strain	
	wild-type	ICT 41*
2-aminoadipate	1.8	13.0
glutamate	1.0	9.0
lysine	5.2	3.6
glutamine	3.5	3.3
threonine	3.2	1.0
alanine	1.1	4.0
proline	1.1	4.2

*Supplements (1 mM) of acetate, succinate or β-hydroxybutyrate included in the growth medium of ICT 41 had no effect on the levels of these amino acids.

and 2-aminoadipate excreted by mutant ICT 41. The excretion of glutamate by the 2-oxoglutarate-deficient mutant is not unexpected and presumably arises by way of transamination or amination of 2-oxoglutarate which cannot be oxidised by the TCA cycle.

The excretion of 2-aminoadipate into the growth medium by mutant ICT 41 is more difficult to explain. A possible route for the biosynthesis of this compound from 2-oxoglutarate is shown in Fig. 5.3. The scheme involves the initial condensation of acetyl-CoA and 2-oxoglutarate in a reaction catalysed by homocitrate synthase. This reaction (1) and reaction (2) are analogous to those catalysed by the TCA cycle enzymes citrate synthase and aconitase. Similarly, the overall effect of reactions (3) and (4) is analogous to the reaction catalysed by isocitrate dehydrogenase. The 2-oxoadipate is transaminated to 2-aminoadipate, the end-product of the sequence. If enzymes are present which catalyse the reactions in Fig. 5.3 it is not clear what their physiological functions are. These enzymes are involved in lysine biosynthesis by the homocitrate-aminoadipate pathway in some algae, yeasts and other higher fungi but not in bacteria where lysine is derived from pyruvate and aspartate with the intermediate formation of diaminopimelic acid (Broquist, 1971; Kurtz and Bhattacharjee, 1975). A possible function of these enzymes in *Pseudomonas* AM1 is the oxidation of acetyl-CoA to glyoxylate by the hypothetical scheme shown in Fig. 5.4.

Preliminary experiments have shown that methanol-grown

Fig. 5.3 Possible sequence involved in the biosynthesis of 2-aminoadipate in Pseudomonas AM1 (and mutant ICT 41)

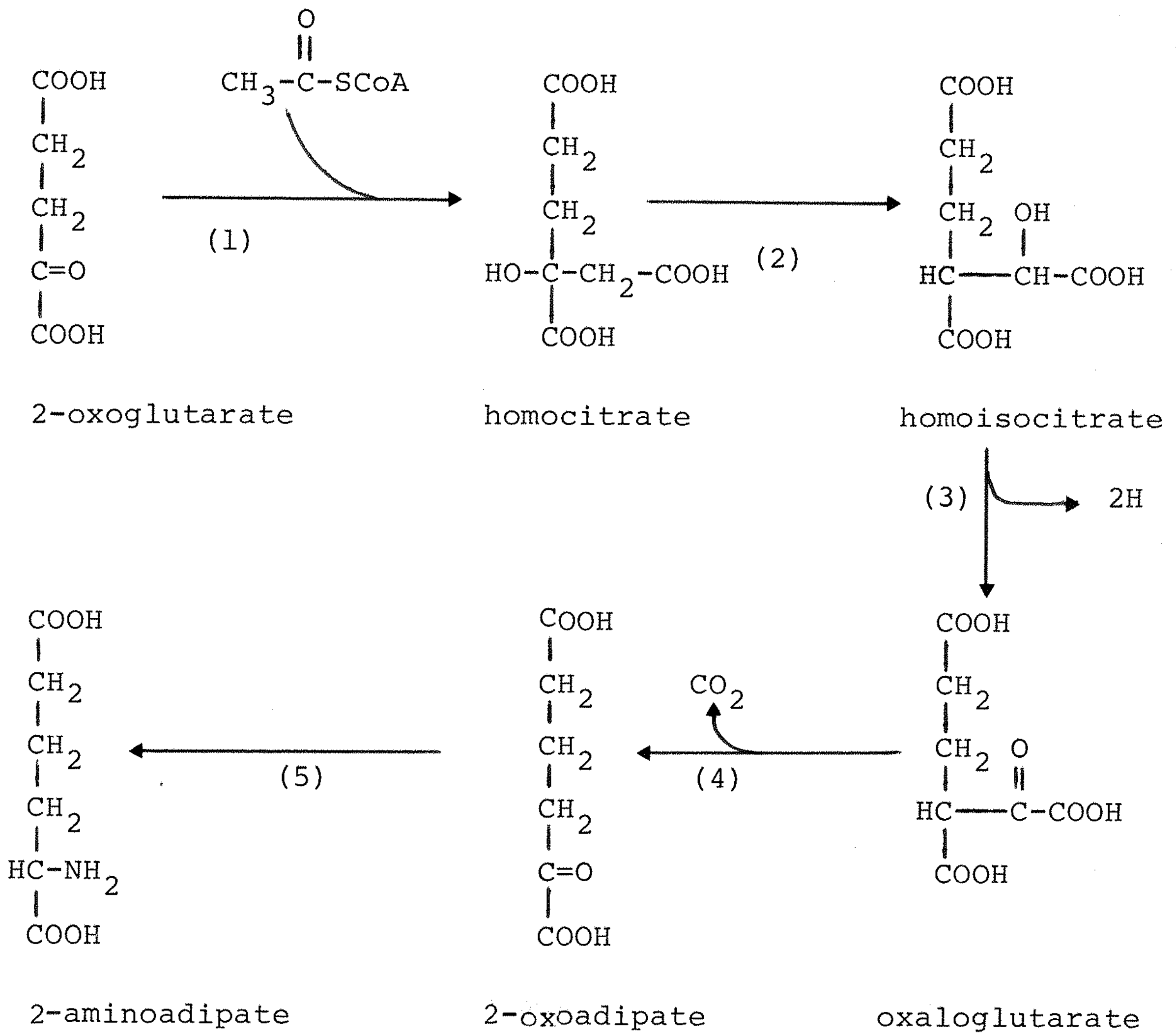
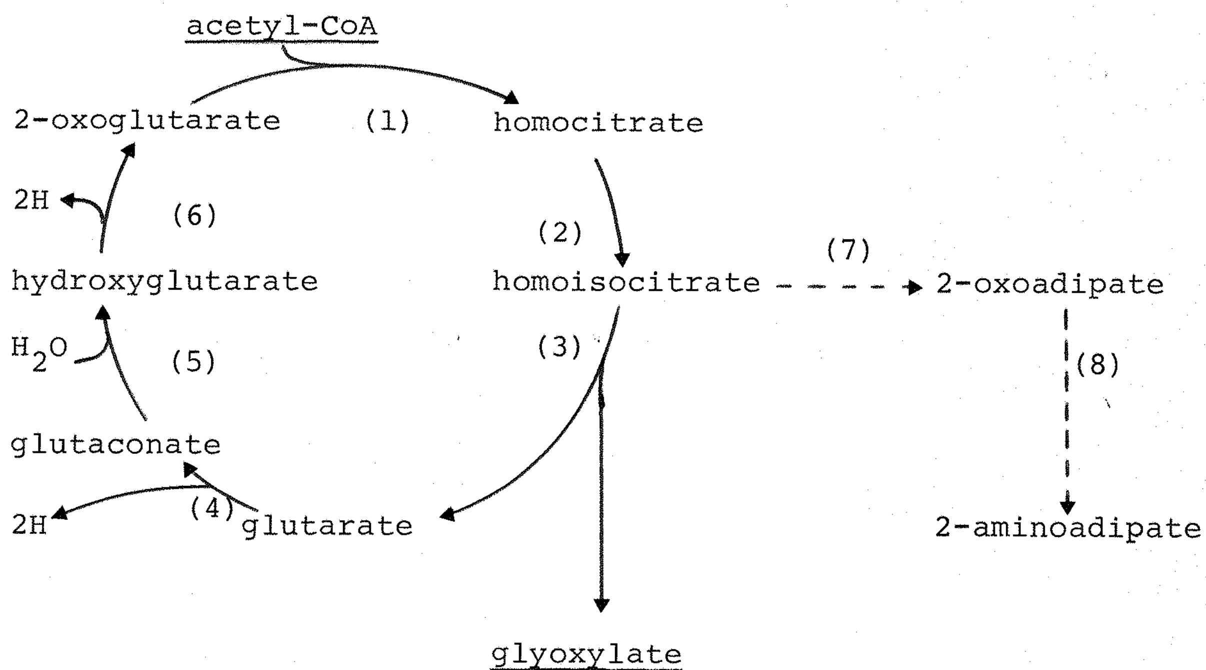


Fig. 5.4 A hypothetical scheme for the oxidation of acetyl-CoA to glyoxylate by a route not involving isocitrate lyase



Enzymes: (1) homocitrate synthase

(2) homoaconitase

(3) homoisocitrate lyase

(4) glutarate dehydrogenase

(5) glutaconate hydratase

(6) hydroxyglutarate dehydrogenase

(7) homoisocitrate dehydrogenase

(8) transaminase

Pseudomonas AM1 and mutant ICT 41 contain low levels of the first enzyme, homoisocitrate synthase. The assay used for this enzyme was the same as that for citrate synthase (Srere, 1969) except that oxaloacetate was replaced by 2-oxoglutarate. Activity (approx. 5 nmoles/min/mg protein) was only detected in crude extracts centrifuged at 4,000 g to remove whole cells and not in high speed (40,000 g) supernatants. Although this activity is not high enough for the enzyme to be involved in a major assimilatory pathway, it is unlikely that optimum assay conditions were used.

