Citric acid cycle enzymes of Methylophilus methylotrophus

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CITRIC ACID CYCLE ENZYMES

OF METHYLOPHILUS METHYLOTROPHUS

Submitted by Adrian J. Lloyd
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For Mum, Dad and Tat.

Abbreviations

Apart from the abbreviations below, all other abbreviations used are as defined in "Policy of the Journal and Instructions to Authors" (1989) Biochem. J. 257, 1-21.

Anti-Mm.IDH: rabbit antiserum raised against M. methylotrophus isocitrate dehydrogenase.

A_o^i: proportion of isocitrate dehydrogenase activity at zero time subject to loss over the initial fast phase of chemical modification.

A_o^m: proportion of isocitrate dehydrogenase thiol residues at zero time subject to loss over the initial fast phase of chemical modification.

B_o^i: analogous to A_o^i, but refers to second slow phase of chemical modification.

B_o^m: analogous to A_o^m, but refers to second slow phase of chemical modification.

CAC: citric acid cycle.

Ches: 2(N-cyclohexyl) ethane sulphonic acid.

CS: citrate synthase.

DALA: delta-aminolevulinic acid.

DCPIP: dichlorophenolindophenol.

DHAP: dihydroxyacetone Phosphate.

DTNB: 5,5'-dithiobis(2-nitrobenzoic acid).

DTNP: 2,2'-dithiobis(5-nitropyridine).

DTT: dithiothreitol.

E: enzyme.

ELig: enzyme-ligand complex.

ES_2: non-productive enzyme:substrate complex.

F6P: fructose 6-phosphate.

$FADH_2$: reduced FAD.

G6P: glucose 6-phosphate.

GA: glycerate.

GAP: glyceraldehyde 3-phosphate

GDH: glutamate dehydrogenase.

GOGAT: glutamate synthase.

GS: glutamine synthetase.

H6P: D-*erythro* L-*glycero* 3-hexulose 6-phosphate.

IAA: iodoacetic acid.

ICL: isocitrate lyase.

IDH: isocitrate dehydrogenase.

IDH K/P: IDH kinase/phosphatase.

$k_{(a)}^{i}$: pseudo first-order rate constant of inactivation of IDH in the initial fast phase of chemical modification.

$k_{(a)}^{m}$: pseudo first-order rate constant of modification of IDH thiol residues in the initial fast phase of chemical modification.

$k_{(b)}^{i}$: pseudo first-order rate constant of inactivation of IDH in the second slow phase of chemical modification.

$k_{(b)}^{m}$: pseudo first-order rate constant of modification of IDH in the second slow phase of chemical modification.

$k_{[-NADH]}$: first-order rate constant of thermal inactivation of CS in the absence of 1 mM-NADH.

$k_{[+NADH]}$: first-order rate constant of thermal inactivation of CS in the presence of 1 mM-NADH.

KDPG: 2-dehydro 3-deoxy 6-phosphogluconate.

Lig: ligand.

MET-8.0: 20 mM-Tris, 2.4 mm-EDTA and 10 mM-$MgCl_2$ adjusted to pH 8.0.

MET-7.2: 20 mM-Tris, 2.4 mM-EDTA and 10 mM-$MgCl_2$ adjusted to pH 7.2.

MET-8.6: 20 mM-Tris, 2.4 mM-EDTA and 10 mM-$MgCl_2$ adjusted to pH 8.6.

NEM: N-ethyl maleimide.

NTP: nucleoside triphosphate.

OAA: oxaloacetate.

2-OG: 2-oxoglutarate.

OGDH: 2-oxoglutarate dehydrogenase.

OHPYR: hydroxypyruvate.

PCMB: *para*-chloromercuribenzoate.

PD: potential difference

PDH: pyruvate dehydrogenase.

PEP: phosphoenolpyruvate.

"PFI": pink facultative isolate of Dahl *et al*. (1972).

2-PGA: 2-phosphoglycerate.

3-PGA: 3-phosphoglycerate.

PQQ: 2,7,9-tricarboxy 1H-pyrrolo [2,3-f] quinoline 4,5-dione.

$PQQH_2$: 2,7,9-tricarboxy 1H-pyrrolo [2,3-f] quinoline 4,5-diol.

RFM: restricted facultative methylotroph.

RubP: ribulose bis phosphate.

RuMP: ribulose monophosphate.

STK: succinate thiokinase.

T-8.0: 20 mM-Tris adjusted to pH 8.0.

T_{50}: temperature (^{o}C) at which 50% of enzyme activity is lost over a specified time.

T-8.6: 20 mM-Tris adjusted to pH 8.6.

TCA: trichloroacetic acid.

TE-8.0: 20 mM-Tris and 1 mM-EDTA adjusted to pH 8.0.

TE-8.6: 20 mM-Tris and 1 mM-EDTA adjusted to pH 8.6.

1/20 TE-8.6: 1 mM-Tris and 1 mM-EDTA adjusted to pH 8.6.

TEMED: N,N,N'N'-tetramethyl ethylenediamine.

TNB: 5-thio 2-nitrobenzoic acid.

TNP: 2-thio 5-nitropyridine.

TPP.Cl: thiamine pyrophosphate.

U: unit of enzyme activity (umol substrate transformed or product produced.min^{-1}).

Z: extent of chemical modification (number of thiol groups modified per dimer of M. methylotrophus isocitrate dehydrogenase) at infinite time.

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Synopsis

(1) Methanol-grown Gram-negative Methylophilus methylotrophus could synthesise 2-oxoglutarate but did not have the dehydrogenases to oxidise 2-oxoglutarate to oxaloacetate. This suggested that this organism did not possess a complete citric acid cycle but used some component enzymes of the latter to synthesise biosynthetic precursors. Oxaloacetate and acetyl-CoA were supplied by pyruvate dehydrogenase and pyruvate carboxylase.

(2) Acetate and acetamide generally increased the activities of citric acid cycle enzymes of methanol-grown M. methylotrophus, but this effect was small relative to those of other organisms that grow on acetate. Activities of enzymes supplying acetyl-CoA were modified, such that acetate or acetamide might partially replace pyruvate as a source of acetyl-CoA.

(3) M. methylotrophus citrate synthase was purified to homogeneity. It was shown to be hexameric (6 X 45,000), exhibited a hyperbolic dependence on both substrates and its affinity for acetyl-CoA was increased by KCl. The response to Mg^{2+} was more complex, with activatory and inhibitory components. The enzyme was inhibited by 2-oxoglutarate, which agrees with the proposed role of the cycle in M. methylotrophus. Unusually, this enzyme was virtually insensitive to NADH. This was confirmed under a variety of conditions using three assays. Further, NADH appeared not to elicit conformational changes associated with NADH-inhibition of the enzyme from other sources.

(4) M. methylotrophus isocitrate dehydrogenase was mainly NAD-linked in extracts. This is unusual among prokaryotes. Extracts also had low NADP-linked activity. The enzyme was purified to homogeneity, making use of its high thermal stability in the presence of isocitrate and its interaction with the immobilised dye Blue HE-RD. The pure enzyme was found to be dimeric (2 X 46,000), exhibited hyperbolic dependences on substrates and also used $NADP^+$ less efficently than NAD^+. This and immuno-inactivation of extract NAD- and NADP-linked activities

suggested M. methylotrophus had one dual-coenzyme specific enzyme. Further immunological studies suggested this enzyme was distinct from those from a range of sources. This enzyme was inactivated by chemical modification of two thiols.

(5) The NADH-insensitivity of citrate synthase and the NAD-linkage of isocitrate dehydrogenase are novel in Gram-negative organisms. A literature review suggests the distribution of these two enzymes may coincide.

CHAPTER 1

Introduction

SECTION A

THE CITRIC ACID CYCLE AND METHYLOTROPHY

1.1. The Discovery of the Citric Acid Cycle

The terminal pathway of carbohydrate oxidation had been partially elucidated by 1935. That year, Szent-Györgi (1935) demonstrated the oxidation of succinate to oxaloacetate (OAA) via fumarate and malate. Both he and Stare and Baumann (1936) showed that these dicarboxylates catalytically supported alpha-glycerophosphate oxidation. In 1937, the formation of 2-oxoglutarate (2-OG) from citrate was observed by Martius and Knoop (1937). That same year, Krebs and Johnson (1937) made the following seminal observations:

(a) Citrate stimulated respiration in minced pigeon heart muscle to a degree that could not be accounted for by the complete oxidation of the added tricarboxylate.

(b) The above effect was further enhanced by addition of alpha-glycerophosphate.

(c) Citrate was produced from OAA.

(d) Metabolic poisons, e.g. malonate or arsenite (known at that time to inhibit oxidation of succinate and 2-OG respectively), attenuated citrate oxidation. Accumulation of 2-OG and succinate was observed that was equivalent to the quantity of citrate metabolised.

(e) The rate of oxygen utilisation during alpha-glycerophosphate oxidation was three times that of citrate synthesis.

In the light of (a)-(e) and the results of their contemporaries, Krebs and Johnson (1937) proposed that citrate oxidation followed the sequence;

Citrate ⟶ 2-OG ⟶ Succinate ⟶ Fumarate ⟶ Malate ⟶ OAA

where citrate was decarboxylated twice to succinate which was oxidised to OAA. The latter then reacted with alpha-glycerophosphate or some other unknown substance to reform citrate, thus establishing a cyclic series of reactions they called "the Citric Acid Cycle" (CAC).

The reasoning behind these proposals was as follows: it was known that succinate was oxidised to OAA so, as inhibition of succinate oxidation caused it to accumulate to an amount equal to the citrate metabolised (d), it was argued that citrate was oxidised to succinate and so, ultimately, to OAA. That poisoning 2-OG oxidation also caused its accumulation to an amount equal to the quantity of metabolised citrate (d), showed that 2-OG resulted from oxidative decarboxylation of citrate and was itself probably oxidised to succinate. Given that citrate was oxidised to OAA, (c) indicated that the pathway of citrate oxidation was cyclic. This explained the catalytic effect of citrate on respiration (a). Observations (b) and (e) suggested that the reformation of citrate involved alpha-glycerophosphate and was responsible for the terminal oxidation of triose-phosphate (and thus carbohydrate) in pigeon muscle.

In 1939 it was shown that oxidation of citrate to 2-OG involved rearrangement of citrate to isocitrate *via* aconitate (Martius, 1939).

By 1940, Krebs had suggested that the substance with which OAA condensed was pyruvate (Krebs and Eggleston, 1940). However, two years later, Lynen (1942) showed that pyruvate did not directly combine with OAA, but instead citrate was formed from acetate and OAA.

The nature of the entry of acetate into the CAC remained elusive until the discovery of coenzyme A (CoA) and its role in the activation of acyl groups (reviewed by Lipmann, 1954). CoA was first implicated in acetate metabolism by Novelli and Lipmann (1947). These workers found that pantothenate (a CoA precursor)-deprivation of yeast caused a reduced capacity for acetate respiration compared with pantothenate-replete yeast. Subsequently, CoA was shown to be a requirement of citrate synthesis from acetate, ATP and OAA in pigeon liver, yeast and *Escherichia coli* (Stern and Ochoa, 1949; Novelli and Lipmann, 1950).

The role played by CoA in acetate metabolism was defined by Lynen *et al.* (1951), who identified the biochemically active form of acetate as the S-acetyl thioester of CoA, acetyl-CoA. Their preparation from yeast could replace ATP, CoA and acetate in the assay for acetylation of sulphanilamide in pigeon liver. That acetyl-CoA replaced ATP showed that the acyl-thioester could provide the free energy used to drive biological acylation reactions that, until then, had required a

nucleoside triphosphate (NTP) *in vitro*.

Thus, by 1950 there was strong if not conclusive evidence that acetate entered the CAC as acetyl-CoA and condensed with OAA to form citrate. This was finally confirmed by the purification of an enzyme from pig heart by Ochoa *et al.* (1951) that catalysed the reaction:

$$\text{Acetyl-CoA} + \text{OAA} + H_2O \longrightarrow \text{Citrate} + \text{CoA} + H^+$$

This enzyme is now known as citrate synthase (CS).

Thus, most of the intermediates and mode of entry of carbon into the CAC had been identified by 1951. Finally, the CAC intermediate succinyl-CoA was discovered when it was found that 2-OG oxidation to succinate proceeded *via* two steps: first, oxidative decarboxylation of 2-OG to succinyl-CoA and second, the hydrolysis of the thioester bond of succinyl-CoA to form succinate and CoA (Kaufmann *et al.*, 1953). The latter reaction also produced NTP, illustrating that the free energy of the thioester could be conserved as a phosphodiester bond in a NTP.

The connection between pyruvate and the CAC proposed by Krebs and Eggleston (1940) was elucidated at the same time as the discovery of acetyl-CoA. Olson and Kaplan (1948) had found that pyruvate oxidation required CoA in duck liver and Korkes *et al.* (1950) had shown that acetate could be formed from pyruvate. Finally, Korkes *et al.* (1951) explained the link between pyruvate, acetate and CoA by the discovery of pyruvate dehydrogenase (PDH):

$$\text{Pyruvate} + \text{CoA} + NAD^+ \longrightarrow \text{Acetyl-CoA} + \text{NADH} + H^+ + CO_2$$

This work demonstrated the route of entry of carbohydrate into the cycle. It was soon shown that the CAC not only oxidised acetyl units from carbohydrates but was also the terminal pathway for fatty and amino acid oxidation (**1.3.1.**). The CAC as it is now known to exist in aerobically respiring plant and animal tissues is shown in Figure 1.

1.2. Confirmation of the Operation of the CAC in Prokaryotes

The methods of Krebs and Johnson (1937) were inadequate for the

Figure Legend to Figure 1

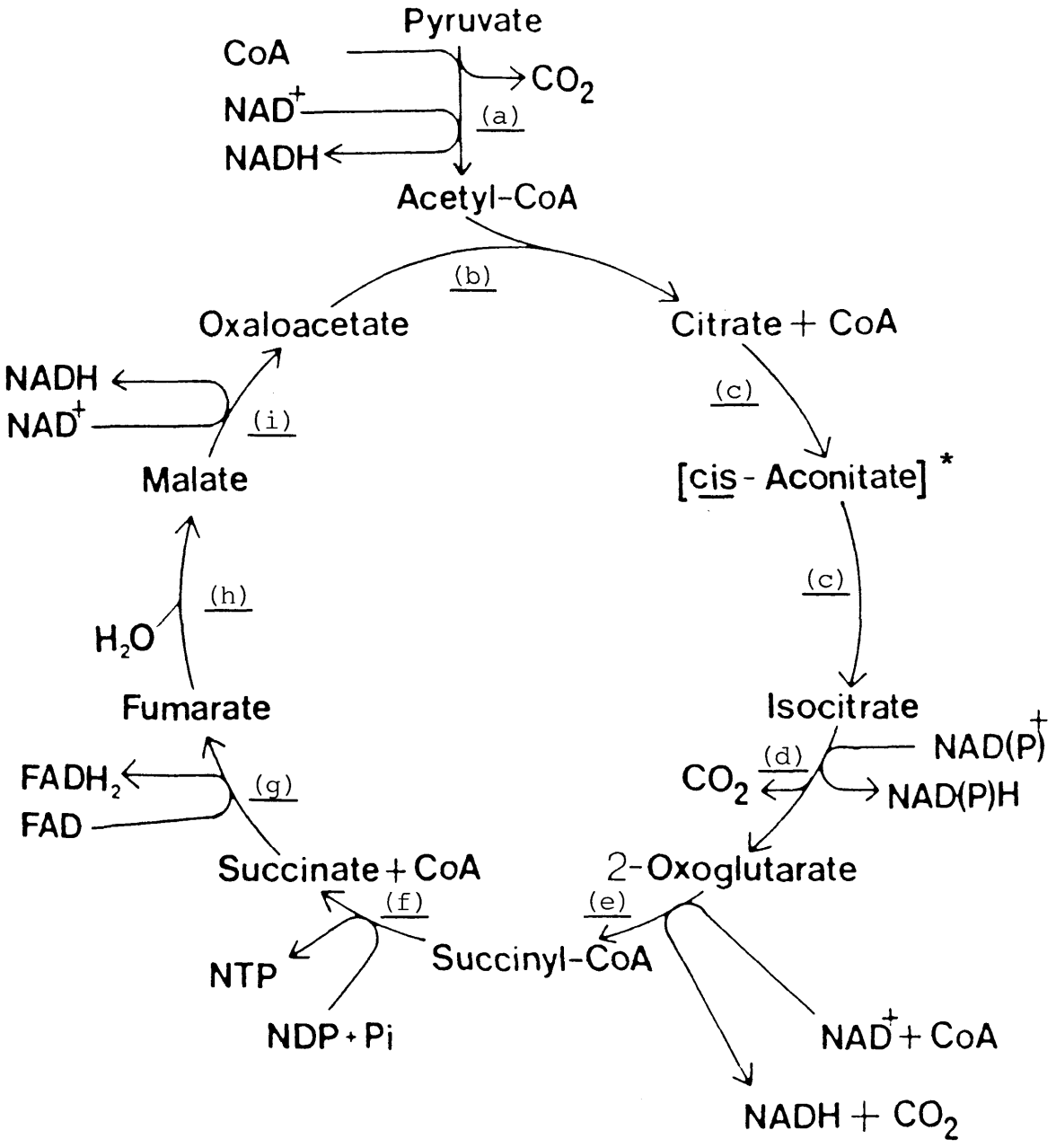
The enzymes responsible for catalysis of the reactions that comprise the CAC and the entrance of pyruvate into the latter are labelled (a) to (i). They are:

(a): Pyruvate Dehydrogenase (PDH)
(b): Citrate Synthase (CS)
(c): Aconitase
(d): Isocitrate Dehydrogenase (IDH)
(e): 2-Oxoglutarate Dehydrogenase (OGDH)
(f): Succinate Thiokinase (STK)
(g): Succinate Dehydrogenase
(h): Fumarase
(i): Malate Dehydrogenase.

Figure 1 is taken from Danson, M.J. (1989): Central Metabolism of the Archaebacteria: An Overview. Can. J. Microbiol. 35, 58-64.

Figure 1

The Citric Acid Cycle



[]* : Denotes an enzyme-bound intermediate

study of the bacterial CAC (Ajl, 1958; Kornberg, 1959). First the bacterial cell wall was impermeable to CAC intermediates and secondly, some of the CAC inhibitors that were employed by Krebs and Johnson (1937) were metabolisable by bacteria.

Thus, it was clear that other methodologies were needed to study the bacterial CAC. Two approaches that were to prove successful were:
(i) Isolation of Isotopically Labelled Cycle Intermediates: Swim and Krampitz (1954) incubated E. coli with [^{14}C]acetate and examined the distribution of label in the CAC intermediates. Citrate, 2-OG and fumarate were labelled to the same order of magnitude. Further, their carboxyl carbons were more heavily labelled than the evolved CO_2, indicating that CO_2 may arise from acetate oxidation via the CAC. Thus the in vivo operation of the CAC was suggested. These results were repeated by Saz and Krampitz (1954) with Micrococcus lysodeikticus.
(ii) Analysis of Glutamate Auxotrophs: Another approach was the use of mutant strains of organisms that were able to produce acetyl-CoA but not 2-OG or glutamate from acetate. Gilvarg and Davies (1956) created such mutants of E. coli and Aerobacter aerogenes. The organisms were CS-deficient and were auxotrophic for glutamate or 2-OG. The E. coli mutant oxidised acetate to CO_2 at less than 0.25% the rate of the wild type. These results showed the importance of citrate synthesis (and by inference the CAC) and also showed that, as in mammals, the bacterial CAC is the major terminal pathway of oxidation of any substance that can be catabolised to acetate.

1.3. The Role of the CAC in Bacterial Metabolism

The CAC is multifunctional. This, perhaps, explains why its component reactions are so widespread. This point is demonstrated by the fact that of the organisms so far examined, CS has been present in all but a few e.g. Streptococcus faecalis (Weitzman and Danson, 1976).

However, superimposed on this almost universal occurrence lie modifications, omissions and additions to the CAC that emphasise a role it is required to play. The role(s) required of the CAC and the way it carries these out are dictated partly by the the way the organism has adapted in order to exploit components of its environment

to grow. Thus it is likely that environmental changes are a driving force behind the diversity of form and function that is displayed by the CAC today. This theme has been pursued by Weitzman (1985).

1.3.1. Catabolism

Any species oxidised by the CAC must, prior to entry into it, be converted to acetyl-CoA or an intermediate of the CAC. It should be noted that the entry of any metabolite into the CAC as a cycle intermediate requires an additional supply of acetyl-CoA to keep the cycle turning. Degradation of fatty acids to acetyl-CoA allows their oxidation by the CAC (Lynen and Ochoa, 1953; Ochoa, 1954). Certain amino acids can be oxidised to acetyl-CoA, whilst others are oxidised to intermediates of the CAC prior to their entry into it (Figure 2a). Thus the CAC allows complete oxidation of major cellular components.

The oxidation of acetyl units yields reducing equivalents (NADH and $FADH_2$) and NTP by substrate level phosphorylation (Figure 1). NADH and $FADH_2$ are used to produce ATP *via* electron transport and oxidative phosphorylation. If the terminal electron acceptor is not O_2, the CAC dehydrogenases may not use NAD^+ but sometimes use alternative electron acceptors more suited to reduction of the terminal electron acceptor. Thus the sulphur and sulphate reducers *Desulphomonas acetoxidans* and *Desulphobacter postgatei* replace NAD^+ with ferredoxin and menaquinone (Thauer, 1988). However, generally, ATP and reducing equivalents such as NADH and $FADH_2$ can be envisaged to be end products of the CAC.

1.3.2. Anabolism

The CAC complements its catabolic function by the production of cycle intermediates for use as carbon skeletons in the formation of precursors of cell material, e.g. amino acid and porphyrins (Figure 2b). In bacteria, the first indications of the biosynthetic function of the CAC were obtained by Cutinelli *et al.* (1951), who showed that *E. coli* could synthesise amino acids during growth on acetate. Krebs *et al.* (1952) then proposed that the CAC made carbon skeletons for biosynthesis. This hypothesis was supported by Roberts *et al.* (1953,

Figure 2

Functions of the CAC

(a) Catabolism

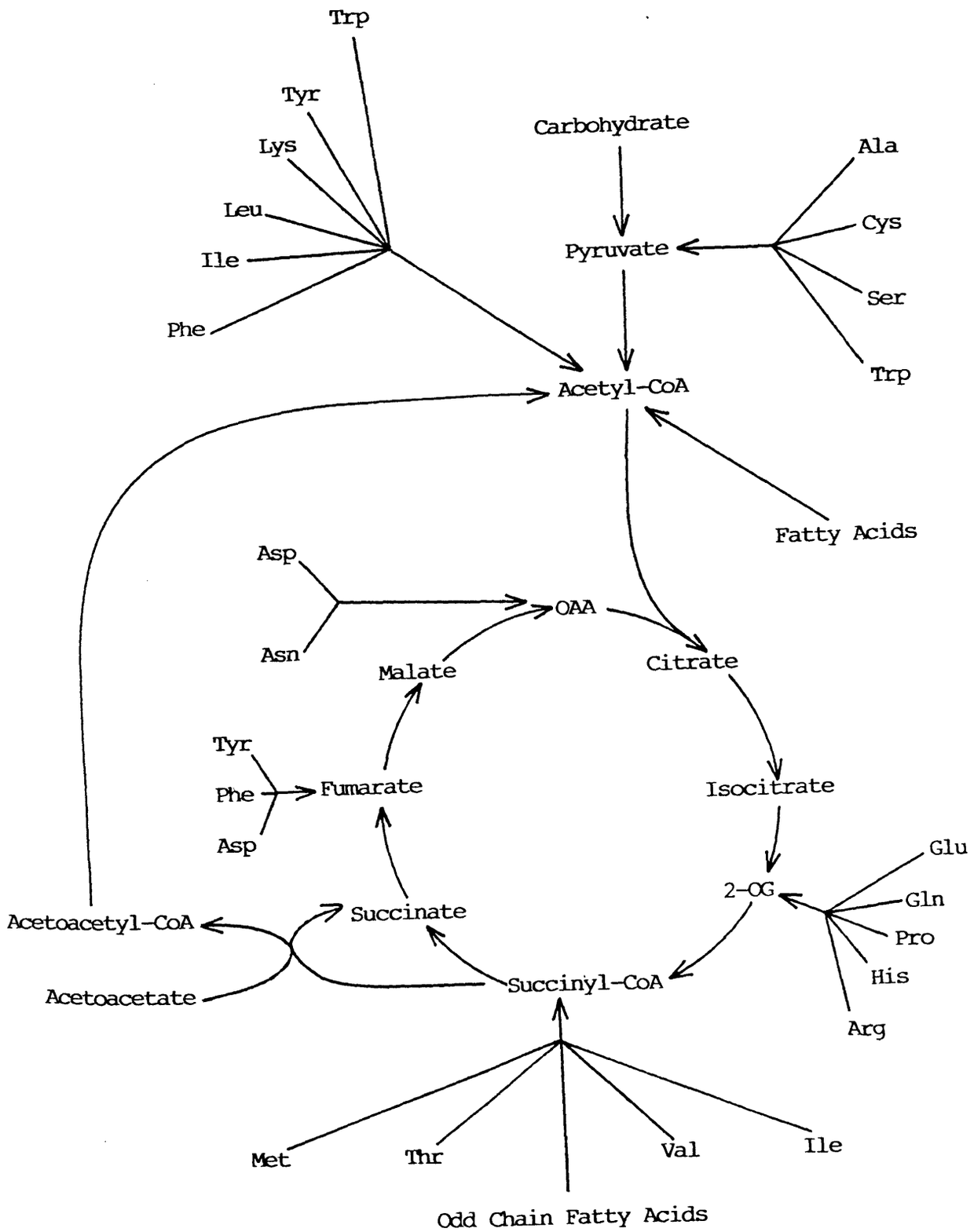
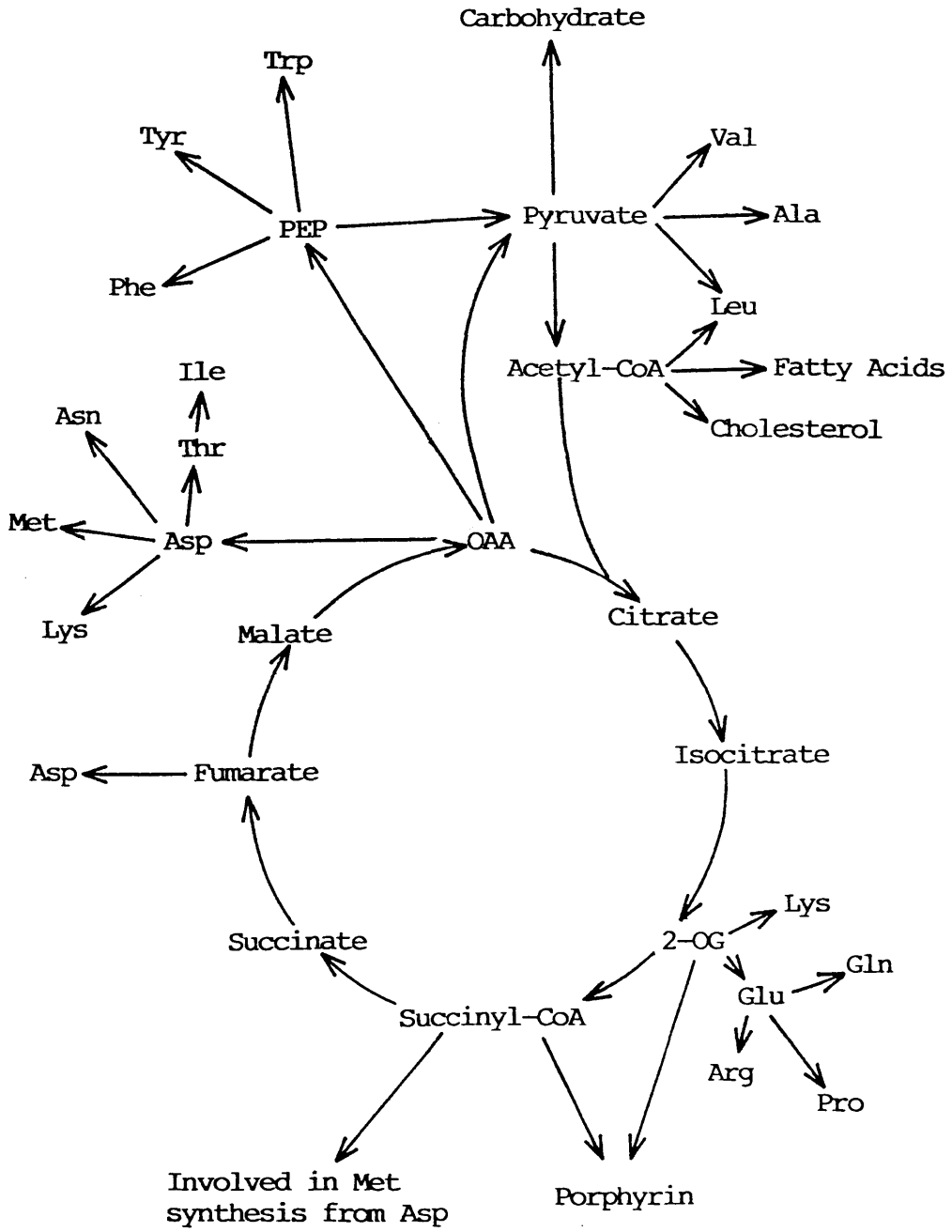


Figure 2

Functions of the CAC

(b) Anabolism



1955) whose results suggested that amino acids may be grouped into families, the 'head' of these being derived from the CAC (Figure 2b).

A demonstration of the necessity of the biosynthetic function of the CAC has come from the study of the auxotrophic requirements of organisms deficient in a CAC enzyme. Gilvarg and Davies (1956; **1.2.**) demonstrated the glutamate dependence of CS-deficient E. coli and Aerobacter aerogenes mutants showing that the operation of the CAC was required for the synthesis of C_5 skeletons for biosynthesis.

The porphyrin precursor - delta-aminolevulinic acid (DALA) is formed by two routes, both of which stem from the CAC. Synthesis of DALA from succinyl-CoA and glycine occurs in mammalian mitochondria, fungi and higher plants. There is also a pathway that is initiated by 2-OG that is novel in that its first step is the activation of glutamate by ligation to $tRNA^{Glu}$ (Kannangara et al., 1988).