It is possible that the activity detected was due to the metabolism of 2-oxoglutarate to oxaloacetate which was the true substrate. However it is unlikely that sufficient amounts of the necessary cofactors were available and furthermore activity was detected in extracts of the 2-oxoglutarate dehydrogenase-deficient mutant (ICT 41).

Another key enzyme is homoisocitrate lyase which cleaves homocitrate to glyoxylate and glutarate by an analogous reaction to that catalysed by isocitrate lyase. The enzymes involved in regenerating the acceptor molecule, 2-oxoglutarate, are analogous to those TCA cycle enzymes involved in the oxidation of succinate to oxaloacetate.

It must be emphasised that this scheme is purely speculative and based only on preliminary observations of the unexpected accumulation of 2-aminoadipate by mutant ICT 41 and the low homocitrate synthase activity measured in both mutant and wild-type bacteria.

Although compounds such as homocitrate and homoisocitrate

are difficult to prepare (see Tuci, Ceci and Bhattacharjee, 1971) a detailed study of this very important area of metabolism in Pseudomonas AM1 is required. This would involve attempts to assay all the enzymes described in Fig. 5.4 in both wild-type Pseudomonas AM1 and mutants such as PCT 48 and ICT 5 which cannot oxidise acetyl-CoA to glyoxylate (Sections 4.2 and 4.3). It would also be advisable to confirm that the normal (bacterial) diaminopimelic acid route operates for lysine biosynthesis in Pseudomonas AM1.

5.6 Summary and discussion

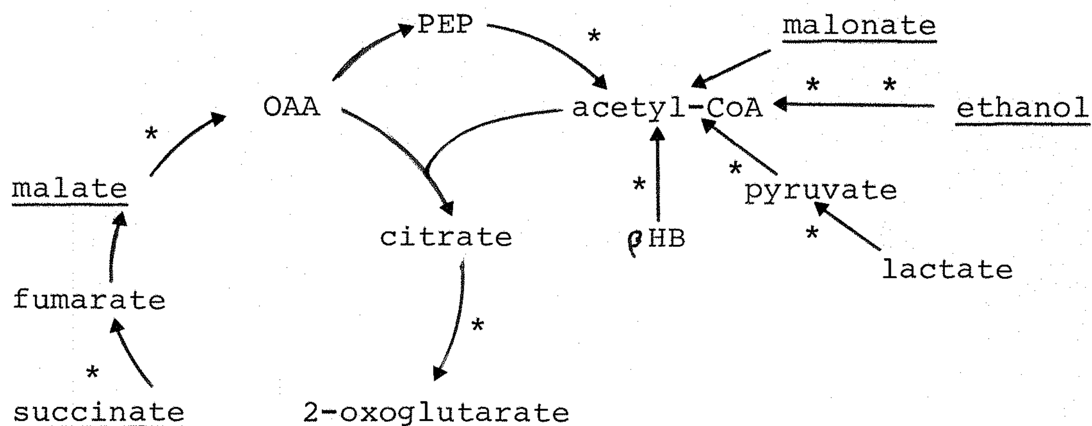
The results presented in this Chapter show that the loss of 2-oxoglutarate dehydrogenase is sufficient to convert a typical facultative methylotroph into an obligate methylotroph. The lack of this enzyme may well be the basis therefore of obligate methylotrophy in some bacteria (Type I methane-utilisers, Organism W1 and Bacterium 4B6) and also the probable reason for the limited range of substrates supporting growth of the restricted facultative methylotrophs (Section 1.12d). These bacteria, however, use the ribulose monophosphate pathway of C_1 assimilation and contrast with the 'obligate' mutant of Pseudomonas AM1 which uses the serine pathway. The mutant is therefore most similar to those obligate methane-utilisers with Type II membranes which also assimilate C_1-compounds by the serine pathway. It has been suggested (Davey et al., 1972) there must be an alternative basis for the obligate methylotrophy of these bacteria because they do have some 2-oxoglutarate

dehydrogenase activity (average specific activity 12 nmoles/min/mg protein). However, this is lower than the specific activity of the dehydrogenase in methanol-grown Pseudomonas AM1 (specific activity 25 nmoles/min/mg protein), where the enzyme is non-essential. It is possible that in Type II methane-utilisers there is no mechanism for induction of the enzyme to the higher levels required for growth on multicarbon compounds (specific activities of 60-140 nmoles/min/mg protein, Table 5.4).

Having shown that lack of 2-oxoglutarate dehydrogenase is able to confer the characteristic of obligate methylotrophy (in Pseudomonas AM1) it is necessary to consider how this one lesion might have such a far-reaching effect. The complete oxidation of multicarbon compounds is not possible without a complete TCA cycle and the resulting energy deficiency may in itself lead to the failure of mutant ICT 41 to grow on these compounds. In addition, the resulting accumulation of intermediates of central metabolic pathways may lead to inhibition of key enzymes. That such accumulations may occur is indicated by the observation that growth of mutant ICT 41 on methanol is inhibited when succinate (0.1%) is also included in the growth medium (an effect not observed with wild-type bacteria).

The apparent oxidation by mutant ICT 41 of some substrates usually oxidised by way of the TCA cycle (ethanol, malonate, succinate and malate; Table 5.2 and Fig. 5.5) is perhaps unexpected. However the rate of oxygen uptake measured in the oxygen electrode depends on a number of

Fig. 5.5 The oxidation pathways of mutant ICT 41



Oxidation reactions are indicated by asterisks.

The compounds underlined do not support growth of mutant ICT 41 but are oxidised by whole cell suspensions.

factors, any one of which may be rate limiting. Such factors include the nature of the primary dehydrogenases and their activities, the electron transport chains, the equilibrium constants of the reactions leading to the TCA cycle and the transport of the substrates into the cell. It is the rate and extent of total oxidation of a substrate that determines the amount of energy available to the bacteria and this will be altered for all these substrates.

CHAPTER 6

The regulation of the tricarboxylic acid cycle and malate synthase of *Pseudomonas* AM1.

6.1 Introduction

The properties of mutant ICT 41 presented in the previous Chapter demonstrated that a complete TCA cycle is not required for growth of *Pseudomonas* AM1 on C_1-compounds but that during growth on multicarbon compounds a complete oxidative cycle is essential. It was thus of interest to investigate any mechanisms involved in the regulation of the TCA cycle in this organism.

The regulation of malate synthase is also of interest. This enzyme is not required for methylotrophic growth (or for growth on lactate or pyruvate) but is present in wild-type *Pseudomonas* AM1 regardless of the carbon source for growth (Section 4.4). This leads to the possibility that the enzyme is inhibited rather than repressed during growth of the organism on substrates not requiring malate synthase for their assimilation.

6.2 Activities of TCA cycle enzymes during growth of *Pseudomonas* AM1 on various substrates

The results in Table 6.1 show that, as expected, the levels of some TCA cycle enzymes are significantly lower during growth of wild-type *Pseudomonas* AM1 on methanol compared with growth on multicarbon compounds; these include isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase and malate dehydrogenase. The differences in the levels however are not very great and suggest that a more important site of regulation

Table 6.1 Specific activities of enzymes of the tricarboxylic acid cycle in extracts of Pseudomonas AM1 grown on various carbon sources

Enzymes were assayed as described in Section 2.12. The average activities for determinations with at least two different extracts are quoted (nmoles/min./mg. protein).
-, not assayed.

Enzyme	Growth substrate			
	methanol	ethanol	β-hydroxy-butyrate	succinate
citrate synthase	51.5	27.1	37.1	41.4
isocitrate dehydrogenase	141	315	268	324
2-oxoglutarate dehydrogenase	24.6	-	60	138
succinyl-CoA synthetase	93	-	100	113
succinate dehydrogenase	28.2	28.2	56.5	65
malate dehydrogenase	1080	4930	2690	3510

of the cycle is at the level of enzyme inhibition.

6.3 Regulation of citrate synthase activity

The regulation of citrate synthase activity has previously been shown to differ in bacteria using the biosynthetic function of the TCA cycle from those which additionally use the cycle in a catabolic role (Section 1.13).

The citrate synthase of methanol-grown *Pseudomonas* AM1 was not inhibited by any of the following compounds (all at 5 mM): 2-oxoglutarate, isocitrate, citrate, glutamate, malate, succinate, fumarate, glycollate, glyoxylate, NAD^+, $NADP^+$, NADPH, ATP or AMP. NADH was, however, an inhibitor of the enzyme with a K_i of 0.7 mM when assayed with 0.2 mM acetyl-CoA and 0.5 mM oxaloacetate.

Inhibition by NADH was competitive with respect to oxaloacetate (Fig. 6.1): the K_m value for oxaloacetate was increased from 10 µM in the absence of NADH to 45 µM in the presence of NADH (1 mM). In both cases the Hill coefficient (n value) was approximately 1. The results in Fig. 6.2 show the sigmoidal nature of NADH inhibition with respect to acetyl-CoA. The Hill coefficient and K_m ($S_{0.5}$) for acetyl-CoA were 0.8 and 124 µM respectively in the absence of NADH and 1.8 and 220 µM in the presence of 1 mM NADH. These results suggest that NADH is an allosteric inhibitor of the citrate synthase of *Pseudomonas* AM1. The 90% inhibition of the enzyme by 2 mM NADH (measured with 0.1 mM acetyl-CoA) was reduced to 15% inhibition in the presence of AMP (0.3 mM) or KCl (50 mM). The molecular weight of the enzyme, estimated by gel-filtration with Sephadex G200 by the method described in Section 2.13

Fig. 6.1 Effect of oxaloacetate on citrate synthase activity in the presence (■) and absence (●) of NADH (1mM). a) Direct plot b) Double-reciprocal plot. The assay was as described in Section 2.13 using 0.2 μmol acetyl-CoA and 90 μg of extract previously heated at 60° for 5 min. to destroy malate dehydrogenase.