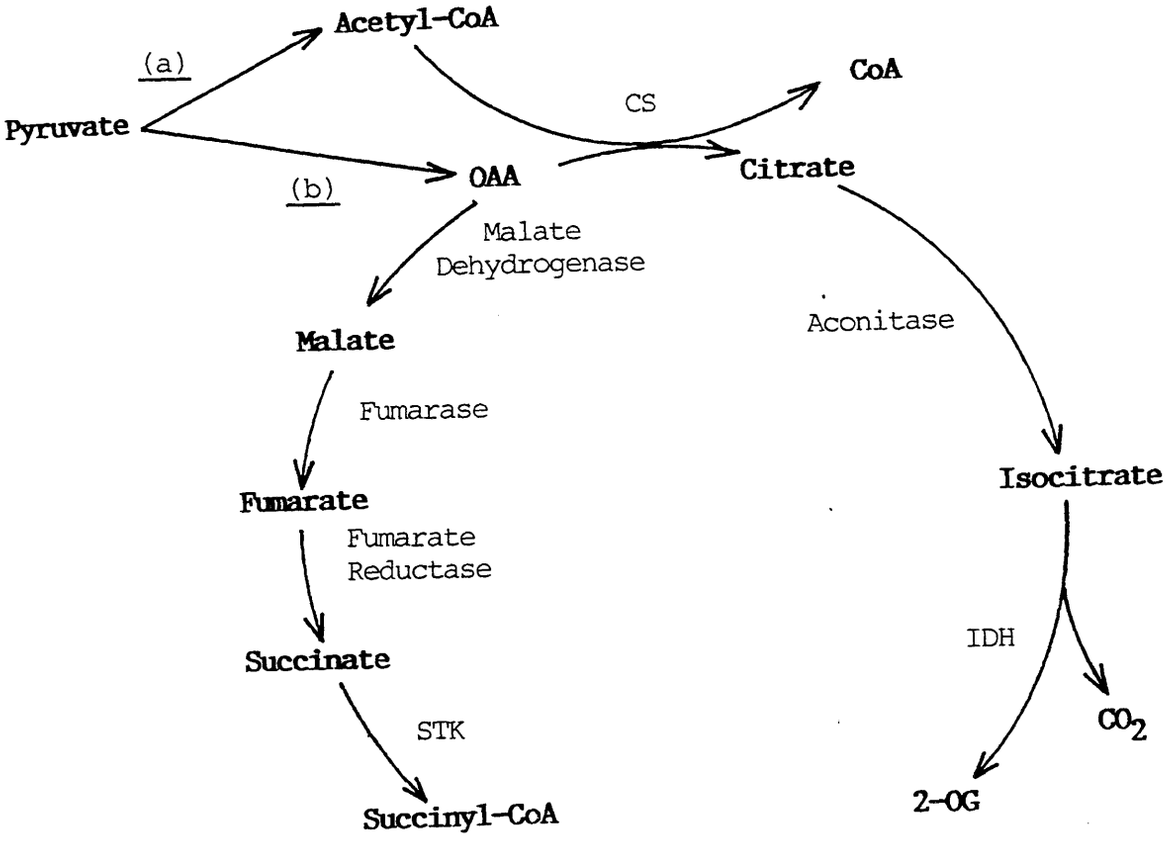
Under conditions where the CAC is not needed for generation of energy and its sole function is anabolic, the CAC is modified to prevent wastage of CO_2. Thus, anaerobically-grown E. coli suppresses synthesis of 2-OG dehydrogenase (OGDH) (preventing CO_2 loss) and succinate dehydrogenase and derepresses fumarate reductase (Amarasingham and Davies, 1965; Spencer and Guest, 1973). Now, the cycle may be visualised as a two-pronged fork (Figure 3); one prong is oxidative leading to the synthesis of 2-OG, while the other is reductive leading to the synthesis of succinyl-CoA. Fumarate reductase directs the carbon flux to succinyl-CoA, i.e. in a direction opposite to that the complete CAC functions in. This makes the synthesis of OAA from C_3 skeletons a necessity. A consequence of these modifications is that the cycle is now a biosynthetic sequence of reactions that produces precursors such as C_5 skeletons and succinyl-CoA but does not conserve energy. It may also be possible that the reductive prong of the CAC thus modified may re-oxidise NADH in E. coli to maintain the supply of NAD^+ to glycolysis (Gest, 1987; Miles and Guest, 1987).

1.3.3. Carbon Assimilation

The CAC can be involved directly in carbon assimilation, e.g. the reductive CAC of Chlorobium thiosulphatophilum is modified to operate

Figure 3

The Biosynthetically Functional OGDH Deficient CAC of Facultatively Anaerobic Organisms During Anerobic Growth



(a): The 'aerobic' route of conversion of pyruvate to acetyl-CoA is catalysed by PDH. Under anaerobic conditions, this enzyme is replaced by pyruvate formate lyase.

(b): Conversion of C_3 compounds to dicarboxylates can be facilitated by a number of enzymes - see Table 1.

in a direction reverse to that of the oxidative CAC and is used to fix CO_2 (Evans et al., 1966; Fuchs et al., 1980; Weitzman, 1985).

A more widespread adaptation to enable the CAC to fix carbon is the glyoxylate cycle. Most organisms with a complete CAC that grows on acetate needs the reactions of this cycle to assimilate carbon. In doing this there are a number of problems to be faced:

a) If the only energy source is acetate, there will be no opportunity to assimilate carbon as it will be oxidised to CO_2 by the CAC.

b) To generate energy in the first place with the CAC, the organism needs to be able to make OAA (C_4) from acetate (C_2).

Thus growth on acetate can only occur if the organism can by-pass the decarboxylation reactions of the CAC to the extent required by the demands of biosynthesis and maintain the level of CAC intermediates.

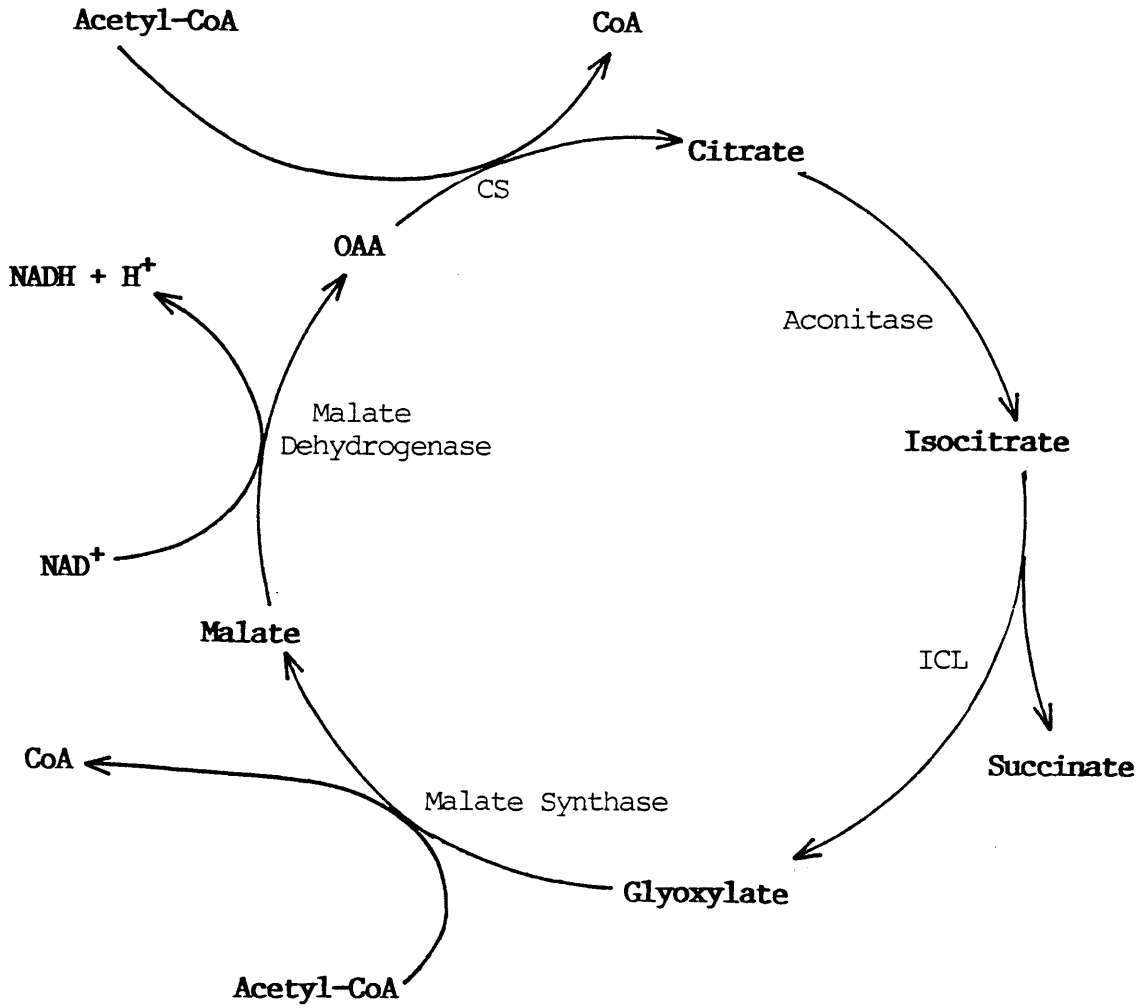
Kornberg (1958) showed that acetate-grown Pseudomonas fluorescens converted acetate to malate. How this occurred became clear with the discovery that acetate-grown Pseudomonas fluorescens expressed isocitrate lyase (ICL), which cleaved isocitrate to succinate and glyoxylate, and malate synthase which converted acetyl-CoA and glyoxylate to malate (Kornberg and Madsen, 1957). As the organism contained CS, aconitase and malate dehydrogenase, this allowed the function of a cycle. One turn of this cycle generated 1 molecule of succinate from 2 molecules of acetyl-CoA (Figure 4). This cycle, (the glyoxylate cycle) uses CS, aconitase and malate dehydrogenase from the CAC plus two ancillary reactions. It replaces the OAA required for the initial fixation of acetate into citrate (and is thus anaplerotic - see **1.3.4.**). Also it by-passes the decarboxylation reactions of the CAC and so allows net carbon assimilation during growth on acetate or substrates initially broken down to it. Possession of the glyoxylate cycle and the remaining reactions of the CAC allows an organism to arrange from acetate the synthesis of C_4 and C_5 biosynthetic precursors and produce energy to support such syntheses.

1.3.4. Anaplerosis

Anaplerosis is not a CAC function, but it is vital for the sustenance of the multifunctional operation of the CAC. The use of the

Figure 4

The Glyoxylate Cycle: Synthesis of C_4 Precursors (Succinate) from C_2 Precursors (Acetyl-CoA)



CAC to supply biosynthetic precursors, oxidise substrates that enter the CAC as intermediates or assimilate acetate causes depletion of intermediates vital to the function of the CAC. So, ancillary reactions to the CAC are needed to support the pool of CAC intermediates. These are the "anaplerotic" reactions (Kornberg, 1966).

Additional to its assimilatory role, the glyoxylate cycle is an anaplerotic sequence as it supplies OAA to the CAC. Transamination reactions that produce CAC intermediates are also anaplerotic.

Also important are enzymes that produce C_4 skeletons from C_3 compounds (Table 1). Studies in mammalian tissues have shown that pyruvate carboxylase is an important anaplerotic enzyme as illustrated by its sensitivity to activation by acetyl-CoA (Wood and Utter, 1965). Salmonella typhimurium and E. coli possess malic enzyme, PEP carboxykinase and PEP carboxylase, all of which could function anaplerotically to produce C_4 CAC intermediates (Table 1). Only the latter enzyme does so, the other enzymes operate anabolically to produce C_3 precursors from CAC intermediates (Kornberg, 1966).

1.4. An Introduction to the C_1-Utilisers

Compounds with no carbon-carbon (C-C) bonds (C1 compounds) occur in the environment at every level of oxidation from CO_2 to methane. Methane and methanol are probably the most biologically widespread reduced C_1 compounds of which methane is the most abundant, being produced by various eco-systems at rates of 5.5×10^{11} to 1.1×10^{12} $kg.year^{-1}$ (Ehhalt, 1976). Methanol is formed from free radical methane oxidation in the upper atmosphere or from degradation of lignin and pectin in plant material (Ehhalt, 1976; Large and Bamforth, 1988).

In view of the availability of reduced C_1 compounds and the diversity and adaptivity of micro-organisms, it is not surprising that bacteria and fungi using such compounds as their sole carbon and energy sources have been isolated. Perhaps the most impressive aspect of the C_1-utilisers is the fact that growth on such compounds means that the organism must make every C-C bond de novo. Thus growth on C_1 compounds requires that complex macro-molecules must be assembled from the most simple carbon sources. For example, Methylobacterium

Table 1

Enzymes Carboxylating C_3 Carbon Skeletons

Enzyme	Reaction	PEP Phosphate Acceptor
Pyruvate Carboxylase	Pyruvate + ATP + CO_2 $\longrightarrow$ OAA + ADP + Pi	-
PEP Carboxylase	PEP + CO_2 $\longrightarrow$ OAA + Pi	H_2O
PEP Carboxykinase	PEP + ADP/GDP + CO_2 $\longrightarrow$ OAA + ATP/GTP	ADP/GDP
PEP Carboxytransphosphorylase	PEP + Pi + CO_2 $\longrightarrow$ OAA + PPi	Pi
Malic Enzyme	Pyruvate + NAD(P)H + H^+ CO_2 $\longrightarrow$ Malate + $NAD(P)^+$	-

extorquens AM1 (Pseudomonas AM1) is capable of the conversion:

CH_3OH ——————→ Methanol Dehydrogenase
$M_r = 32$ M_r = 120-146,000 (Anthony, 1986; Nunn et al., 1989).

It is these synthetic abilities that have provided a stimulus to much of the research carried out on the C_1 utilisers in the past 30 years, not least because of the biotechnological opportunities they provide.

1.5. Nutritional Groups of C_1-Utilisers

The two groups of C_1-utilisers are the METHYLOTROPHS and the AUTOTROPHS. The definitions of these terms are from Anthony (1982):
Methylotrophy is the ability of an organism (a methylotroph) to grow at the expense of reduced C_1 compounds.
Autotrophy is the ability of an organism (an autotroph) to exploit CO_2 as the major carbon source.

1.5.1. Nutritional Subdivisions of the Methylotrophs

The methylotrophs are nutritionally divided into two groups:
The Obligate Methylotrophs: organisms growing exclusively on reduced C_1 compounds.
The Facultative Methylotrophs: organisms that, as well as reduced C_1 compounds, grow on multi C-C bond (heterotrophic) substrates.

Another classification based on nutritional characteristics is also used here: Colby and Zatman (1975a) have isolated "restricted" facultative organisms (RFMs) which use a small range (<6) of heterotrophic carbon sources. There are two types: "Type M" RFMs grow only on one or two heterotrophic carbon sources, while "Type L" RFMs grow on a larger range (up to 6) of heterotrophic substrates.

1.5.2. Methylotrophic Growth Substrates

The C_1 compounds used by methylotrophs encompass many functional

groups (Table 2; from Anthony, 1982; Large and Bamforth, 1988).

Methylotrophs that grow on methane are methanotrophs. The terms "facultative" and "obligate" classify methanotrophs that can or cannot grow on heterotrophic substrates. These terms do not reflect the ability or otherwise to exploit C_1 compounds other than methane.

1.6. General Characteristics of Methylotrophs

Methylotrophic growth is a characteristic displayed both by prokaryotes and eukaryotes. Eukaryotic methylotrophs (yeasts) are not further discussed, and the reader is referred to Anthony (1982) and Large and Bamforth (1988).

The following characteristics are generally observed:

(i) All obligate methylotrophs are Gram-negative. Most facultative methylotrophs and those also capable of autotrophy are also Gram-negative (Anthony, 1982).

(ii) Most methylotrophs are obligately aerobic. Exceptions to this are Paracoccus denitrificans and the Hyphomicrobia which also use NO_3^- as the terminal electron acceptor, and the methanogenic methylotrophs (Anthony, 1982). This latter group is not considered here - see Anthony (1982), Keltjens and van der Drift (1986), Wood et al. (1986), Noll et al. (1987) and Large and Bamforth (1988).

(iii) The methanotrophs are distinct from other methylotrophs. Thus, they have a methane mono-oxygenase, a complex membrane system and form spores (Davies and Whittenbury, 1970; Anthony, 1982). There are two types of membrane arrangement: TYPE I organisms possess bundles of disc shaped-vesicles formed from invagination of the cytoplasmic membrane; TYPE II organisms have paired membranes on the periphery of the cytoplasm. Associated with this division is the type of C_1 assimilation pathway used and the presence and absence of certain key enzymes. This is so for the CAC enzymes (Davey et al., 1972) and is considered in more detail later.

1.7. Methylotrophic Carbon Assimilation and Dissimilation

There are two sets of problems facing organisms that grow on

Table 2

Reduced C_1 Compounds Used as Carbon Sources by Methylotrophs

Compound	Formula
Methane	CH_4
Methanol	CH_3OH
Formaldehyde	HCHO
Formate	HCOOH
Carbon monoxide	CO
Foramide	$HCONH_2$
Dimethylforamide	$HCON(CH_3)_2$
Methylamine	CH_3NH_2
Dimethylamine	$(CH_3)_2NH$
Trimethylamine	$(CH_3)_3N$
Tetramethylammonium	$(CH_3)_4N^+$
Trimethylamine N-oxide	$(CH_3)_3NO$
Monochloromethane	CH_3Cl
Dichloromethane	CH_2Cl_2
Dimethylether	$(CH_3)_2O$
Dimethylsulphide	$(CH_3)_2S$
Dimethylsuphoxide	$(CH_3)_2SO$
Trimethylsulphonium	$(CH_3)_3S^+$
Methylsulphate	CH_3OSO_3H
Dimethylsulphate	$(CH_3O)_2SO_2$
Hydrogen Cyanide	HCN

From Anthony (1982) and Large and Bamforth (1988)

reduced C_1 compounds. First, methane or methanol oxidation generates formaldehyde which for many, if not most, methylotrophs stands at a branch point between carbon assimilation and carbon dissimilation for energy generation. The methylotroph is thus placed in a critical situation: not only does it have correctly to apportion formaldehyde to assimilatory and dissimilatory pathways commensurate with the cell's needs, but also the reactivity of formaldehyde with cellular components means the control of its intracellular concentration is crucial (Attwood and Quayle, 1984). How intracellular formaldehyde is handled is unknown. However, Attwood and Quayle (1984) have proposed that those enzymes making formaldehyde, i.e. methanol dehydrogenase, and those using it, i.e. hexulose monophosphate synthase, are on the periplasmic and cytoplasmic sides of the cell membrane respectively. This would allow production and consumption of formaldehyde before it reaches the cytoplasm. Recent results concerning the intracellular locations of methanol dehydrogenase and hexulose monophosphate synthase in Methylophilus methylotrophus support this proposal (Jones et al., 1982; Carver et al., 1984; Burton et al., 1986).

The second set of problems concerns triose synthesis and energy generation from C_1 compounds. After formation of metabolites such as PEP or pyruvate, the synthesis of the major cell components may be accomplished by established metabolic pathways. Catabolism of C_1 compounds requires a highly specialised sequence of enzymes. This section describes how triose synthesis and C_1 catabolism are achieved.

1.7.1. Carbon Assimilation

In the prokaryotic methylotrophs, there are three pathways of reduced C_1 compound assimilation. These are:

a) The Ribulose bisphosphate (RubP) cycle (Calvin cycle).

b) The Ribulose Monophosphate (RuMP) cycle.

c) The Serine Pathway.

The RubP cycle is responsible for C_1 assimilation after oxidation of the C_1 compound to CO_2. The RuMP cycle assimilates reduced C_1 compounds at the oxidation state of formaldehyde. The serine pathway assimilates C_1 carbon as a mixture of formaldehyde and CO_2.

1.7.1.1. The RubP Cycle. Methylotrophs using this cycle must oxidise the reduced C_1 compound to CO_2 before it is assimilated *via* this route. The RubP cycle causes the net reduction of 3 molecules of CO_2 to 1 molecule of triose phosphate which is then used for biosynthesis. In methylotrophic growth, the reducing equivalents for this process are provided by oxidation of the C_1 substrate to CO_2. The RubP cycle was first observed in methylotrophs by Quayle and Keech (1959*a*, *b*) in formate-grown *Pseudomonas oxalaticus*. The cycle has also been found in methylotrophs that use C_1 compounds more reduced than formate, e.g. *Paracoccus denitrificans*, (Cox and Quayle, 1975; Anthony, 1982). The RubP cycle is also used by most autotrophic and photosynthetic bacteria ([c.f. **1.3.3.**]; Kelly, 1971; Smith and Hoare, 1977; Tabita, 1988). The cycle is illustrated in Figure 5. It has three phases:

(i) *Fixation*: CO_2 is fixed by condensation with ribulose 1,5-*bis*phosphate to produce two molecules of 3-phosphoglycerate. This is catalysed by ribulose *bis*phosphate carboxylase/oxygenase.

(ii) *Reduction*: Reduction of 3-phosphoglycerate yields glyceraldehyde 3-phosphate. For every three molecules of CO_2 fixed, six molecules of 3-phosphoglycerate are reduced to glyceraldehyde 3-phosphate, one of which is assimilated.

(iii) *Rearrangement*: The remaining triose phosphate is used to regenerate three molecules of ribulose 5-phosphate. There are two probable modes of rearrangement; one involves transaldolase, the other involves sedoheptulose 1,7-*bis*phosphatase (illustrated) and both use transketolase. This phase ends with the phosphorylation of ribulose 5-phosphate by phosphoribulokinase to reform ribulose 1,5-*bis*phosphate.

Of the methylotrophic C_1 assimilation pathways, the RubP cycle is energetically the most costly means of making triose phosphate (Quayle and Ferenci, 1978; Anthony, 1982; Dijkhuizen, *et al.*, 1985). This is because synthesis of cell material with a redox potential similar to that of carbohydrate from CO_2 is endergonic. This prompts the question - why is the RubP cycle used in methylotrophy, when more economic pathways are available? Sahm *et al.* (1976) and Quayle and Ferenci (1978) suggest that the gain in nutritional versatility achieved by use of the RubP cycle justifies the disadvantage of using the most energetically costly C1 assimilation pathway.

1.7.1.2. The RuMP Cycle. The inability of Methylomonas methanica to assimilate $^{14}CO_2$ or express ribulose bisphosphate carboxylase/oxygenase suggested that the RubP cycle was not the only C_1 assimilation route (Johnson and Quayle, 1965). Further, Methylomonas incorporated $^{14}CH_4$, $^{14}CH_3OH$ or $H^{14}CHO$ initially into hexose phosphates thus eliminating operation of the RubP cycle. Kemp and Quayle (1965; 1966) showed that Methylomonas hydroxymethylated ribulose 5-phosphate to give the hexose D-erythro L-glycero 3-hexulose 6-phosphate (H6P; Kemp, 1972, 1974). Subsequently, it was shown by Strøm et al. (1974) that H6P was cleaved to two triose molecules which were rearranged to form ribulose 5-phosphate. This led Strøm et al. (1974) to propose a complete formulation of the RuMP cycle of formaldehyde fixation.

Like the RubP cycle, the RuMP cycle is divisible into three phases: fixation, cleavage and rearrangement. There is no reductive phase because formaldehyde (which is of a similar redox potential to carbohydrate) and not CO_2 is condensed in the fixation phase. Thus, this cycle is energetically more economic than the RubP cycle.

(i) Fixation: The first phase of the cycle is formaldehyde fixation. Three molecules of formaldehyde condense with ribulose 5-phosphate to give three H6P molecules. The reaction is catalysed by H6P synthase. H6P is then isomerised by H6P isomerase to form three molecules of fructose 6-phosphate (F6P).

(ii) Cleavage: One molecule of F6P is cleaved to two triose phosphates in one of two ways: (a) glycolytic-type cleavage of F6P to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate via phosphofructokinase and fructose 1,6 bisphosphate aldolase (Kemp and Quayle, 1966; Quayle, 1972); or (b) the Entner-Doudoroff-type cleavage where F6P is isomerised to glucose 6-phosphate (G6P) which is oxidised and dehydrated to 2-dehydro 3-deoxy 6-phosphogluconate (KDPG) by G6P dehydrogenase and 6-phosphogluconate dehydratase. KDPG is then cleaved to pyruvate and glyceraldehyde 3-phosphate by KDPG aldolase. Depending upon the type of cleavage, either dihydroxyacetone or pyruvate is assimilated into cell material.

(iii) Rearrangement: The modes of rearrangement of the remaining F6P and glyceraldehyde 3-phosphate to three molecules of ribulose 5-phosphate probably resemble the two modes used by the RubP cycle.

There are thus four possible variants of the RuMP cycle (listed as cleavage/rearrangement):

(1) KDPG aldolase/Transaldolase
(2) KDPG aldolase/Sedoheptulose 1,7-bisphosphatase
(3) Fructose 1,6-bisphosphate aldolase/Transaldolase
(4) Fructose 1,6-bisphosphate aldolase/Sedoheptulose 1,7-bisphosphatase

The two most common variants are (1) and (4) (Anthony, 1982; Stirling and Dalton, 1985; Figures 6a, b). Variant (1) is mainly found in obligate type I methanotrophs such as Methylomonas methanica and the Texas and Bath strains of Methylococcus capsulatus, as well as other obligate methylotrophs and RFMs, e.g. Methylomonas L3, M. methylotrophus, Organisms T15, 4B6, C2A1, W3A1 and W6A (Taylor, 1977; Hirt et al., 1978; Zatman, 1981; Beardsmore et al, 1982; Anthony, 1982; Stirling and Dalton, 1985; Bussineau et al., 1987). Variant (4) occurs mostly in facultative methylotrophs like Bacillus PM6, Organism S2A1 and Nocardia SP-239 (Zatman, 1981; Hazeau et al., 1983). The energetically least favoured variant (2) has not, unsurprisingly, been observed (Quayle and Ferenci, 1978; Anthony, 1982). The remaining variant (3) has only been found in the facultative organism Arthrobacter P1 (Levering et al., 1982).

The function of the RuMP and RubP cycles is not just limited to triose production. Recirculation of a triose phosphate in a second revolution of both cycles will, with the assimilation of two further C_1 units, result in net pentose phosphate synthesis (Quayle and Ferenci, 1978; Anthony, 1982). Net synthesis of hexose phosphates can also be achieved by the RuMP cycle by condensation of the newly formed pentose phosphate with a further C_1 unit. Additionally, C_4 sugars (erythrose phosphate) can be made by any variant of the RuMP or RubP cycles from four C_1 units (Quayle and Ferenci, 1978; Anthony, 1982). Thus, both cycles can synthesise aromatic amino acids, carbohydrate precursors and nucleic acids and the RuMP cycle can also synthesise polysaccharide precursors for the cell wall or for storage.

1.7.1.3. The Serine Pathway. An initial indication that there was a third C_1 assimilation pathway was the ability of cold CO_2 to reduce

Figure Legend to Figures 5, 6a and 6b

The enzymes that catalyse the reactions of the RubP and RuMP cycles have been labelled 1 to 19. They are:

1: RubP carboxylase/oxygenase
2: Phosphoglycerate kinase
3: Glyceraldehyde 3-phosphate dehydrogenase
4: Triose phosphate isomerase
5: Aldolase; 5a is fructose 1,6 bisphosphate aldolase and 5b is sedoheptulose 1,7 bisphosphate aldolase activity
6: Fructose 1,6 bisphosphatase
7: Sedoheptulose 1,7 bisphosphatase
8: Transketolase
9: Pentose phosphate epimerase
10: Phosphoribulokinase
11: Pentose phosphate isomerase
12: Transaldolase
13: H6P synthase
14: H6P isomerase
15: Phosphofructokinase
16: Glucose phosphate isomerase
17: G6P dehydrogenase
18: Phosphogluconate dehydratase
19: KDPG aldolase

Abbreviations not mentioned in the text are: GAP: glyceraldehyde 3-phosphate and DHAP: dihydroxyacetone phosphate.

Figures 5, 6a and 6b are taken from Anthony (1982).

Figure 5

The RubP Cycle of Carbon Fixation from CO_2 or Reduced C_1 Compounds Oxidised to CO_2 Prior to Carbon Fixation