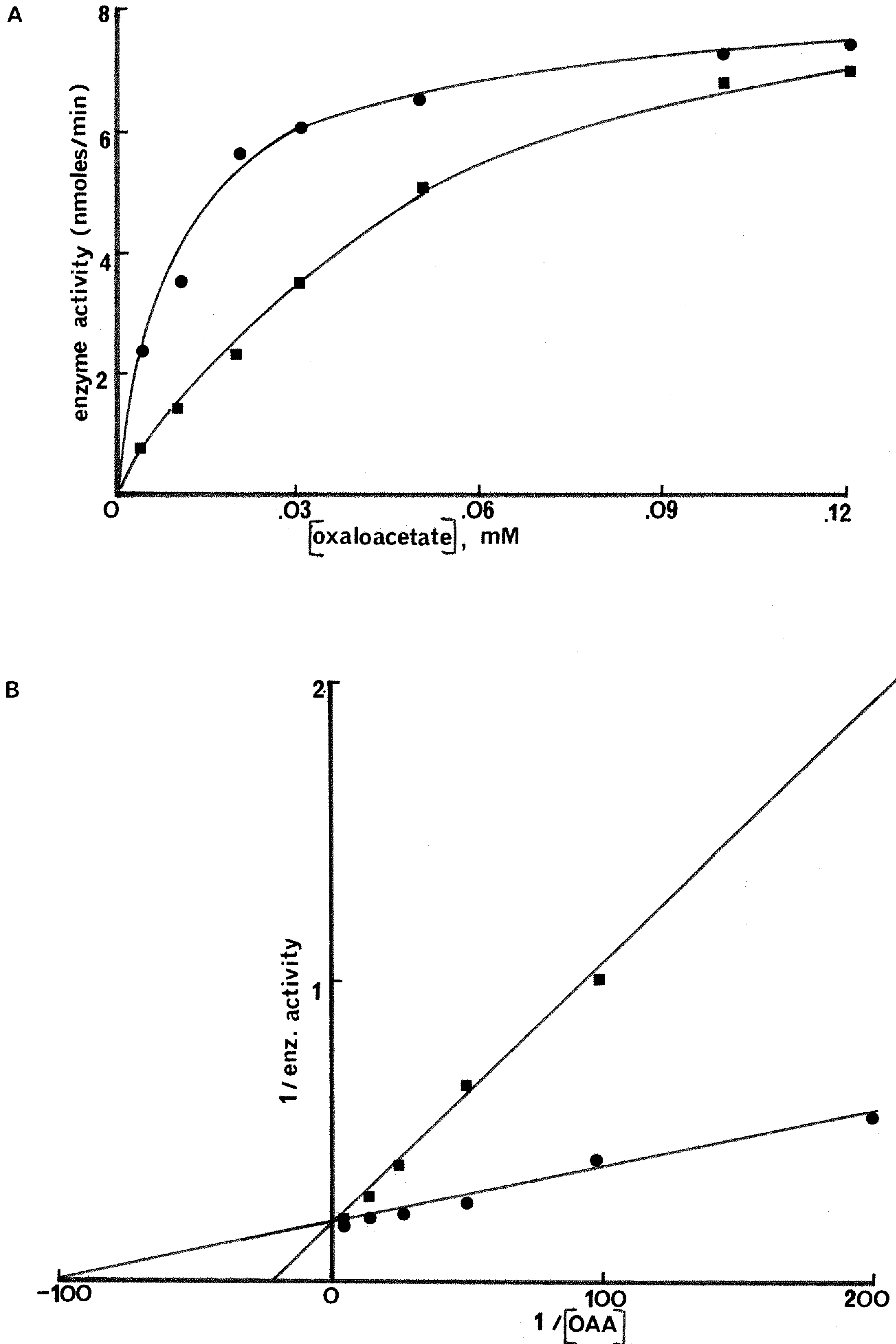
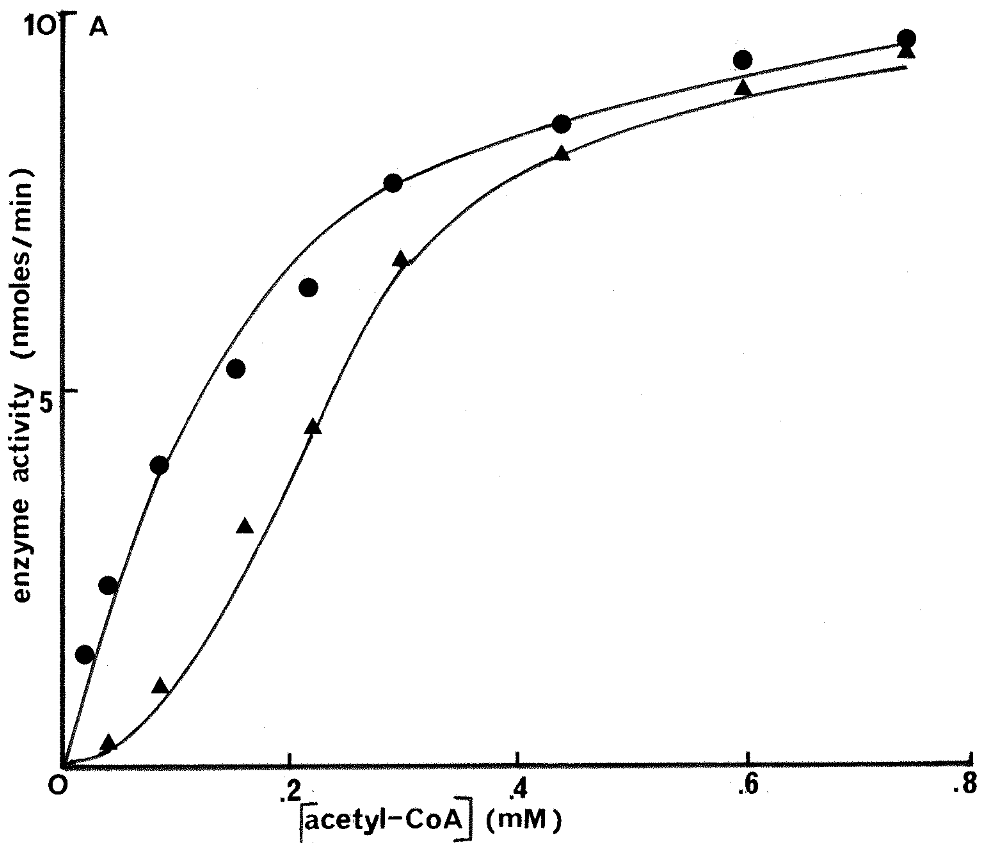
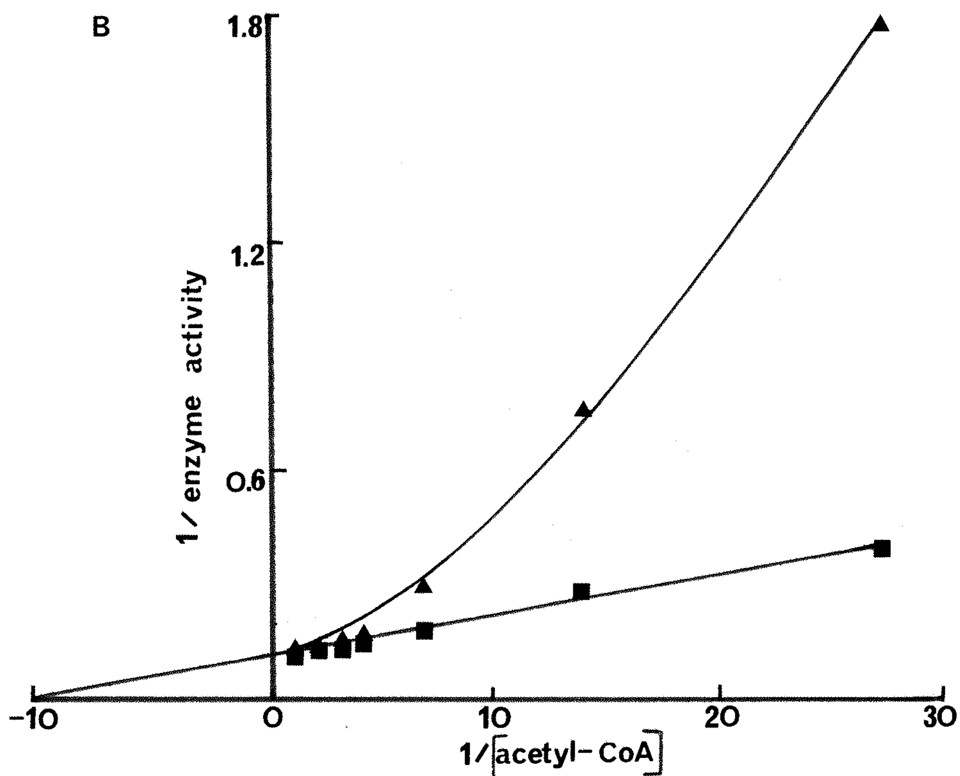


Fig. 6.2 Effect of acetyl-CoA on citrate synthase activity in the presence (▲) and absence (●) of NADH (1mM). a) Direct b) Double-reciprocal plot. The assay was as described in Section 2.13 using 0.5 µmol OAA and 90 µg of extract previously heated at 60° for 5 min.





was 250,000.