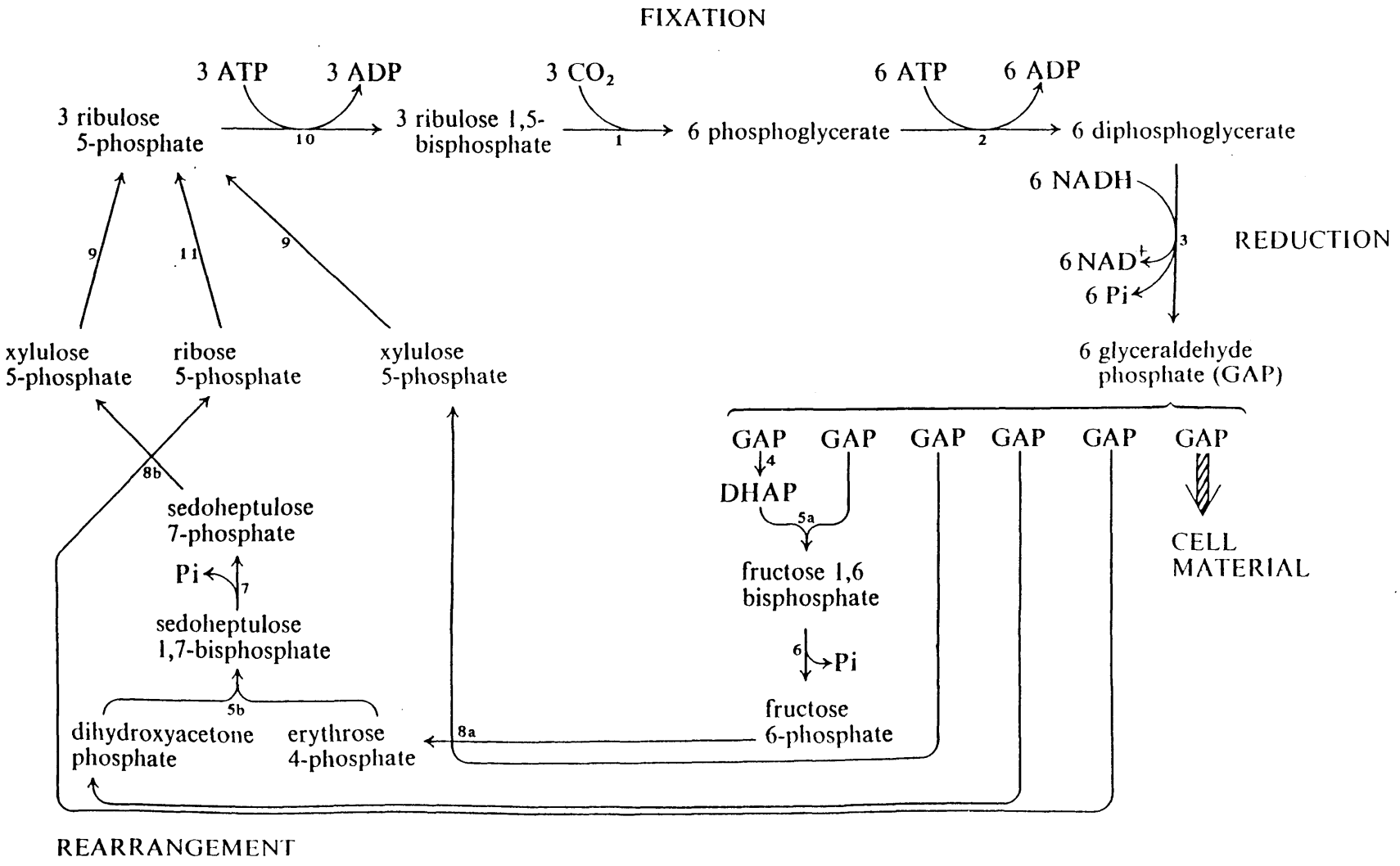


Figure 6

The RuMP Cycle of C_1 Assimilation

(a) The KDPG Aldolase/Transaldolase Varient

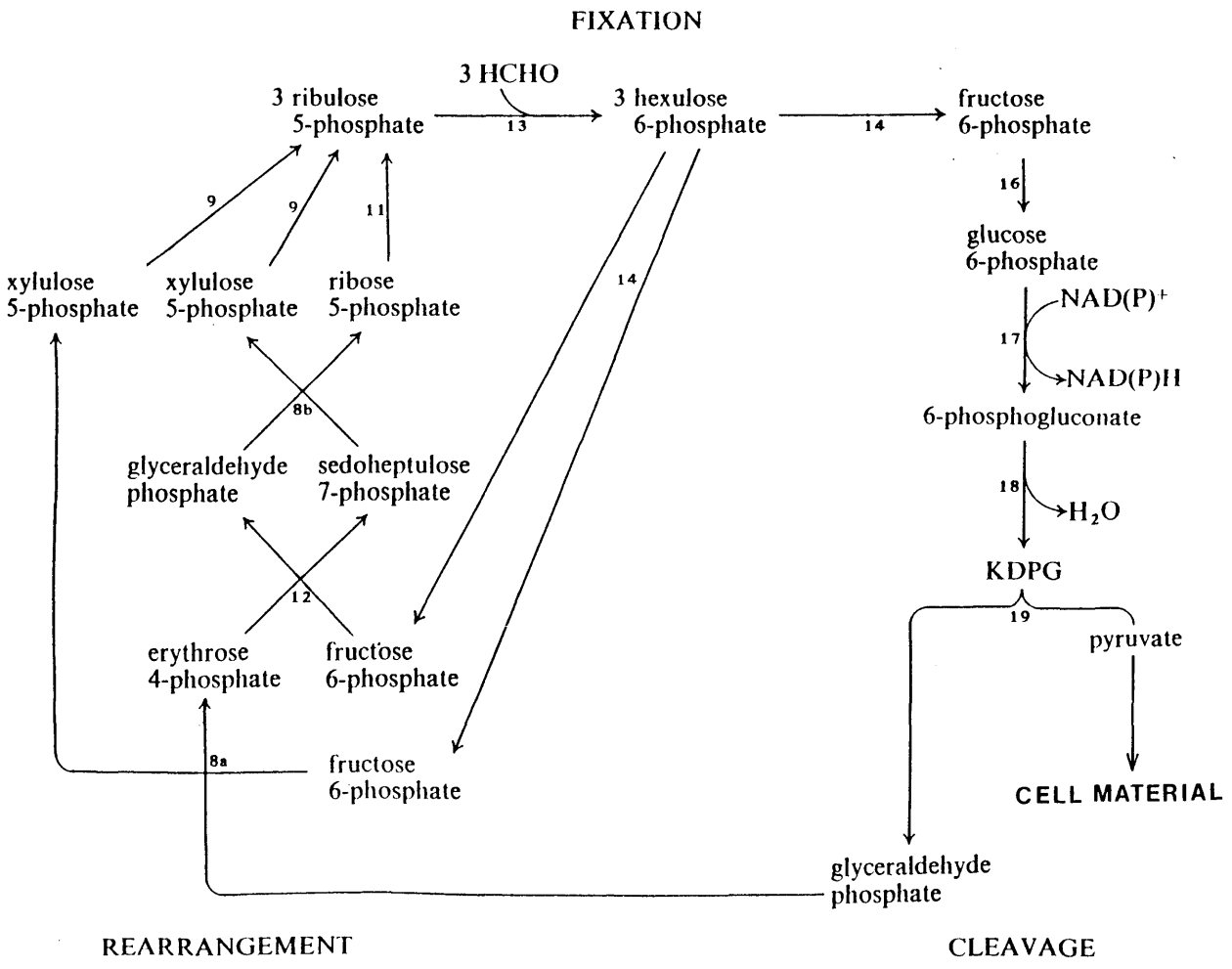
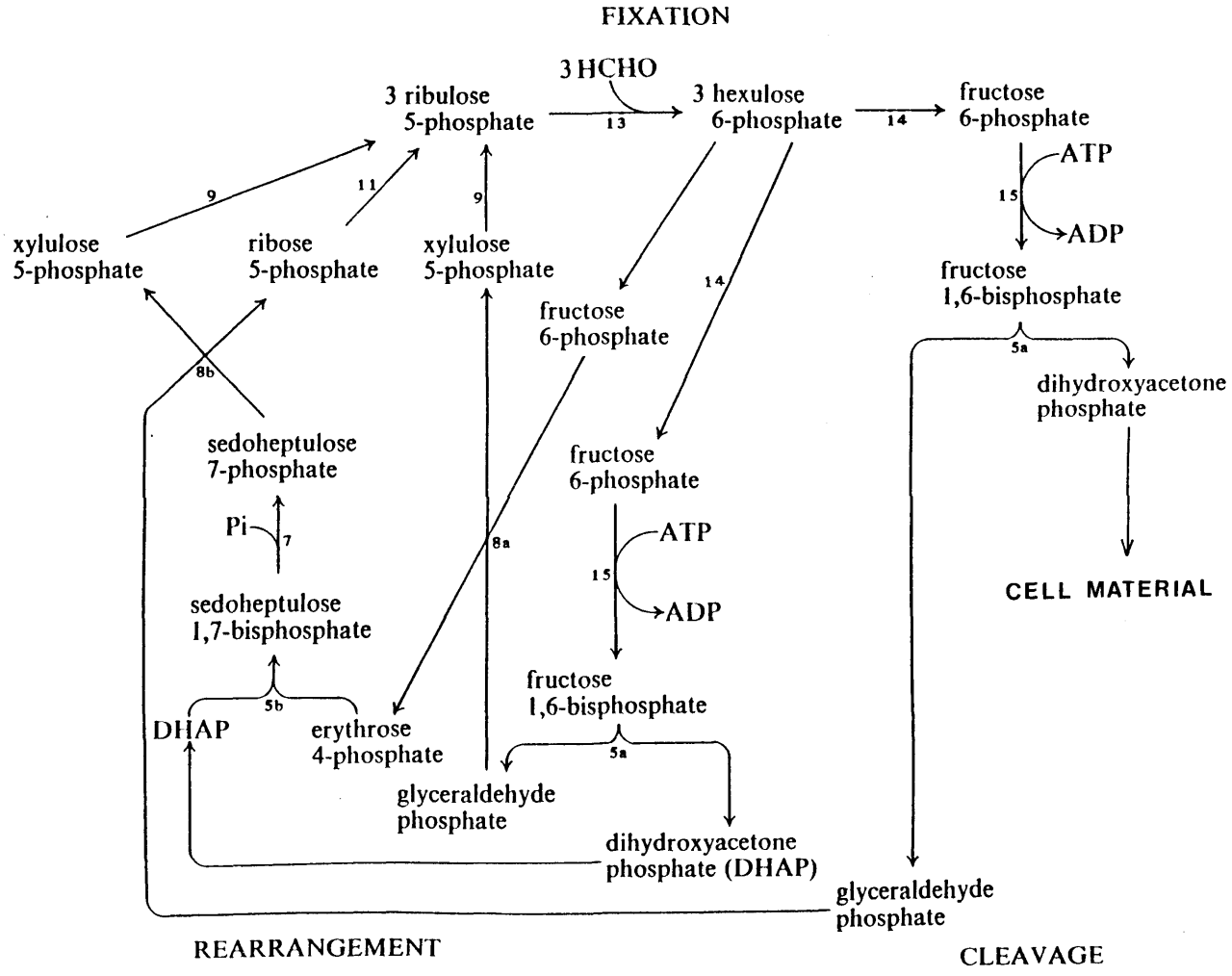


Figure 6

The RuMP Cycle of C_1 Assimilation

(b) The Fructose 1,6 bis Phosphate Aldolase/Sedoheptulose 1,7 bis Phosphatase Varient



the specific activity of cell material of Methylobacterium extorquens AM1 grown on $^{14}CH_3OH$ by about 50% (Large et al., 1961). This showed that roughly half the assimilated carbon arose from CO_2. This was not consistent with the operation of the RuMP or RubP cycles. Large et al. (1961) also found that ^{14}C was initially incorporated into serine, glycine, aspartate and malate instead of phosphorylated intermediates.

This work suggested that Methylobacterium extorquens AM1 used a novel C_1 assimilation pathway and prompted further work (see Anthony, 1982) which led to the proposal of a cycle (Figure 7): two molecules of glycine are hydroxymethylated by formaldehyde to produce two serine molecules. The reaction is catalysed by serine transhydroxymethylase. Both serines are reduced to 2-phosphoglycerate, one of which is assimilated into cell material. The other triose is carboxylated to OAA by PEP carboxylase and reduced to malate by malate dehydrogenase. The malate is then esterified and cleaved by malyl-CoA lyase to one molecule each of glyoxylate and acetyl-CoA. The latter is oxidised to another molecule of glyoxylate. Both glyoxylate molecules are aminated, reforming the pathway's C_1 acceptor-glycine.

There are two serine pathway variants that differ principally in how the acetyl-CoA is oxidised to glyoxylate:

(a) The icl^+ Variant: Bacteria using this pathway (Figure 7) generate two glyoxylate molecules from malate via malate thiokinase and malyl-CoA lyase. The glyoxylate cycle enzymes (CS, ICL, aconitase and those CAC enzymes that oxidise succinate to OAA) are used to oxidise the remaining acetyl-CoA to glyoxylate (Figure 4, 7; Colby et al., 1979; Hanson, 1980; Anthony, 1982; Large and Bamforth, 1988). The involvement of ICL was first shown by Bellion and Hersch (1972).

(b) The icl^- Variant: Probably most serine pathway utilisers have no ICL (Anthony, 1982; Stirling and Dalton, 1985). Even so, they still oxidise acetyl-CoA to glyoxylate. Further, how malate is cleaved to the two C_2 units that are then converted to glycine in these organisms is also unclear (Quayle, 1975). A recent proposal for acetyl-CoA oxidation has been made by Shimuzu et al. (1984), based on the observation that Protaminobacter ruber catalyses the cleavage of mesaconyl-CoA to propionyl-CoA and glyoxylate. The following sequence is proposed which also involves the CAC enzymes (except OGDH):

Figure Legend to Figure 7

The figure shows the net fixation of carbon from formaldehyde and CO_2 into triose phosphate. The dashed line represents the as yet unknown and unconfirmed sequence of reactions used by the icl^- serine pathway to oxidise acetyl-CoA to glyoxylate. The abbreviations in this figure that have not been used until now are:

OHPYR: Hydroxypyruvate
GA: Glycerate
2-PGA: 2-phosphoglycerate
3-PGA: 3-Phosphoglycerate

The enzymes involved in this pathway of C_1 assimilation are labelled a to o. These enzymes are:

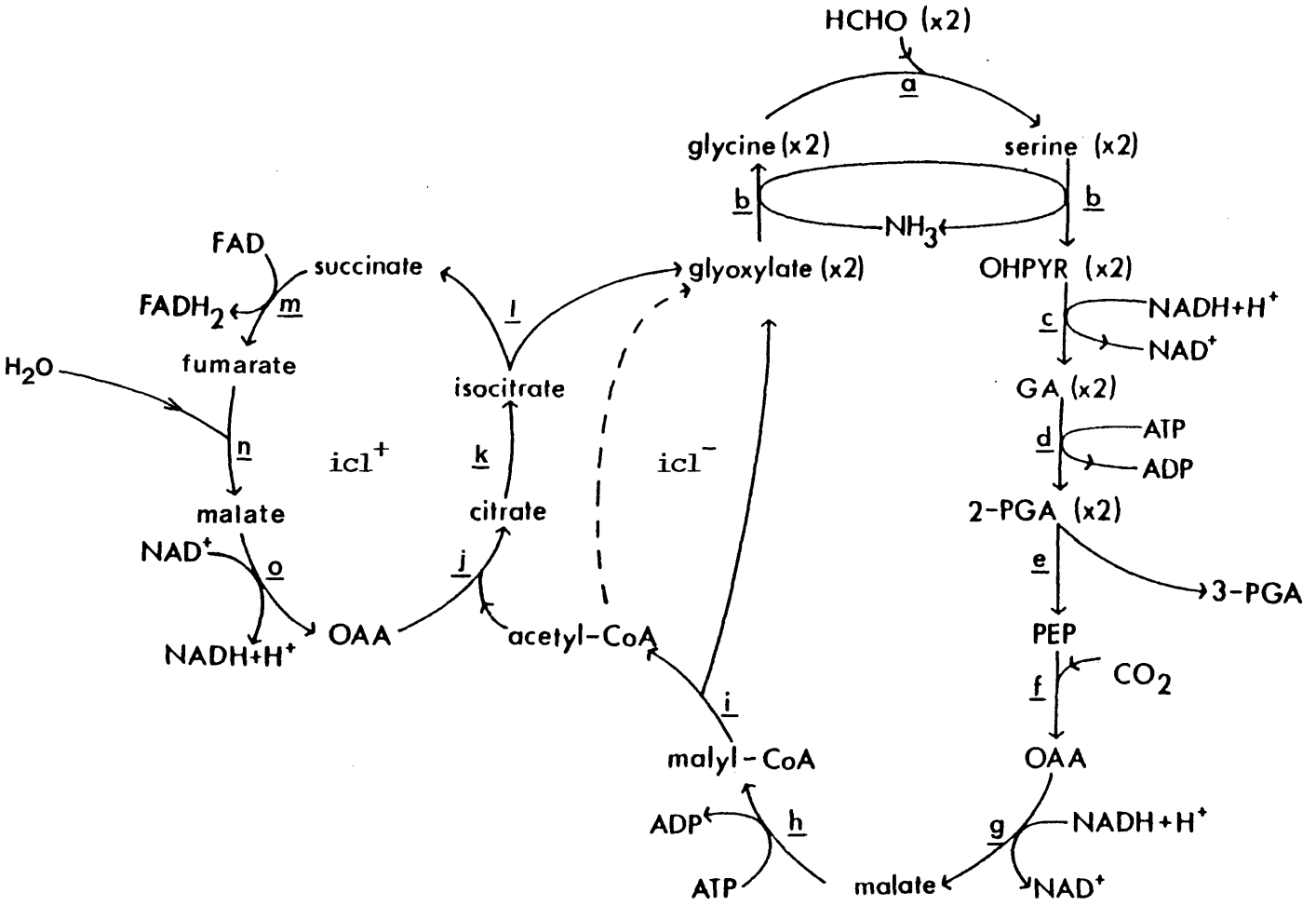
a: Serine Transhydroxymethylase
b: Serine Glyoxylate Amino-transferase
c: OHPYR Reductase
d: GA Kinase
e: Enolase
f: PEP Carboxylase
g: Malate Dehydrogenase
h: Malate Thiokinase
i: Malyl-CoA Lyase
j: CS
k: Aconitase
l: ICL
m: Succinate Dehydrogenase
n: Fumarase
o: Malate Dehydrogenase

2-PGA is isomerised to 3-PGA by phosphoglycerate mutase.

Figure 7 is taken from Colby et al. (1979).

Figure 7

The Serine Pathway of C_1 Assimilation



Acetyl-CoA + OAA ⟶ ⟶ ⟶ 2-OG + CO_2

2-OG ⟶ Glutamate ⟶ 3-Methylaspartate ⟶ Mesaconate

Mesaconate ⟶ Mesaconyl-CoA ⟶ **Glyoxylate** + Propionyl-CoA

Propionyl-CoA + CO_2 ⟶ Methylmalonyl-CoA ⟶ Succinyl-CoA

Succinyl-CoA ⟶ ⟶ ⟶ ⟶ OAA

SUM: **Acetyl-CoA ⟶ Glyoxylate**

Whether this mode of acetyl-CoA oxidation occurs more generally in other icl^- bacteria remains to be seen. However, use of CAC enzymes in the icl^- serine pathway is controversial as Cox and Zatman (1976) have found that C_1-growth of icl^+ organisms is more sensitive to inhibition by fluoroacetate (which forms fluorocitrate - a CAC inhibitor) than C_1-growth of icl^- organisms. This may show the unimportance of the CAC in the serine pathway of icl^- organisms.

Like other C_1 assimilation mechanisms, the serine pathway must provide precursors of all cell components. Anthony (1982) has pointed out that this pathway can synthesise OAA or succinate from two molecules each of formaldehyde and CO_2 and can generate acetyl-CoA. Serine pathway utilisers require the gluconeogenic enzymes to synthesise carbohydrate from assimilated triose.

The serine pathway occurs mostly in facultative methylotrophs. The only obligate organisms that use this pathway are the obligate type II methanotrophs i.e. Methylomonas sp. (Lawrence and Quayle, 1970). The icl^+ variant of the serine pathway occurs in Gram-negative bacteria whose sole C_1 substrates are methylated amines, e.g. Pseudomonas MA (Bellion and Hersch, 1972; Anthony, 1982). The icl^- variant, which is more common than the icl^+ variant, is found in facultative methylotrophs such as Methylobacterium extorquens AM1 and the methanotrophic Methylobacterium organophilum XX (O'Conner and Hanson, 1977; Anthony, 1982).