The regulation of the enzyme was similar in succinate-grown bacteria and was unchanged in the 2-oxoglutarate dehydrogenase-deficient mutant (ICT 41).

These properties of the citrate synthase of Pseudomonas AM1: NADH inhibition and relief of inhibition by AMP, no inhibition by 2-oxoglutarate and a molecular weight of 250,000, are typical of Gram-negative, strictly aerobic bacteria in which the TCA cycle serves both biosynthetic and oxidative functions (Section 1.13).

6.4 Regulation of isocitrate dehydrogenase activity

The isocitrate dehydrogenase of Pseudomonas AM1 was $NADP^+$-specific and required Mn^{++} ions for maximum activity. The enzyme was present with a lower activity during growth of the organism on methanol compared with multicarbon compounds (Table 6.1).

The enzyme from methanol-grown bacteria was 90% inhibited in a concerted fashion by a combination of glyoxylate (1 mM) and oxaloacetate (1 mM); separately these compounds inhibited the enzyme by only 23%. Less than 13% inhibition was observed with NADH, 2-oxoglutarate, L-glutamate or DL-malate (all at 1 mM) and none of these compounds significantly altered the inhibition obtained with glyoxylate or oxaloacetate (Table 6.2).

These results are essentially similar to those observed with the $NADP^+$-specific enzymes of Escherichia coli, Brevibacterium flavum, Bacillus subtilis, pig-heart (Shiio and Ozaki, 1968; Bennet and Holms, 1975) and the protozoan Crithida fasciculata (Marr and Weber, 1969). No evidence for

Table 6.2 Concerted inhibition of isocitrate dehydrogenase by glyoxylate and oxaloacetate

Isocitrate dehydrogenase was assayed in extracts of methanol-grown bacteria as described in Section 2.12b. Potential inhibitors were incubated in the assay mixture containing 120 µg protein for 1 min before the addition of isocitrate to initiate the reaction.

1st addition (1 mM)	2nd addition (% inhibition)		
	glyoxylate (1 mM)	oxaloacetate (1 mM)	none
oxaloacetate	90	-	20
glyoxylate	-	90	23
2-oxoglutarate	24	16	10
glutamate	26	19	12
DL-malate	21	20	10
NADH	24	21	13

concerted inhibition by oxaloacetate and glyoxylate has been described for isocitrate dehydrogenases specific for NAD^+ and this inhibition therefore appears to be a general property of $NADP^+$-specific enzymes.

It has been suggested that the significance of the concerted inhibition of isocitrate dehydrogenase is the diversion of isocitrate from the TCA cycle into the glyoxylate bypass by way of isocitrate lyase (Marr and Weber, 1969). However, it is unlikely that the products of isocitrate lyase (glyoxylate and C_4 dicarboxylic acids) would act to increase its activity (by increasing the supply of isocitrate) and furthermore neither pig heart nor *Pseudomonas* AM1 have any isocitrate lyase. The regulatory significance of this concerted inhibition thus remains unclear.

6.5 Regulation of malate synthase activity

The effects of a number of compounds on the activity of malate synthase in crude extracts of methanol-grown *Pseudomonas* AM1 and ethanol-grown *Hyphomicrobium* X are presented in Table 6.3. Although *Hyphomicrobium* X contrasts with *Pseudomonas* AM1 in that its malate synthase *is* repressed during growth on methanol (Attwood and Harder, 1974), the pattern of inhibition by these compounds was similar: in both cases hydroxypyruvate (1 mM) inhibited enzyme activity by between 55% and 66% when assayed in the presence of DTNB. Succinate had no effect on either of the enzyme activities although the other compounds (except serine) inhibited the enzymes by between 5% and 25%. The effect of serine differed in that it significantly inhibited malate synthase of

Table 6.3 The effect of potential inhibitors on the activity of malate synthase from methanol-grown *Pseudomonas* AM1 and ethanol-grown *Hyphomicrobium* X

Malate synthase was assayed in extracts by the DTNB method described in Section 2.12e. Potential inhibitors were incubated in the assay mixtures containing 80 μg (*Pseudomonas* AM1) and 280 μg protein (*Hyphomicrobium* X) for 1 min before the addition of glyoxylate to initiate the reaction.

inhibitor (1 mM)	% inhibition	
	Ps AM1	Hyphomicrobium
hydroxypyruvate	56	66
serine	0	33
glycine	5	20
phospho(enol) pyruvate	14	20
malate	12	18
succinate	0	0
pyruvate	23	25

The specific activities in the absence of any inhibitor were 53 and 10 nmoles/min/mg. protein for *Pseudomonas* AM1 and *Hyphomicrobium* X respectively.

Hyphomicrobium, but had no effect on the enzyme from Pseudomonas AM1.

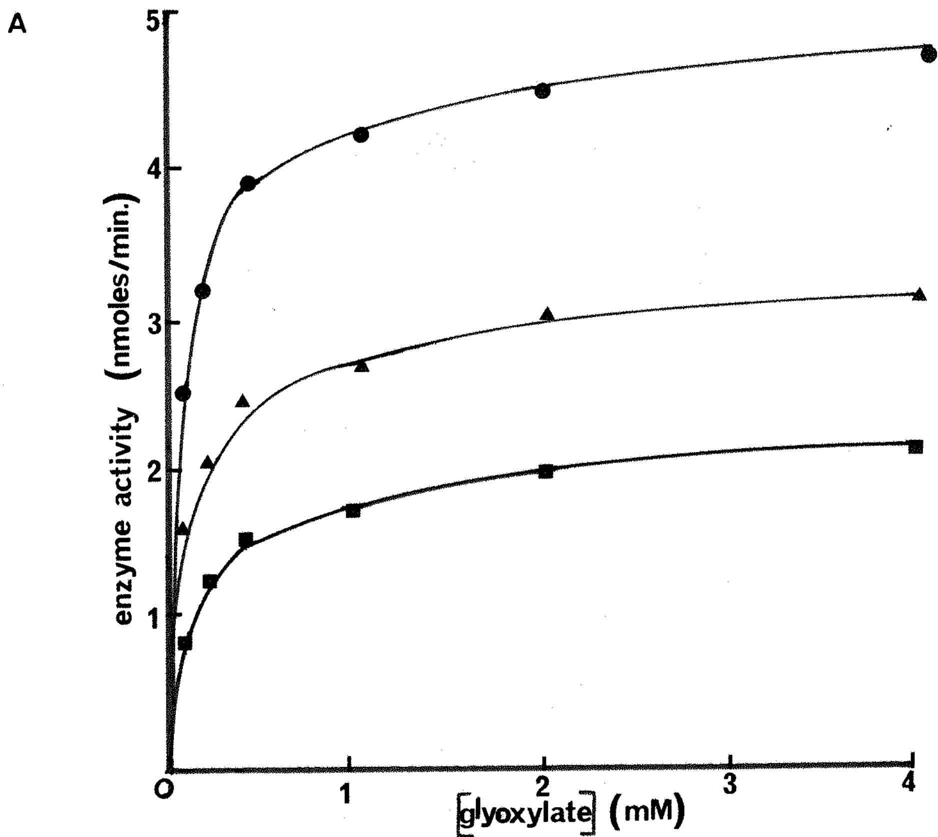
The physiological significance of the inhibition of malate synthase by the serine pathway intermediate hydroxypyruvate was further investigated with extracts of methanol-grown Pseudomonas AM1.

In the presence of hydroxypyruvate the K_m for glyoxylate (140 μM) was virtually unchanged although the V_{max} decreased from 5 nmoles/min to 2.1 in the presence of 2 mM hydroxypyruvate (Fig. 6.3). This is characteristic of non-competitive inhibition despite the similarity of the glyoxylate and hydroxypyruvate molecules. The effect of acetyl-CoA on the inhibition of malate synthase by hydroxypyruvate is shown in Fig. 6.4. The apparent K_m for acetyl-CoA was 8 μM and the V_{max} was 4.8 nmoles/min. In the presence of 2 mM hydroxypyruvate the apparent K_m was increased to 10 μM and the V_{max} decreased to 2.3 nmoles/min. Fig. 6.4 shows that malate synthase is additionally inhibited by its substrate, acetyl-CoA (at concentrations above 0.06 mM) indicating that the enzyme only functions maximally under low concentrations of acetyl-CoA and relatively high concentrations of glyoxylate. The significance of these regulatory properties are discussed in Section 6.6.

When assayed by the method of Dixon and Kornberg (1959), in the absence of DTNB, hydroxypyruvate was a much stronger inhibitor of malate synthase activity (Fig. 6.5). This may be due to the removal of coenzyme A by DTNB, thus pulling the equilibrium of the reaction in favour of malate synthesis or

Fig. 6.3 Effect of glyoxylate concentration on malate synthase activity in the presence and absence of hydroxypyruvate

The enzyme was assayed in extracts of methanol-grown bacteria by the DTNB method (Section 2.12c) using 0.05 μmol acetyl-CoA and 80 μg protein. a) Direct plot b) Double-reciprocal plot.
●, no hydroxypyruvate. ▲ and ■ , 1 and 2 mM hydroxypyruvate respectively.