1.7.2. Carbon Dissimilation

1.7.2.1. Oxidation of Reduced C_1 Compounds to Formaldehyde. Apart from formate utilisers, most methylotrophs have to oxidise reduced C_1

compounds to formaldehyde for both energy conservation and carbon assimilation. The oxidation of methane to formaldehyde is outlined below. For additional information regarding oxidation of this and other C_1 substrates, see Anthony (1982) and Large and Bamforth (1988).

(i) Oxidation of Methane to Methanol: All methanotrophs hydroxylate methane to methanol by means of a methane mono-oxygenase which uses molecular oxygen and NADH:

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

Use of this reaction obliges the organism to generate at least two further reducing equivalents during C_1 oxidation in order to grow.

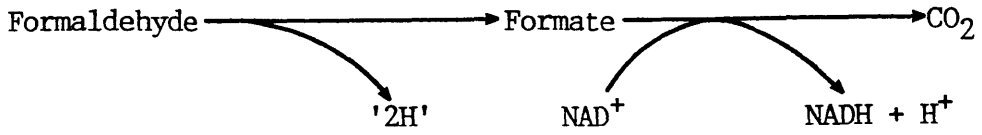
Methane mono-oxygenase exists in soluble and insoluble membrane-bound forms in Type I (e.g. Methylococcus capsulatus [Bath]) and type II (e.g. Methylosinus trichosporium OB3B) methanotrophs (Scott et al., 1981; Stanley et al., 1983). The soluble enzyme from both organisms consists of three proteins, two of which are responsible for substrate hydroxylation and NADH oxidation. The third component has a regulatory role, directing electrons from NADH to methane and O_2 on the substrate hydroxylation site, thus converting the enzyme from an oxidase to an oxygenase (Colby et al., 1979; Anthony, 1986; Fox et al., 1989).

(ii) Oxidation of Methanol to Formaldehyde: All Gram-negative methylotrophs oxidise methanol to formaldehyde using an NAD(P)-independent methanol dehydrogenase first described by Anthony and Zatman (1964). The enzyme has a bound prosthetic group: pyrrolo-quinoline quinone (PQQ; 2,7,9-tricarboxy 1H-pyrrolo [2,3-f] quinoline 4,5-dione; Duine and Frank, 1981; Anthony, 1982, 1986). PQQ is reduced to $PQQH_2$ during the oxidation of methanol to formaldehyde. It has been shown in vitro that the reduced $PQQH_2$ of methanol dehydrogenases from Acetobacter methanolicus or M. methylotrophus can be re-oxidised by transfer of electrons to the electron transport chain at the level of cytochrome c (Beardmore-Gray et al., 1983; Beardmore-Gray and Anthony, 1984; Elliott and Anthony, 1988). It has recently been shown that Gram-positive methylotrophs such as Bacillus C1 possess an NAD-linked methanol dehydrogenase (Arfman et al., 1989).

Methanol dehydrogenase also oxidises formaldehyde to formate, and

this must be controlled to prevent loss of assimilatable carbon. This is achieved in M. methylotrophus and Methylobacterium extorquens AM1 by a "modifier protein" which decreases the affinity of methanol dehydrogenase for formaldehyde over 60-fold and halves its V_{max} of formaldehyde oxidation (Page and Anthony, 1986). It is also probable that disruption of the interaction between the dehydrogenase and the modifier protein may be used to remove excess formaldehyde.

1.7.2.2. Oxidation of Formaldehyde. Two routes are known:
(i) via Formate: This is the linear sequence of oxidations:



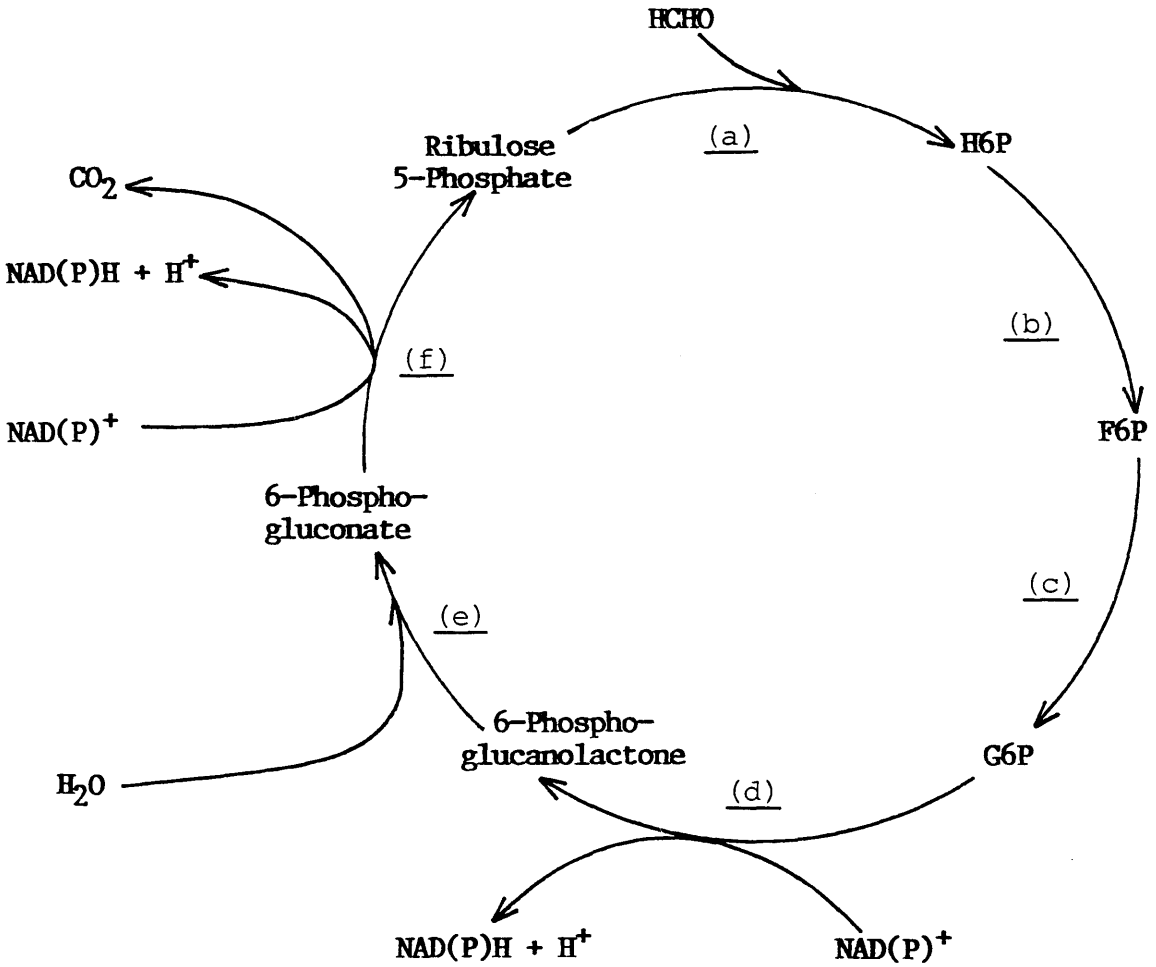
Formaldehyde dehydrogenase is linked either to NAD^+ or an alternative electron acceptor which must be replaced in vitro with an artificial electron acceptor (Zatman, 1981; Anthony, 1982). Zatman (1981) has expressed doubt that dye-linked formaldehyde dehydrogenase is active enough to support formaldehyde oxidation in vivo, although this opinion has been contested by Anthony (1982).

Formate oxidation is generally NAD-linked (Zatman, 1981; Anthony, 1982). It is often the only source of NADH from reduced C_1 oxidation (although see (ii) below). In methanotrophs it is crucial for supply of NADH for methane hydroxylation. Formate dehydrogenases are most common amongst the methanotrophs and the serine pathway-utilising organisms (Zatman, 1981); however an exception to this trend is M. methylotrophus-see below.
(ii) via Reactions of the RuMP Cycle: This route was proposed by Strøm et al. (1974). It is shown in Figure 8. This cyclic sequence uses some of the reactions of the KDPG aldolase/transaldolase variant of the RuMP cycle, 6-phosphogluconate dehydrogenase and probably, 6-phosphoglucanolactonase. The former enzyme has been found in RuMP-cycle bacteria such as Pseudomonas C and M. methylotrophus (Ben-Bassat and Goldberg, 1977; Large and Haywood, 1981; Beardsmore et al., 1982).

This cycle causes the oxidation of formaldehyde to CO_2 and two

Figure 8

Cyclic Oxidation of Formaldehyde to Carbon Dioxide via A Derivative of the RuMP Cycle



The reactions of this cycle are catalysed by:

(a): H6P Synthase
(b): H6P Isomerase
(c): Hexose Phosphate Isomerase
(d): G6P Dehydrogenase
(e): 6-Phosphogluconolactonase
(f): 6-Phosphogluconate Dehydrogenase

The summary equation of this cycle is:

$$HCHO + 2\ NAD(P)^+ \longrightarrow CO_2 + 2\ NAD(P)H + H^+$$

NAD(P)H. Zatman (1981) has noted that this cycle is absent from the methanotrophs and serine pathway-utilisers. This cycle probably removes dependence on formaldehyde and formate dehydrogenases, and indeed these enzymes are usually absent in those organisms believed to use this cycle. An exception to this is M. methylotrophus which has comparable levels of NAD-linked formaldehyde, formate and 6-phosphogluconate dehydrogenases (Beardsmore et al., 1982). Using ^{13}C-NMR it has been shown that the formaldehyde dehydrogenase may be active in vivo (Jones et al., 1987), although its function in routine substrate oxidation is uncertain. However, as the affinity of H6P synthase (the enzyme allowing entry of formaldehyde into the dissimilatory cycle) for formaldehyde is 4 times that of formaldehyde dehydrogenase, it may be that the linear route of formaldehyde oxidation operates to prevent toxic accumulations of formaldehyde (Aperghis, 1981; Beardsmore et al., 1982).

1.8. The State and Operation of the CAC in Methylotrophs

The CAC in methylotrophs has been studied by both analysis of the fate of labelled acetate and the assay of CAC enzymes. The results of such studies are summarised here:

1.8.1. Metabolism of ^{14}C-Acetate

The function of a complete CAC is indicated by the following redistribution of ^{14}C when an organism is exposed to labelled acetate:
(1) The label should be oxidised to $^{14}CO_2$.
(2) The label should appear in all cellular components and amino acids derived from the intermediates of the cycle.
(3) ^{14}C derived from [1-^{14}C]acetate should be found in glutamate, the degradation of which should show the C-1 and C-5 positions are labelled. The activity of C-5 should be twice that of C-1.

The observations that were made were not all in accord with the above, but could be divided into two groups:
(i) Obligate Methylotrophs and Type M RFMs (No Serine Pathway): Acetate oxidation was not observed in Methylococcus capsulatus

[Texas], Pseudomonas C, Methylococcus mobilis [nov] M. methylotrophus, organisms 4B6, C2A1, and the type M RFM organism W3A1 (Patel et al., 1969, 1975; Colby and Zatman, 1975b; Ben-Bassat and Goldberg, 1977; Taylor, 1977; Hazeau, 1980).

The distribution of ^{14}C amongst cellular components of Methylococcus capsulatus [Texas], 4B6, C2A1, W1 and W3A1 was more limited than expected for the operation of the complete CAC. Label was only found in lipids and proteins. In the latter, only leucine, glutamate, arginine and proline were labelled (Patel et al., 1969; 1975; Dahl et al., 1972; Eccleston and Kelly, 1973; Colby and Zatman, 1975b). This showed that acetate could only be used to synthesise amino acids derived from acetyl-CoA and 2-OG.

Glutamate carboxyls from Methylococcus capsulatus [Texas] grown in the presence of [1-^{14}C]acetate were labelled only at C-5.

(ii) Facultative and Obligate Serine Pathway Utilising Methylotrophs: The results obtained with these organisms contrast with those in (i):

The obligately methylotrophic serine pathway utiliser Methanomonas methano-oxidans (obligate type II methanotroph) and facultative organisms Methylobacterium extorquens AM1, Pseudomonas 1 and Methylobacterium R6 could oxidise acetate or substrates catabolised via acetyl-CoA (Wadzinski and Ribbons, 1975; Ben-Bassat and Goldberg, 1977; Patel et al., 1978; Dunstan and Anthony, 1973; Taylor and Anthony; 1976a, b).

Labelling of Methanomonas methano-oxidans, Pseudomonas 3A2, organism 5B1, Methylobacterium extorquens AM1, Arthrobacter 2B2, "PFI" and various Hyphomicrobia with ^{14}C-acetate showed that the label was more widely distributed in these organisms, being found in protein, lipid, nucleic acid and carbohydrate fractions. Analysis of the protein and soluble fractions of these organisms showed that ^{14}C was incorporated into all amino acids expected from the operation of the complete CAC (Dahl et al., 1972; Dunstan et al., 1972; Wadzinski and Ribbons, 1975; Colby and Zatman, 1975b; Taylor and Anthony, 1976b; Harder and Attwood, 1978).

On examination of the labelling pattern in glutamate from Methanomonas methano-oxidans grown with [1-^{14}C]acetate, it was evident that one third of the isotope was incorporated into C_1 and two thirds

were incorporated into C-5 (Wadzinski and Ribbons, 1975). Such a labelling pattern is indicative of a complete CAC.

1.8.1.1. Conclusions from the Labelling Experiments. The foregoing suggested that the RuMP cycle utilising obligate methylotrophs had an incomplete CAC. The lesion was at OGDH which would account for the limited distribution of the label amongst cellular components. This conclusion was also consistent with the labelling pattern of the carboxyl carbons of glutamate which indicated that 2-OG could not be converted to succinate. If it was, the label would have been subsequently incorporated into both OAA carboxyls and this would have led to labelling of glutamate at C-1 and C-5.

In contrast, the data from all facultative organisms and obligate serine pathway utilisers fulfilled criteria (1)-(3) in **1.8.1.** and indicated that the CAC in these organisms was complete.

An apparent exception to these conclusions was the type L RFM Bacillus PM6 which redistributed label from [1-^{14}C]acetate as would be expected if the CAC was complete. However, no acetate oxidation was found. Bacillus PM6 has a low level of OGDH (Colby and Zatman, 1975a). Thus, possibly, the CAC activity in this RFM was not great enough to be detected by O_2 consumption (Colby and Zatman, 1975b).

1.8.2. The Distribution of CAC Enzymes in the Methylotrophs

1.8.2.1. Interconversion of OAA to Succinyl-CoA. All types of methylotroph, viz the obligate organisms 4B6 and C2A1, facultative organisms such as Methylobacterium extorquens AM1 and Arthrobacter 2B2 and type I and type II methanotrophs such as Methylococcus capsulatus [Bath] and Methylobacterium R6, contain all three enzymes (CS, aconitase and isocitrate dehydrogenase [IDH]) required to synthesise 2-OG from acetyl-CoA and OAA (Davey et al., 1972; Patel et al., 1978; Bolbot and Anthony, 1980; Paddon et al., 1985). For a more complete list of occurrence of these enzymes see Anthony (1982). The universal observation of these enzymes is consistent with, in the methylotrophs, the universal incorporation of acetate carbon into glutamate (**1.7.1.**).

Conversion of 2-OG to succinyl-CoA requires OGDH. The

distribution of this enzyme is generally that predicted from the acetate incorporation data (**1.7.1.**). Thus the enzyme is found in facultative methylotrophs, type L RFMs and serine pathway utilisers (obligate and facultative) and is absent from all obligate RuMP cycle-utilising methylotrophs and type M RFMs (Table 3). Interestingly, with the exception of Pseudomonas oleovorans, OGDH is also present in facultative methylotrophs during methylotrophic growth (Table 3).

1.8.2.2. Interconversion of Succinyl-CoA and OAA. In the complete CAC, this process regenerates OAA. How those organisms without OGDH synthesise OAA is discussed in **1.9.2.**

OAA is typically made from succinyl-CoA by succinate thiokinase (STK), succinate dehydrogenase, fumarase and malate dehydrogenase. All four enzymes are present in facultative organisms and type L RFMs like Bacillus PM6, Methylobacterium extorquens AM1, Pseudomonas 3A2, organisms S2A1 and 5B1 during methylotrophic and heterotrophic growth (Dunstan et al., 1972; Dunstan and Anthony, 1973; Colby and Zatman, 1972, 1975a; Taylor and Anthony, 1976a, b). Similar but incomplete surveys of CAC enzymes in this part of the cycle of other facultative organisms confirm this view (Dahl et al., 1972; Bellion and Hersch, 1972; Attwood and Harder, 1974; Patt et al., 1974; Patel et al., 1978; Loginova and Trotsenko, 1979a; Paddon et al., 1985). In obligate methylotrophs with OGDH, i.e. type II methanotrophs, activities of succinate and malate dehydrogenases and fumarase compare well with those of facultative methylotrophs (Davey et al., 1972; Trotsenko, 1976; cf. data from references quoted previously in this paragraph). It seems likely, then, that these organisms have a complete CAC.

STK, succinate and malate dehydrogenases and fumarase have been found to be absent or present in either negligible or far lower amounts in the OGDH-deficient obligate methylotrophs and type M RFMs than in the organisms discussed in the previous paragraph (Dahl, 1972; Davey et al., 1972; Colby and Zatman, 1972, 1975a; Trotsenko, 1976).

1.8.3. General Conclusions about the State of the CAC in Methylotrophs

Generally, both radiochemical and enzymological data show that:

Table 3

The Occurance of 2-OGDH Amongst the Methylotrophs

Organism	Type*	Presence of OGDH	References
Methylomonas methanica	1	No	Davey et al. (1972)
Methylomonas albus	1	No	Davey et al. (1972)
Methylococcus minimus	1	No	Davey et al. (1972)
Methylococcus capsulatus [Bath]	1	No	Davey et al. (1972)
Methylococcus mobilis [Nov]	1	No	Hazeau et al. (1980)
Organism W1	1	No	Dahl et al., (1972)
Organisms C2A1, W3A1 and W6A	1	No	Colby and Zatman (1975a)
Organism 4B6	1	No	Colby and Zatman (1972)
Methylobacter vinelandii	1	No	Trotsenko (1976)
Methylobacter Bovis	1	No	Trotsenko (1976)
Methylobacter chroococum	1	No	Trotsenko (1976)
M. methylotrophus	1	No	Large and Haywood (1981)
Organism W6	1	No	Babel and Hofmann (1975)
Pseudomonas C	1	No	Ben-Bassat and Goldberg (1977)
Methylobacterium extorquens AM1	2^+	Yes	Taylor and Anthony (1976a)
Methylobacterium organophilum XX	2^+	Yes	Patt et al. (1974)
Organism S2A1 and Bacillus PM6	2	Yes	Colby and Zatman (1975a)
Organism 5B1 and Pseudomonas 3A2	2^+	Yes	Colby and Zatman (1972)
Methylosinus trichosporium OB3B	2^+	Yes	Davey et al. (1972)
Methylosinus trichosporium PG	2^+	Yes	Davey et al. (1972)
Methylosinus sporium	2^+	Yes	Davey et al. (1972)
Methylosinus parvus	2^+	Yes	Davey et al. (1972)

Table 3 Continued

Organism	Type*	Presence of OGDH	References
PFI	2+	Yes	Dahl et al. (1972)
Methylomonas methano-oxidans	2+	Yes	Wadzinski and Ribbons (1975)
Hyphomicrobium X	2+	Yes	Attwood and Harder (1974)
Pseudomonas MA	2+	Yes	Bellion and Hersch (1972)
Achromobacter IL	2	Yes	Loginova and Trotsenko (1979a)
Pseudomonas 8	2	Yes	Loginova and Trotsenko (1979a)
Mycobacterium 50	2	Yes	Loginova and Trotsenko (1979a)
Arthrobacter 2B2	2	(Yes)*	Paddon et al. (1985)
Methylobacterium R6	2+	Yes	Patel et al. (1978)
Pseusomonas oleovorans	2	(Yes)*	Loginova and Trotsenko (1977)
Pseudomonas 1	2+	Yes	Ben-Bassat and Goldberg (1977)

*: **Type 1** are obligate methylotrophs or type M RFMs that do not use the serine pathway of C_1 assimilation. **Type 2** are serine pathway pathway utilising (marked +) and/or facultative methylotrophs with the exception of Bacillus PM6 and organism S2A1 which are type L RFMs.

: Pseudomonas oleovorans only has an OGDH during heterotrophic growth.

: Only component activities (2-OG decarboxylase and lipoamide dehydrogenase) of Arthrobacter 2B2 OGDH demonstrated.

In addition to the references quoted in this Table see Anthony (1982).

(1) The CAC of all RuMP cycle utilising obligate methylotrophs (including the type I methanotrophs and type M RFMs) is incomplete.
(2) If incomplete, the CAC always lacks OGDH and often also one or more of the enzymes that convert succinyl-CoA to OAA.
(3) All serine pathway utilisers including the obligately methylotrophic type II methanotrophs have a complete CAC.
(4) All facultative methylotrophs including the type L RFMs can express a complete CAC irrespective of the C_1 assimilation pathway.

1.9. The Supply of Carbon to the CAC in Methylotrophs

1.9.1. The Supply of Acetyl-CoA

During methylotrophic growth, serine pathway utilisers may exploit the malyl-CoA lyase reaction:

$$\text{Malyl-CoA} \longrightarrow \text{Glyoxylate} + \text{Acetyl-CoA}$$

as a source of acetyl-CoA. This mechanism of acetyl-CoA synthesis is open to organisms that use this pathway, i.e. type II methanotrophs and the facultative methylotrophs (Anthony, 1982). Additionally, many serine pathway utilisers have PDH, e.g. the facultative methylotroph Methylobacterium extorquens AM1 and the type II methanotrophs (Davey et al., 1972; Bolbot and Anthony, 1980); however, the presence of PDH in the latter organisms is disputed by Trotsenko (1976).

The relative contributions malyl-CoA lyase and PDH make to the acetyl-CoA supply in C_1-growth in organisms with both enzymes are not known. However, the ratio of malyl-CoA lyase: PDH in Methylobacterium extorquens AM1 is about 180:1 (Cox and Quayle; 1976; Bolbot and Anthony, 1980). Thus the major acetyl-CoA supplier may be the lyase. That PDH^- mutants of Methylobacterium can grow methylotrophically (Bolbot and Anthony, 1980) supports this conclusion.

A similar situation occurs naturally in the Hyphomicrobia. These organisms are facultative methylotrophs but are also PDH deficient and during methylotrophic growth probably use malyl-CoA lyase to supply acetyl-CoA to the CAC (Attwood and Harder, 1978). A result of this PDH

lesion is limited use of heterotrophic carbon sources; Hyphomicrobia only grow on carbon sources that do not require pyruvate oxidation to acetyl-CoA. It has recently been shown that the range of carbon sources that can be used can be increased if the PDH deficiency is reversed by genetic engineering. The resulting organism, unlike the wild-type exploited succinate and pyruvate (Dijkhuizen et al. (1984).

In RuMP and RubP cycle utilisers, the acetyl-CoA supply may be mediated by PDH during C_1-growth. This enzyme is found amongst those methylotrophs that use these cycles irrespective of nutritional status. PDH has been found in the RuMP cycle utilising type I obligate methanotrophs, obligate methylotrophs like organisms W1 and W6 and facultative methylotrophs, e.g. Arthrobacter globiformis B-175 and Achromobacter 1L (Davey et al., 1972; Dahl et al., 1972; Babel and Hofmann, 1975; Trotsenko, 1976; Loginova and Trotsenko, 1976, 1979a). An anomaly to this is the facultative organism Arthrobacter 2B2. This RuMP cycle-utiliser lacks overall PDH and pyruvate decarboxylase activity under methylotrophic or heterotrophic growth conditions (Paddon et al., 1985). This result is, however, inconsistent with the ability of this organism to grow on succinate, citrate, glutamate, pyruvate and glucose (Colby and Zatman, 1973).

1.9.2. Supply of OAA to Methylotrophs with an Incomplete CAC

In organisms with an incomplete CAC, there must be another source of OAA as the CAC cannot generate its own. Likely candidates are the glyoxylate cycle or the carboxylation of a C_3 skeleton.

Although Colby and Zatman (1972, 1975a) may have observed ICL in the OGDH-deficient organisms 4B6, C2A1, W6A and W3A1, it was present at very low activity and Trotsenko (1976) was unable to detect ICL in the OGDH-deficient type I methanotrophs. These results together with the inability of methylotrophs with an incomplete CAC to incorporate ^{14}C-acetate into aspartate, seem to rule out operation of a glyoxylate cycle (Patel et al., 1969, 1975; Dahl et al., 1972; Eccleston and Kelly, 1973; Colby and Zatman, 1975b).

What then of C_3 carboxylation? Those enzymes catalysing this type of reaction are listed in Table 1. All except malic enzyme can produce

OAA directly, while the latter enzyme (1) requires the participation of malate dehydrogenase (2) to produce OAA:

(1) $\text{Pyruvate} + CO_2 + \text{NAD(P)H} \longrightarrow \text{Malate} + \text{NAD(P)}^+$

(2) $\text{Malate} + \text{NAD(P)}^+ \longrightarrow \text{OAA} + \text{NAD(P)H} + H^+$

(1)+(2) $\text{Pyruvate} + CO_2 \longrightarrow \text{OAA} + H^+$

In the obligate methylotroph M. methylotrophus, Aperghis (1981) has shown that only pyruvate carboxylase is present at levels that would enable it to supply OAA to the CAC. PEP carboxykinase is also present but in smaller amounts. Aperghis (1981) has suggested that this latter enzyme may have an anaplerotic function for the RuMP cycle. Pyruvate carboxylase has also been found in other obligate RuMP cycle utilisers, e.g. organism W6 (Babel and Loffhagen, 1977). Of the type I methanotrophs Methylomonas methanica and Methylobacter bovis, the latter contained PEP carboxykinase and both contained PEP carboxylase and malic enzyme. However, these enzymes had very low activities compared with the activity of CS which they would have had to supply (Davey et al., 1972; Patel et al., 1975; Trotsenko, 1976).

Although Pseudomonas oleovorans is a facultative RuMP cycle utiliser with OGDH during heterotrophic growth, in C_1-growth it has no OGDH and like the above organisms needs another OAA supply. This organism interconverts C_3 and C_4 compounds in a way similar to M. methylotrophus (Loginova and Trotsenko, 1977, 1979b; Anthony, 1982).

1.9.3. Supply of OAA to Methylotrophs with a Complete CAC

The serine pathway can generate OAA for use in the CAC. The enzyme that does this is PEP carboxylase, and this is the only enzyme that converts C_3 to C_4 compounds in Methylobacterium extorquens AM1, Hyphomicrobium X and other serine pathway utilisers during methylotrophic growth (Salem et al., 1973; Attwood and Harder, 1974; Anthony, 1982). Heterotrophically grown Hyphomicrobium X also contains malic enzyme and PEP carboxykinase. The latter is reversible, unlike PEP carboxylase where carboxylation is irreversible. It is thus

possible that during heterotrophy PEP carboxylase (and possibly malic enzyme) fulfil anaplerotic roles while PEP carboxykinase may function in gluconeogenesis (Wood and Utter, 1965; Attwood and Harder, 1974).

1.10. The Role of the CAC in Methylotrophs

1.10.1. OGDH-Deficient CAC

This type of CAC is found in all obligate methylotrophs that do not use the serine pathway including all type I methanotrophs. It occurs in association with the RuMP cycle (**1.7.**).

The nature of methylotrophic catabolism and the CAC of anaerobically grown E. coli (**1.3.2.**; **1.7.2.**) suggests that in obligate methylotrophs and type M RFMs, the CAC is not catabolic. This has been proposed before (Smith and Hoare, 1977; Zatman, 1981). Further, Zatman (1981) has suggested the OGDH lesion is an economy measure to conserve carbon. The CAC is not involved in carbon assimilation in the RuMP cycle utilising type M RFMs and obligate methylotrophs (**1.7.1.2.**).

The incomplete CAC probably functions biosynthetically. The precursors it produces are 2-OG and perhaps succinyl-CoA. C_4 dicarboxylates maybe supplied by triose carboxylation (**1.9.2.**).

As the CAC of all methylotrophs can function to generate C_5 skeletons, i.e. 2-OG, it follows that the CAC may also function to assimilate nitrogen through the amination of 2-OG to form glutamate (PART C). Thus the CAC may make CAC skeletons for the formation of amino acids metabolically related to glutamate (glutamine, proline and arginine). As much is suggested by the distribution of ^{14}C-acetate in OGDH-deficient methylotrophs [**1.8.1.** (i)]. Glutamate is a precursor of DALA in one of the pathways of porphyrin biosynthesis (**1.3.2.**). The obligately aerobic nature of the methylotrophs discussed here (Anthony, 1982) makes cytochrome and therefore porphyrin biosynthesis crucial, and the OGDH-deficient CAC may produce 2-OG for this.

Succinyl-CoA is involved in methionine synthesis in the obligate methylotroph organism OM33 (Moringa et al., 1982). Succinyl-CoA may also be used in lysine synthesis or, with glycine, it may initiate porphyrin synthesis by the other route of porphyrin biosynthesis.

However, neither process has been studied in a methylotroph. Without OGDH, two possible routes of succinyl-CoA synthesis are from OAA by reversal of the CAC reactions or via ICL and STK:

(i) Succinyl-CoA Production from OAA: Methylotrophs without OGDH might synthesise succinyl-CoA by reversal of the reactions that oxidise succinyl-CoA to OAA. This would require substitution of fumarate reductase for succinate dehydrogenase and operation of malate dehydrogenase, fumarase and STK in reverse relative to their operation in the oxidative CAC (**1.3.2.**). This has been suggested for the obligate methylotroph organism W6 (Babel and Hofmann, 1975). However, in other obligate organisms, e.g. C2A1, W6A and 4B6, one or more of the enzymes required are virtually undetectable (Colby and Zatman, 1972, 1975b). It thus seems difficult to draw conclusions regarding this method of succinyl-CoA production.

(ii) Supply of Succinyl-CoA from Isocitrate: The obligately autotrophic cyanobacteria cannot incorporate ^{14}C-acetate into aspartate and thus have an incomplete CAC. The results also suggest that these organisms have no functional glyoxylate cycle (Smith et al., 1967). However Pearce and Carr (1967) have confirmed that these organisms have ICL and malate synthase. This may suggest that C_4 skeletons made in these organisms by ICL and malate synthase may not be used to produce OAA. However, the presence of STK (Weitzman and Kinghorn, 1980) in the cyanobacteria may allow ICL and malate synthase to supply C_4 skeletons for succinyl-CoA synthesis from which precursors such as methionine and porphyrins could be derived. The foregoing suggests a modification of the CAC where a sequence of reactions initiated by CS is ended by 2-OG and succinyl-CoA (Figure 9). Similarly, as obligate methylotrophs C2A1 and 4B6 and type M RFMs W3A1 and W6A have low levels of STK and perhaps even ICL, and as ICL cannot make C_4 skeletons for OAA synthesis (Colby and Zatman, 1975b), succinyl-CoA may also originate from isocitrate in these organisms.