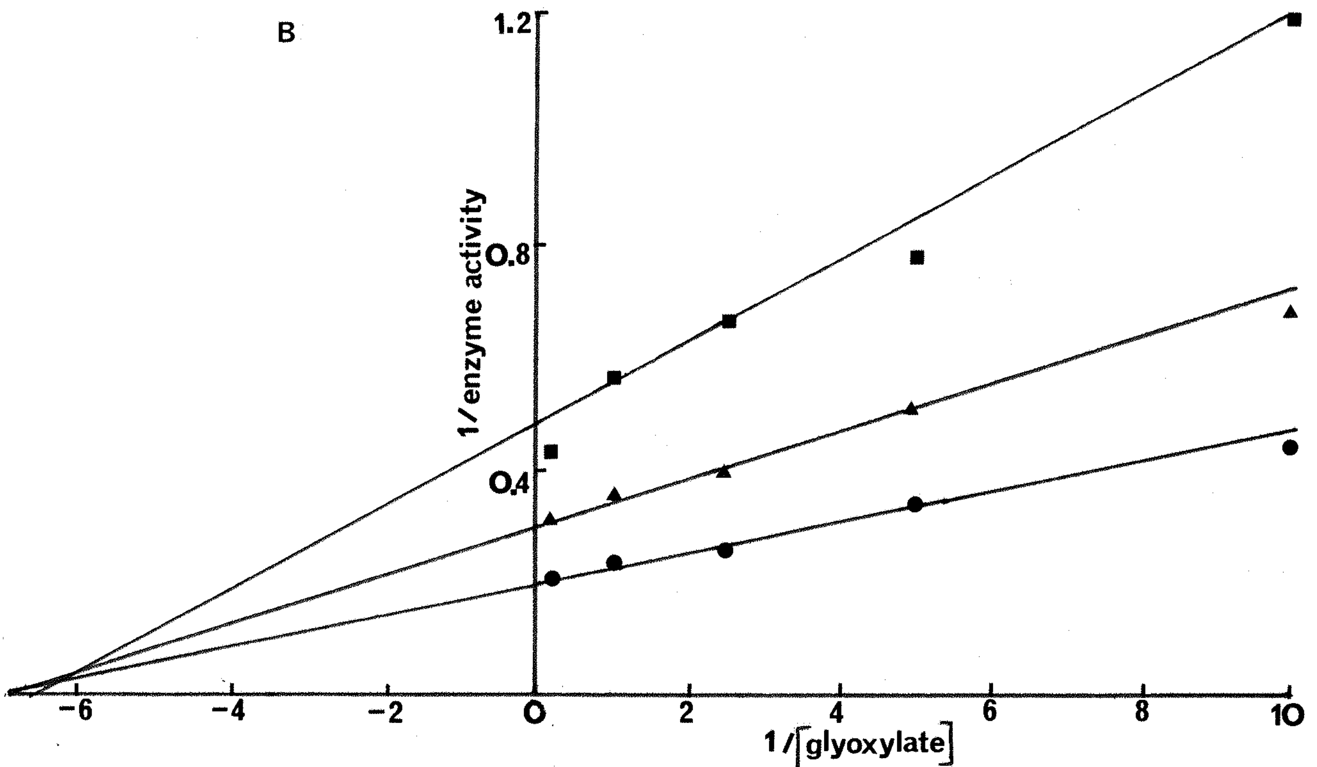


Fig. 6.4 Effect of acetyl-CoA concentration on malate synthase activity in the presence and absence of hydroxypyruvate

The enzyme was assayed by the DTNB method (Section 2.12c) using 2 μmole glyoxylate and 80 μg protein. a) Direct plot b) Double-reciprocal plot. ●, no hydroxypyruvate ▲, 1 mM hydroxypyruvate.

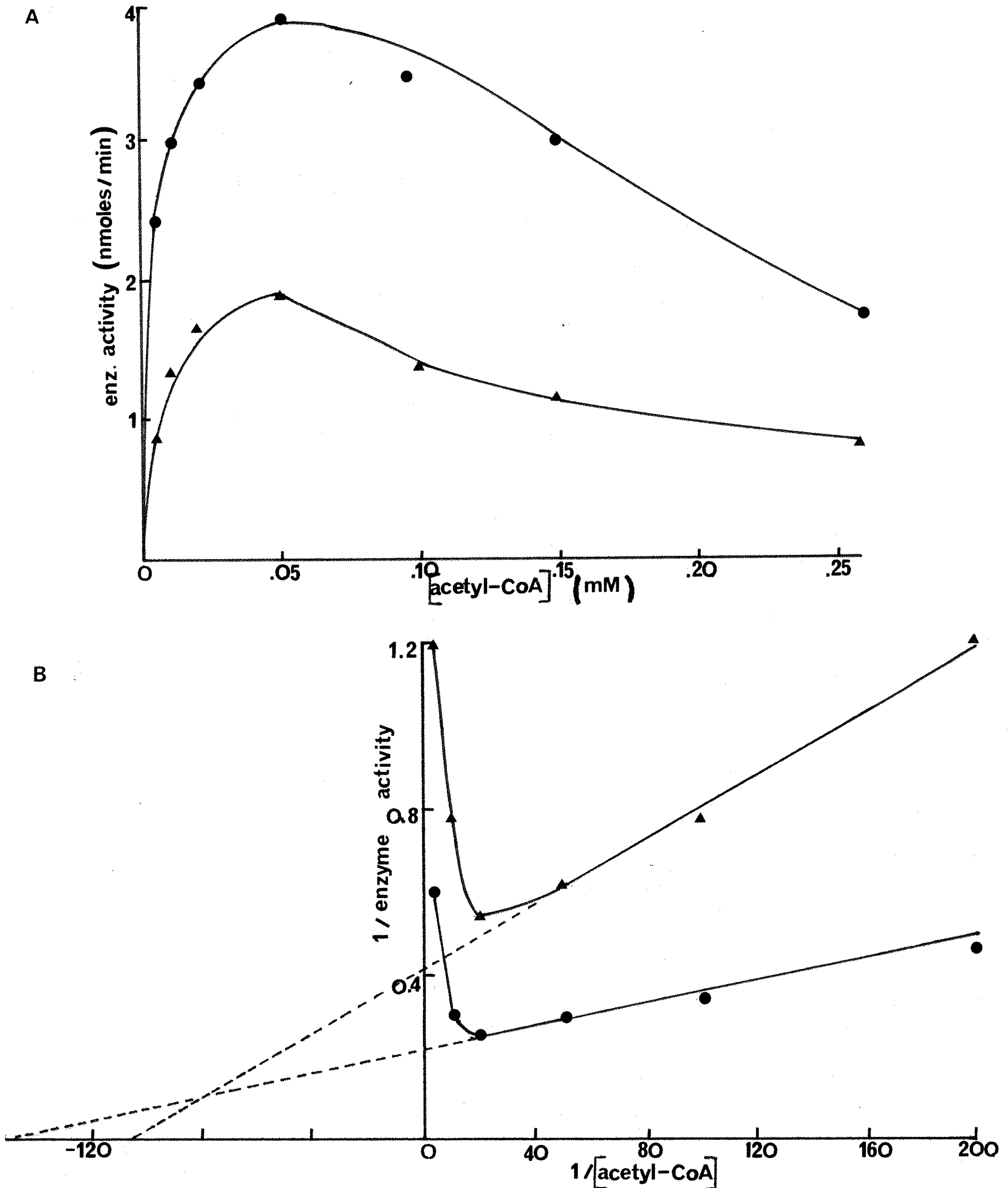
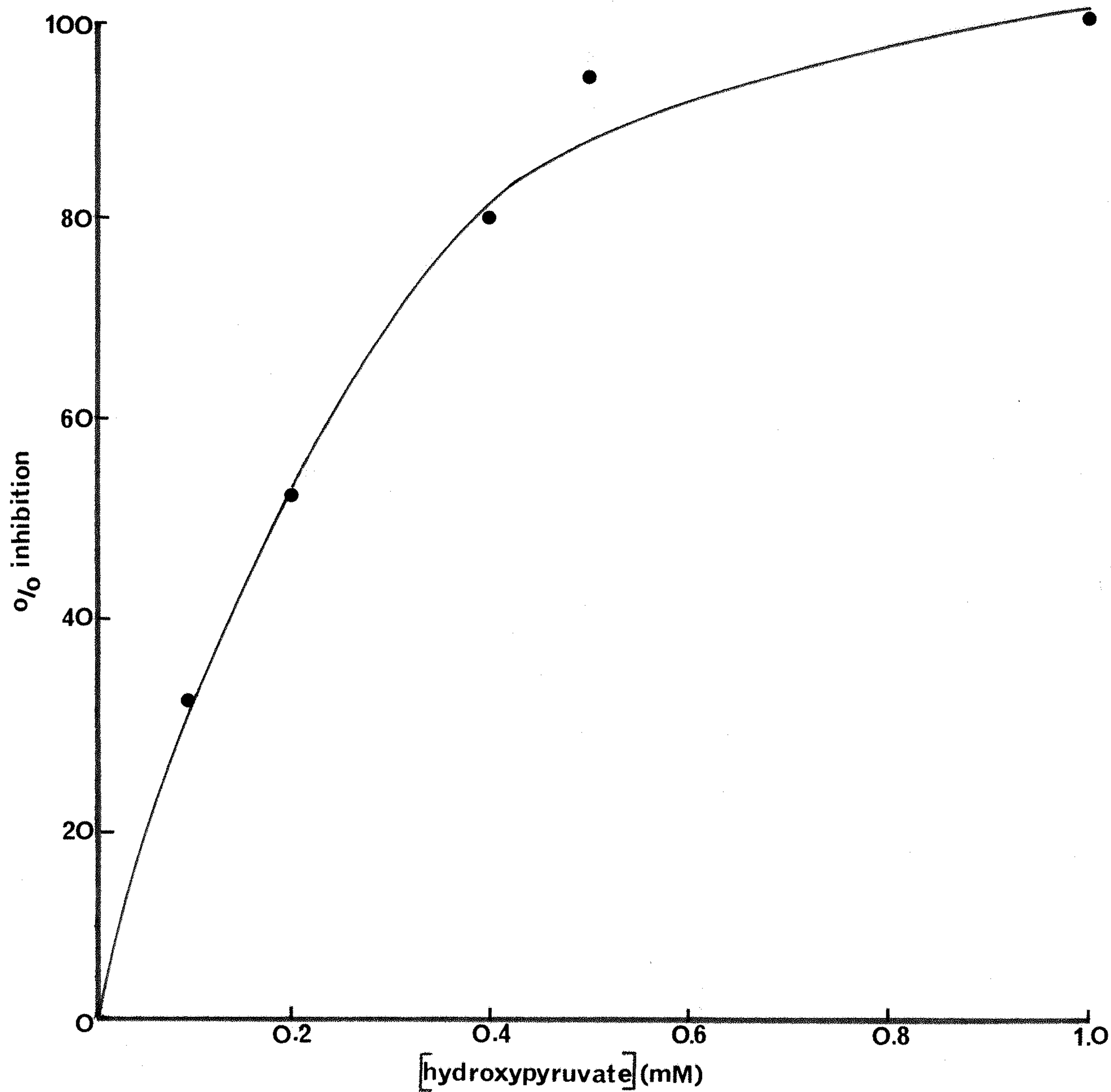


Fig. 6.5 Inhibition of malate synthase by hydroxypyruvate

The enzyme was assayed by the method of Dixon and Kornberg (1959).



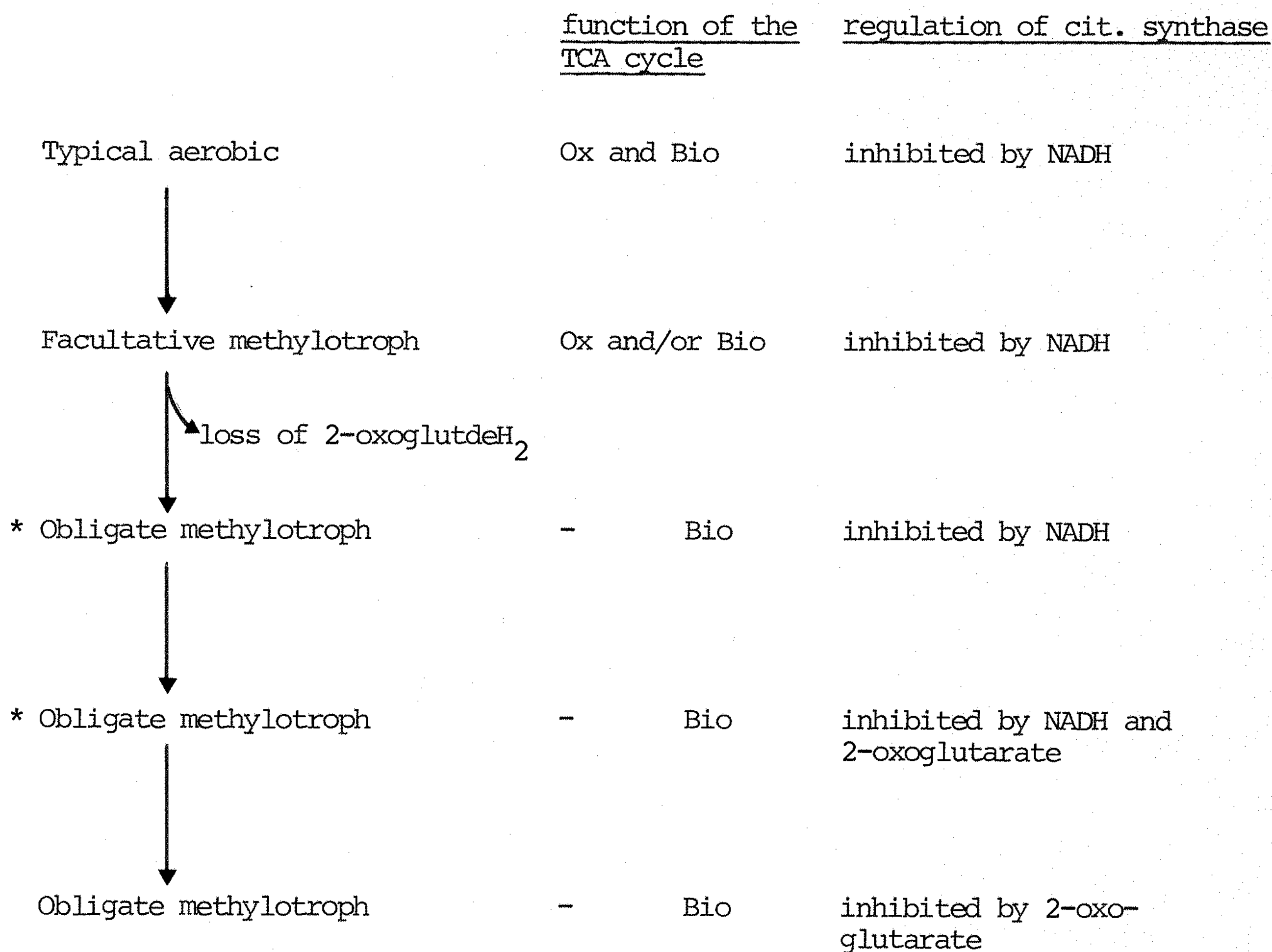
DTNB itself may diminish the inhibition of malate synthase by hydroxypyruvate. An analogous situation to this has been reported for the citrate synthase of Azotobacter beijerinckii. In the presence of DTNB the catalytic activity of the enzyme was unchanged but regulation of activity by AMP and NADH was abolished indicating that an essential thiol group is present at the regulatory site (Senior and Dawes, 1971).

6.6 Summary and discussion

The results with citrate synthase of Pseudomonas AM1 are in agreement with those of Colby and Zatman (1975c) who recently demonstrated that the citrate synthases of the facultative methylotrophs 5B1 and 3A2 are regulated by NADH and AMP. By contrast, the enzyme of obligate and restricted facultative methylotrophs (lacking 2-oxoglutarate dehydrogenase) were insensitive to NADH inhibition. The citrate synthase from two of the obligate methylotrophs (4B6 and C2A1) and a restricted facultative methylotroph (W3A1, type M) were inhibited by high concentrations (10 mM) of 2-oxoglutarate (Colby and Zatman, 1975c).

These observations, together with the demonstration that the loss of 2-oxoglutarate dehydrogenase is sufficient to convert a typical facultative methylotroph into an obligate methylotroph (mutant ICT 41) suggest a sequence of evolution to obligate methylotrophy by the scheme shown in Fig. 6.6. In this scheme some typical Gram-negative aerobic bacteria acquired the ability to grow on C_1-compounds (facultative methylotrophs); these organisms, once subjected to environmental conditions not requiring an oxidative TCA cycle, lost

Fig. 6.6 Possible evolutionary sequence to obligate methylotrophy



*To date no obligate methylotrophs with these properties have been described.

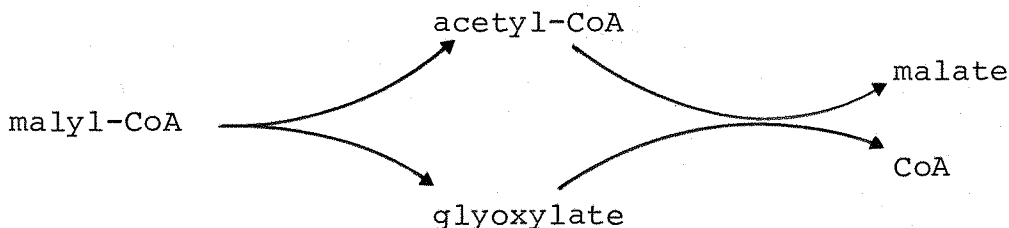
the ability to produce sufficient 2-oxoglutarate dehydrogenase required for growth on multicarbon compounds thus becoming obligate methylotrophs; regulation of citrate synthase by NADH was then unnecessary and was lost in favour of regulation by the 'biosynthetic' end-product 2-oxoglutarate. It is therefore possible that the obligate methylotrophs examined by Colby and Zatman (1975c) are in the process of acquiring the ability to regulate their citrate synthases by 2-oxoglutarate inhibition. Some of these oganisms (4B6, C2A1 and W3A1) already possess this property although at this stage of their evolution high concentrations of 2-oxoglutarate are required. An extended study of the citrate synthases of methylotrophs would probably show that certain obligate methylotrophs do possess citrate synthases which are inhibited by physiological concentrations (1-2 mM) of 2-oxoglutarate.

There is some diversity amongst methylotrophs with respect to the type of isocitrate dehydrogenase they possess. The obligate methane-utilisers which use the serine pathway of C_1 assimilation (Type II) contain $NADP^+$-specific isocitrate dehydrogenases, (Davey _et_ _al_., 1972). By contrast, the majority of obligate methylotrophs with the ribulose monophosphate pathway have both $NADP^+$- and NAD^+-specific enzymes. Such bacteria include Bacterium C2A1 and Bacterium 4B6 in which the NAD^+-specific enzyme predominates (Colby and Zatman, 1975a) and the methane-utilisers _P. methanica_, _Methylomonas albus_ and _Methylococcus minimus_ which have equal levels of the NAD^+- and $NADP^+$-specific enzymes (Davey _et_ _al_., 1972). The isocitrate dehydrogenase from _M. capsulatus_ is an exception in being

strictly dependent on NAD^+. Type 'M' restricted facultative methylotrophs also have both NAD^+- and $NADP^+$-specific isocitrate dehydrogenases whereas the type 'L' organisms have $NADP^+$-specific enzymes (Colby and Zatman, 1975a). The enzymes from all the facultative methylotrophs are $NADP^+$-specific.

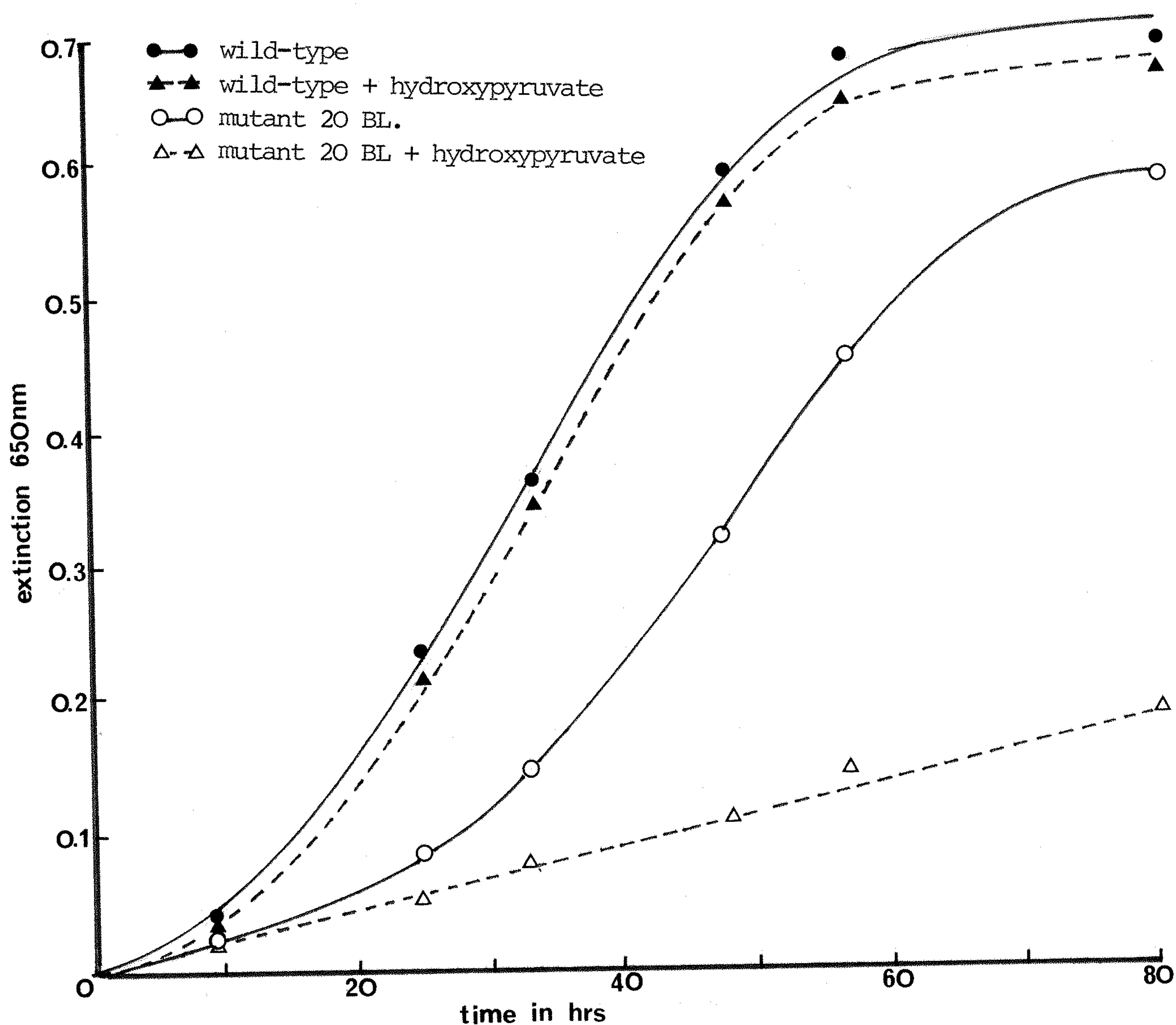
All the bacteria which use the serine pathway therefore contain isocitrate dehydrogenase activity strictly dependent on $NADP^+$ while the cofactor requirement of the isocitrate dehydrogenases from ribulose monophosphate pathway methylotrophs appears to depend on the 'degree' of their obligate methylotrophy.

The inhibition of malate synthase by hydroxypyruvate and the substrate inhibition of the enzyme by acetyl-CoA may be of significance during growth of <u>Pseudomonas</u> AM1 on C_1-compounds. During growth on C_1-compounds the high malyl-CoA lyase activity would provide acetyl-CoA which is not rapidly oxidised because of the low TCA cycle activity. This may lead to a high acetyl-CoA concentration which will inhibit malate synthase and prevent futile 'malyl-CoA deacylase' activity:



Conversely, during growth of <u>Pseudomonas</u> AM1 on compounds requiring malate synthase for their assimilation (e.g. ethanol and β-hydroxybutyrate; Section 4.4) the higher TCA cycle

Fig. 6.7 Effect of hydroxypyruvate (2 mM) on the growth of mutant 20 BL and wild-type Pseudomonas AM1 on β-hydroxybutyrate



activity may lower the intracellular acetyl-CoA concentration and prevent substrate level inhibition of the enzyme.

The results in Fig. 6.7 indicate that hydroxypyruvate inhibits malate synthase *in vivo* as well as *in vitro*. Growth of the hydroxypyruvate reductase-deficient mutant (20 BL) on β-hydroxy-butyrate was severely inhibited in the presence of 2 mM hydroxypyruvate although growth of wild-type *Pseudomonas* AM1 on this compound was unaffected. It can be assumed that hydroxypyruvate is not rapidly metabolised by mutant 20 BL and inhibits growth of the mutant by inhibition of malate synthase. By contrast, the relatively high level of hydroxy-pyruvate reductase in wild-type bacteria grown on β-hydroxy-butyrate precludes inhibition of malate synthase by removing the hydroxypyruvate.

Having shown that acetyl-CoA and hydroxypyruvate inhibit malate synthase activity, it is necessary to confirm the physiological significance of these inhibitory mechanisms by measuring the intracellular concentrations of these com-pounds during growth of *Pseudomonas* AM1 on various carbon sources. It would also be advantageous to confirm that the same regulatory mechanisms operate with a purified malate synthase preparation.

CHAPTER 7

Unsolved problems and areas for further investigation

7.1 Introduction

The functions of this final Chapter are twofold; first, to discuss some of the fundamental problems concerned with the metabolism of C_1-compounds by *Pseudomonas* AM1 and related organisms, and secondly to discuss areas where further research is necessary.

7.2 Unsolved problems of metabolism of C_1-compounds by Pseudomonas AM1

Two major problems remain in the study of the group of serine pathway methylotrophs exemplified by *Pseudomonas* AM1:

a) The activation of malate (or precursor) to malyl-CoA

b) the oxidation of acetyl-CoA to glyoxylate in the absence of isocitrate lyase. This problem is common to growth of the organisms on both C_1- and C_2-compounds.

From the data presented in Table 7.1, it is evident that these problems are very closely linked. All the organisms lacking isocitrate lyase activity (*Pseudomonas* AM1, *Pseudomonas* 3A2, P. *extorquens* and Bacterium 5β1) during growth on C_1-compounds also lack malyl-CoA synthetase and consequently overall ATP-malate lyase activity is absent. These organisms however do contain high levels of malyl-CoA lyase during growth on C_1-compounds. Conversely, the organisms which do possess isocitrate lyase (*Pseudomonas* MS, *Pseudomonas* MA and P. *aminovorans*) also contain malyl-CoA synthetase activity. It would be of interest to extend this survey of serine pathway methylotrophs to see if organisms do exist which contain isocitrate lyase but not malyl-CoA synthetase or vice versa.

Table 7.1 Activities of malate- and isocitrate lyases in C_1 utilisers (modified from Quayle, 1975)

Organism	Growth substrate	Enzyme activity (nmoles/min/mg. protein)				Reference
		isocitrate lyase	malate synthase	malyl-CoA lyase	ATP-malate lyase	
Pseudomonas MA	acetate	219	206		0.3	Bellion & Hersh (1972)
" "	methylamine	111	6.6		38	Bellion & Hersh (1972)
Pseudomonas MS	methylamine	10.6		1150	134; 27	Large and Carter (1973); Salem et al. (1973b)
Pseudomonas aminovorans	methylamine	10.6			111	Large and Carter (1973); Salem et al. (1973b)
Pseudomonas AM1	ethanol	4.3 (n.d)*	31(42)	(210)	n.d	Dunstan et al. (1972a); Salem et al. (1973b)
" "	methanol	n.d. (n.d.)	20(44)	1650(850)	n.d(n.d)	Dunstan et al. (1972a); Salem et al. (1973b)
Pseudomonas 3A2	acetate	n.d	n.d	220	n.d	Hacking & Quayle (unpublished)
" "	methylamine	n.d	n.d	770	n.d	Hacking & Quayle (unpublished)
Pseudomonas extorquens	methylamine	1	26	771	n.d	Hacking & Quayle (unpublished)
Bacterium 5B1	acetate	143				Colby & Zatman (1972)
" "	methylamine	8			n.d	R. Cox, personal communication

* Figures in parentheses are results obtained during the present study

The solution to the inability of these organisms to activate malate to malyl-CoA in the absence of a malyl-CoA synthetase may be found in CoA transfer from a suitable donor. However, several CoA-thioesters have been tested for this ability without success using Pseudomonas AM1 (Quayle, 1975). These include the CoA derivatives of the following compounds; acetate, succinate, propionate, butyrate, β-hydroxybutyrate, glycollate, oxalate, citrate, β-hydroxy β-methylglutarate, formate, methylmalonate and acetoacetate.

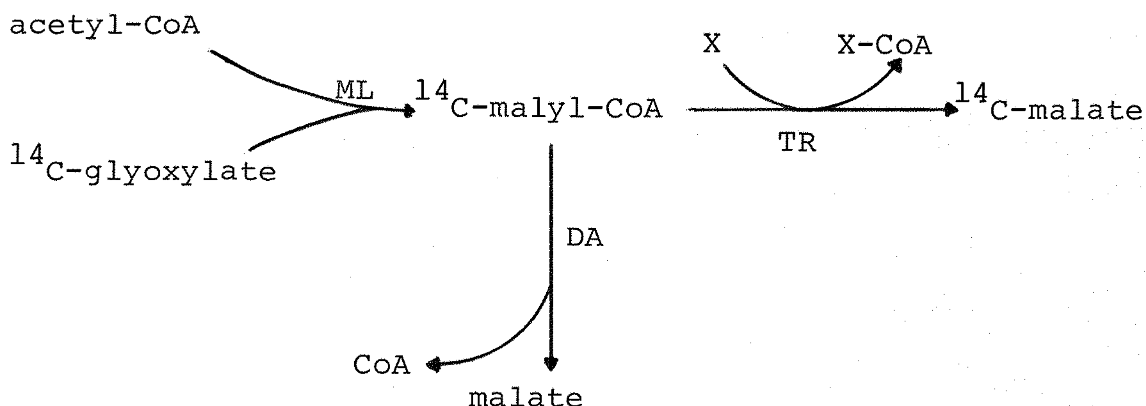
Whilst the activation of malate to malyl-CoA by this group of bacteria may be regarded as a minor problem, a more fundamental problem is posed by their lack of isocitrate lyase. No direct evidence has so far been obtained for the hydroxylation of acetate to glycollate or glyoxylate by cell-free extracts. The results presented in Sections 4.8 and 4.9 indicate that acetyl-CoA is the substrate for 'oxidation' and that glycollate may not be involved. These results are consistent with the hypothetical scheme presented in Fig. 5.4 in which acetyl-CoA condenses with 2-oxoglutarate to give homocitrate, which, after conversion to homoisocitrate is cleaved to glyoxylate and glutarate. The 2-oxoglutarate would be regenerated from glutarate by a series of enzymes analogous to those TCA cycle enzymes involved in oxaloacetate formation from succinate.

7.3 Areas for further investigation

The problems discussed above clearly require further investigation. A more detailed search for either a CoA-transferase or an acyl-CoA synthetase capable of activating

malate or a precursor must be undertaken using a variety of assay conditions. As discussed in Section 4.5 the presence of a reversible CoA-transferase would explain the ability of mutant ICT 51R to grow on C_2-compounds in the absence of any detectable malate synthase activity.

This mutant may be useful in attempts to elucidate the possible CoA-donor to malate and experiments could be designed such as the one outlined below.



In the presence of acetyl-CoA, ^{14}C-glyoxylate and an extract of ICT 51R the production of ^{14}C-malyl-CoA would be catalysed by a reverse of the 'normal' malyl-CoA lyase reaction. If a CoA-transferase was present, ^{14}C-malate would only be detected in the presence of a suitable CoA-acceptor molecule and various compounds could be tested for their ability to catalyse the formation of free ^{14}C-malate. Malyl-CoA deacylase which would interfere with such an assay system is absent from extracts of mutant ICT 51R. Similar considerations to these could also apply to Pseudomonas 3A2 which also lacks malate synthase activity but is able to grow on C_2-compounds (Table 7.1).

Providing the CoA-acceptor is also the physiological CoA-donor to malate, such an approach has the distinct advantage that an infinite number of CoA-thioesters need not be synthesised.

Attempts must also be made to assay the enzymes possibly involved in the scheme given in Fig. 5.4. If such enzymes are present they must have sufficiently high activities to account for a role in a major assimilatory pathway. Assuming that 50% of the dry weight of the cell is carbon, it can be calculated that the specific activities of these enzymes must be in the range of 100-120 nmoles/min/mg. protein in order to allow a doubling time of 5 hr. during growth on C_1-compounds.

Several other areas exist where further investigation is required. These include the route for assimilation of lactate and pyruvate by Pseudomonas AM1 (Section 4.11), the assimilation of β-hydroxybutyrate and ethanol by mutant ICT 51R (lacking malate synthase) and, in particular, the assimilation of these compounds by mutant PCT 57 which apparently lacks both malate synthase and malyl-CoA lyase.

It would also be of interest to isolate and investigate the properties of mutants of Pseudomonas AM1 lacking TCA cycle enzymes other than 2-oxoglutarate dehydrogenase.

For convenience, a summary of the properties of most of the mutants used in this study is given in Table 7.2.

7.4 Mutants unable to oxidise methanol

A group of mutants have been isolated whose properties have not been previously described. The growth properties of

these mutants (selected for their ability to grow on β-hydroxybutyrate but not methanol) are presented in Table 7.3. Mutant ICT 111 is similar to mutant PCT 29 (Dunstan _et al_., 1972a) and lacks methanol dehydrogenase. As expected this mutant is unable to grow on methanol or ethanol. The other three mutants lack cytochrome c and are interesting in that they have different growth properties. Mutant ICT 31 is similar to mutant PCT 761 (Anthony, 1975b) and cannot grow on methanol, ethanol or methylamine. These compounds also fail to support the growth of mutants ICT 21 and ICT 112 which are also unable to grow on malonate and formate respectively. These cytochrome c deficient mutants may be useful in a study of the role of cytochrome c during methylamine oxidation. Mutant PCT 761 lacks methylamine dehydrogenase as well as cytochrome c and thus the involvement of cytochrome c during methylamine oxidation has not yet been established (see Anthony, 1975b). It would also be of interest to determine whether or not cytochrome c is involved in malonate and formate metabolism.

Table 7.2 Summary of the properties of mutants used in this work

Mutants	wt	ICT 41	ICT 41R	ICT 51	ICT 51R	ICT 54	ICT 5 (PCT 48)	PCT 57	20 BL
Growth substrate									
Malate	5	0	4	5	5	5	5	5	5
Succinate	5	0	5	5	5	5	5	5	5
Methanol	5	5	4	5	5	5	0	0	0
Ethanol	3	0	3	0	3	0	0	3	1
β-hydroxybutyrate	5	0	5	0	5	4	0	5	4
Malonate	3	0	3	0	3	0	0	3	1
Lactate	5	0	5	4	4	4	5	4	4
Pyruvate	4	0	-	4	-	4	4	4	-
Acetate + glyoxylate	3	0	-	0	3	0	-	-	-
Ethanol + glycollate	5	-	-	0	-	-	2	-	0
Ethanol + glyoxylate	5	-	-	0	-	-	4	-	4
Selection procedure	-	$MeOH^+$	$MeOH^+$	$malate^+$	$malate^+$	$malate^+$	$succ^+$	$succ^+$	$succ^+$
		BHB^-	BHB^+	Ac + $glyox^-$	Ac + gly^+	Ac + $glyox^-$	BHB^-	$MeOH^-$	$MeOH^-$
Metabolic lesion	-	2-ogdeH_2	-	malate synthase	-	acetyl-CoA synth(et)ase	?	malyl-CoA lyase (malate synthase)	OHPyr reductase
No. of mutants isolated	-	1	2	1	1	1	6	1	-

Table 7.3 Properties of mutants unable to utilise methanol

The growth properties were investigated in liquid media as described in Section 2.7.

Growth Substrate	Strain				
	Wt	ICT 111	ICT 112	ICT 21	ICT 31
methanol	5	0	0	0	0
methylamine	5	5	0	0	0
formate	4	4	0	3	3
oxalate	3	3	2	3	3
ethanol	3	0	0	0	0
malonate	3	3	3	0	3
β-hydroxy-butyrate	5	5	5	5	5
lactate	5	5	4	4	4
succinate	5	5	5	5	5
metabolic lesion	-	MDH	cyt c	cyt c	cyt c
No. of mutants isolated	-	2	1	1	1

MDH - methanol dehydrogenase

cyt c - cytochrome c

All the mutants are unable to oxidise methanol

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