1.10.2. Methylotrophs with a Complete CAC

1.10.2.1. The Role of the CAC During Heterotrophic Growth. A complete CAC is present in most facultative organisms in methylotrophic and

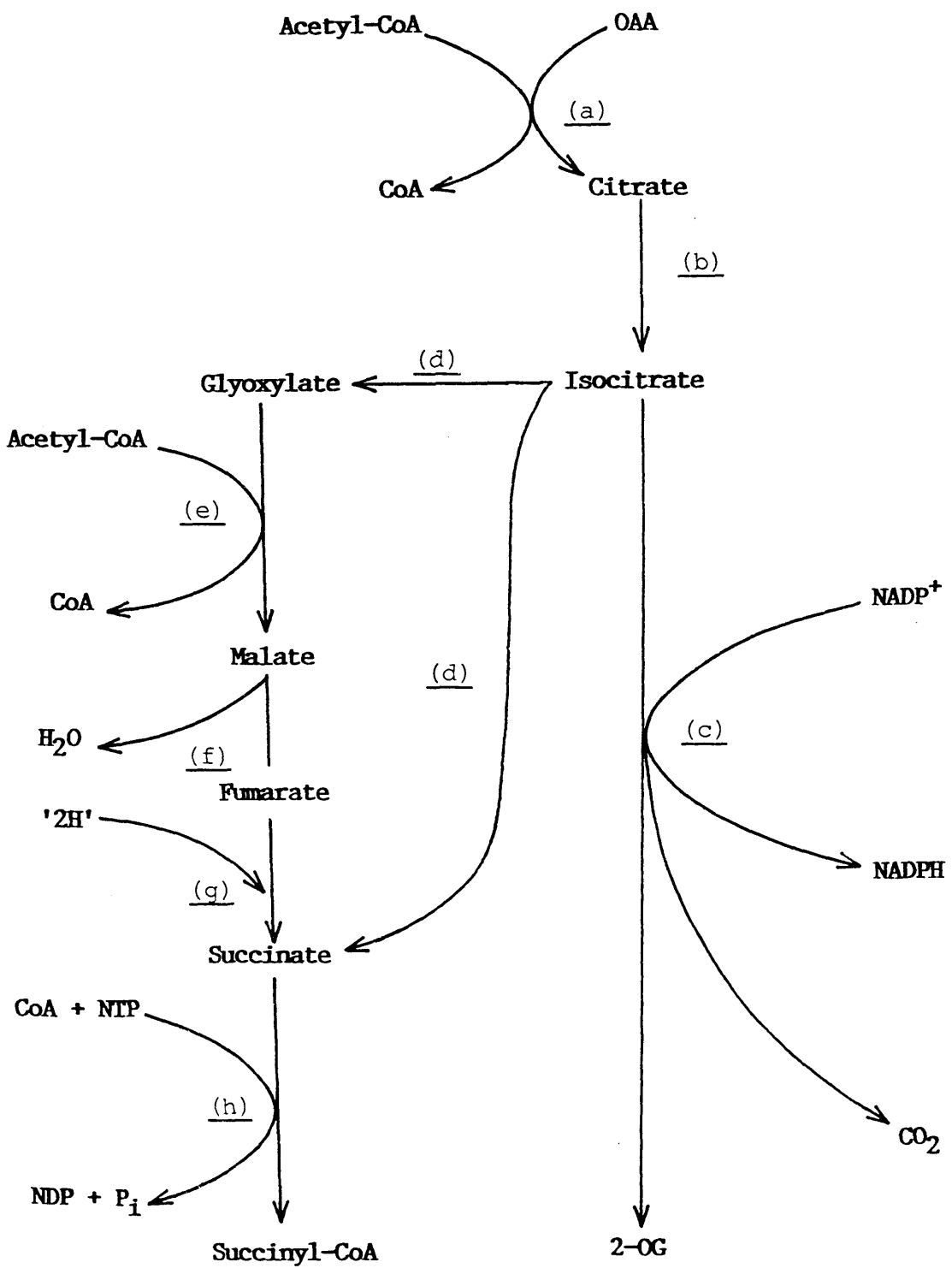
Figure Legend to Figure 9

The enzymes thought to catalyse the reactions in the metabolic sequence shown in Figure 9 are:

(a): CS
(b): Aconitase
(c): NADP-IDH
(d): ICL
(e): Malate Synthase
(f): Fumarase
(g): Fumarate Reductase
(h): STK

Figure 9

The End-Product Nature of Succinyl-CoA and 2-OG in the Cyanobacterial Varient of the CAC



heterotrophic growth. In the latter condition, the organism relies on the CAC to perform tasks required of it by any aerobic heterotroph. Additional to biosynthesis, the CAC will have a catabolic role during heterotrophy. This increasing work-load may be reflected on transition to heterotrophic growth from methylotrophic growth by the general but variable rise in the activity of the CAC enzymes, e.g. Pseudomonas 3A2, organism 5B1, Pseudomonas 8, Arthrobacter 2B2, Mycobacterium 50, Hyphomicrobium X, Achromobacter 1L, Methylobacterium extorquens AM1, Methylobacterium R6, Methylobacterium organophilum XX and Pseudomonas MA (Large and Quayle, 1963; Bellion and Hersch, 1972; Colby and Zatman, 1972; Attwood and Harder, 1974; Cox and Quayle, 1976; O'Connor and Hanson, 1977; Patel et al., 1978; Loginova and Trotsenko, 1979a; Paddon et al., 1985).

The variation of activation of the CAC enzymes may result from the ability of the organism to use a substrate that would otherwise have been a product of the CAC. For example, heterotrophic growth requires the first three enzymes of the CAC to conserve energy and to produce 2-OG for biosynthesis. Transfer of methylotrophically growing organism 5B1 to growth on acetate increases the levels of CS, aconitase and IDH 4.7, 5.9 and 8.6-fold. However, if trimethylamine-grown 5B1 is supplied with glutamate (a substrate making production of 2-OG redundant), the increases are now only 1.4, 1.5 and 3.1-fold (Colby and Zatman, 1972, 1975a) as the CAC only has to fulfil its new catabolic role. Similar behaviour is shown by Arthrobacter 2B2 and Bacillus PM6 (Colby and Zatman, 1975b; Paddon et al., 1985) where repression of some enzymes on glutamate is so severe that their levels are lower than during methylotrophic growth.

1.10.2.2. The Role of the Complete CAC During Methylotrophy. As well as C_5 skeleton production, the CAC may perform other roles during methylotrophy:

1.10.2.2.1. Carbon Assimilation. The involvement of the CAC enzymes in carbon assimilation in the icl^+ serine pathway and the proposed function of the CAC enzymes in the icl^- serine pathway have already been outlined (see **1.7.1.3.**). In this context it is interesting to

note that the facultative methylotroph Pseudomonas MA which uses the icl^+ serine pathway for C_1 assimilation and grows on acetate has two ICL isozymes, one for use in growth on C_1 compounds, the other for growth on C_2 compounds (Bellion and Woodson, 1975).

1.10.2.2.2. Energy Generation. The absence of OGDH from obligate methylotrophs (except type II methanotrophs - see Davey et al., 1972; Trotsenko, 1976) has led to the idea that OGDH and thus a complete CAC only function in heterotrophy (Colby and Zatman, 1975a; Smith and Hoare, 1977; Anthony, 1982). However, this view is questionable in view of the persistence of OGDH during methylotrophic growth of facultative organisms and the presence of OGDH in the obligately methylotrophic members of the type II methanotrophs. Indeed, the activity of OGDH in some obligate type II methanotrophs (e.g. Methylosinus spp.) approaches that found in heterotrophically growing facultative organisms such as Hyphomicrobium X, Pseudomonas 3A2, Arthrobacter 2B2 and others (Davey et al., 1972; Colby and Zatman, 1972; Attwood and Harder, 1974; Trotsenko, 1976; Loginova and Trotsenko, 1979a; Patel et al., 1978; Paddon et al., 1985). Further, the CAC of the obligate type II methanotroph Methanomonas methanooxidans can oxidise acetate to CO_2 and distribute acetate carbon amongst cell components in a manner that is suggestive of a complete and functional CAC (Wadzinski and Ribbons, 1975).

These results suggest that a complete CAC might have a catabolic role in methylotrophic growth in the provision of NADH. However, it would be unlikely that methylotrophs oxidising compounds as reduced as methanol would require the CAC to function like this because they can conduct net synthesis of NADH from methanol oxidation (**1.7.2.**). As much has been shown by Taylor and Anthony (1976a). These workers isolated OGDH-deficient mutants of Methylobacterium extorquens AM1. The mutant's ability to oxidise methanol was undiminished, suggesting that the CAC did not oxidise reduced C_1 compounds.

There are, however, other organisms whose ability to produce NADH is reduced by their C_1 source, and so might benefit from the operation of the CAC during methylotrophy, e.g. type II methanotrophs. Use of methane requires 1 molecule of NADH per molecule of methane for

methane hydroxylation (**1.7.2.1.**). Thus, to provide NADH, the organism must generate > 1 molecule of NADH during catabolism. As methanol oxidation is not generally NAD-linked (**1.7.2.1.**), formaldehyde oxidation is crucial to the supply of NADH. However, this process yields at most 2 molecules of NAD(P)H per molecule of formaldehyde, and this yield may not be realised in some of the serine pathway utilisers like the type II methanotrophs (Zatman, 1981). This NADH shortage is exacerbated in the type II methanotrophs as the serine pathway also consumes NADH. In such circumstances it seems reasonable to suggest that the CAC may serve a useful catabolic function in the type II methanotrophs where it could oxidise formaldehyde after its conversion to acetyl-CoA by the serine pathway. This may help reduce the shortfall of NADH caused by the demands of the serine pathway and methane mono-oxygenase. This has also been suggested by Zatman (1981).

Newaz and Hersch (1975) have also proposed that the CAC may avert NADH-deficiency in Pseudomonas MA. These authors failed to find enzymes that could oxidise the C_1 source directly. They concluded that Pseudomonas MA assimilates and dissimilates carbon "via common intermediates". As Pseudomonas MA had PDH, malic enzyme and pyruvate kinase, Newaz and Hersch (1975) suggested that PEP from the serine pathway could be converted to pyruvate and then oxidised (i) directly to acetyl-CoA or (ii) indirectly via malate which could be cleaved to acetyl-CoA. By route (i), acetyl-CoA could be oxidised to glyoxylate via the glyoxylate cycle; by route (ii), to achieve the same yield of ATP and NADH, the full CAC would be used to oxidise acetyl-CoA. These pathways are shown in Figure 10. Although plausible, Anthony (1982) considered that there was no conclusive evidence for their operation.

For the moment, a role for the complete CAC in methylotrophic oxidation remains hypothetical. If it has no role, it is not easy to see how OGDH expression in methylotrophy, with its potential to cause unnecessary loss of assimilated carbon, could not be deleterious.

1.11. The CAC and Obligate Methylotrophy

It is intriguing that despite their extreme synthetic ability, many methylotrophs cannot grow on compounds with C-C bonds. It has

Figure Legend to Figure 10

Routes (i) and (ii) refer to those metabolic sequences [(i) and (ii)] discussed in the text in **1.10.2.2.2.**

Both sequences involve the serine pathway enzymes that convert glycine and formaldehyde to PEP (enzymes labelled a to e of Figure 7). However, route (i) oxidises PEP to acetyl-CoA via the enzymes pyruvate kinase and PDH, while route (ii) oxidises PEP to acetyl-CoA via pyruvate kinase, malic enzyme, malate thiokinase and malyl-CoA lyase.

Route (i) oxidises acetyl-CoA to glyoxylate via the glyoxylate cycle enzyme ICL and the CAC enzymes CS, aconitase, succinate dehydrogenase, fumarase and malate dehydrogenase. The glyoxylate is recirculated to reform the C_1 acceptor - glycine.

Route (ii) Completely oxidises acetyl-CoA to CO_2 via the complete CAC. The glyoxylate required to reform glycine is generated by malyl-CoA lyase.

For both routes, formaldehyde is oxidised to CO_2 with conservation of energy as reducing equivalents according to the equation:

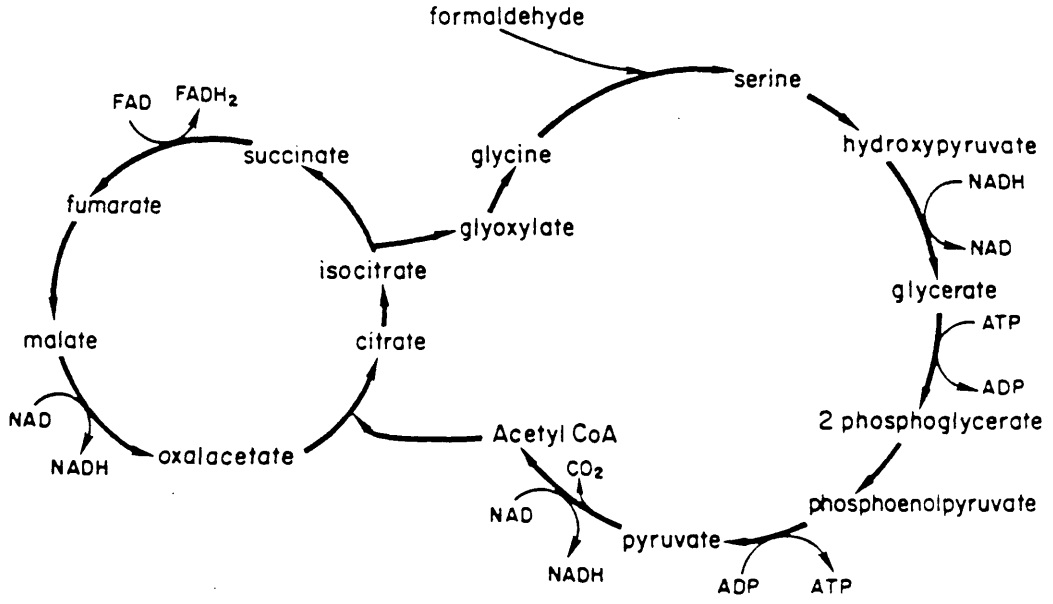
$$\text{Formaldehyde} + NAD^+ + FAD + H_2O \longrightarrow CO_2 + FADH_2 + NADH + H^+$$

Figure 10 is taken from Newaz and Hersch (1975).

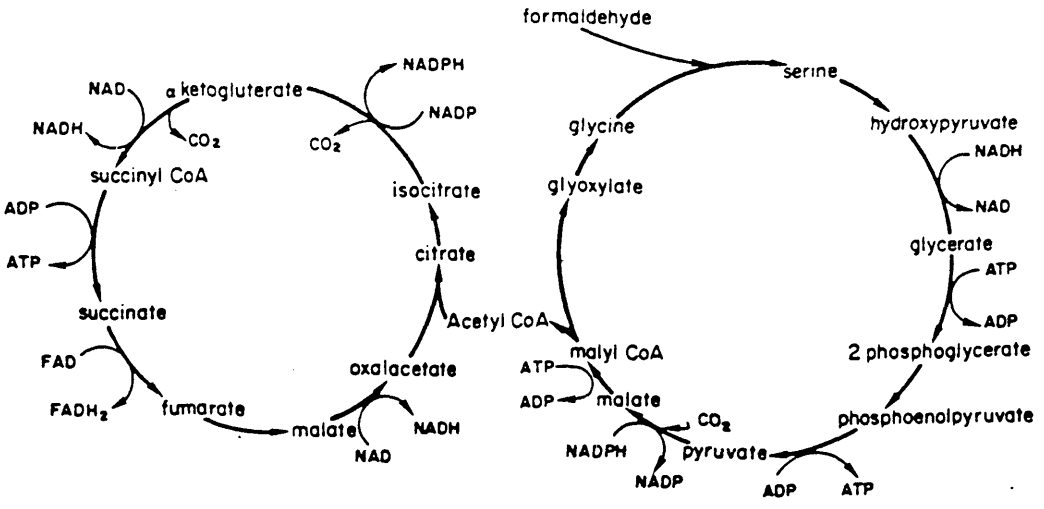
Figure 10

Proposed Partial or Complete Involvment of the CAC in the Oxidation of Formaldehyde to CO_2 by Pseudomonas MA

Route (i): Partial Involvment of the CAC in the Oxidation of Formaldehyde to CO_2



Route (ii): Complete Involvment of the CAC in the Oxidation of Formaldehyde to CO_2



been speculated that this may be due to an OGDH-deficiency. Evidence for this is provided by an OGDH-deficient mutant of Methylobacterium extorquens AM1 (ICT41) which is an obligate organism, the reversion of which restores the organism's heterotrophic ability (Taylor and Anthony, 1976a). This shows that an OGDH lesion may cause obligate methylotrophy. Taylor and Anthony, (1976a) proposed the lesion had its effect by stopping acetyl-CoA oxidation, so compromising energy conservation, or by causing harmful accumulation of metabolites.

However, obligate methylotrophy does not always result from an OGDH lesion, e.g. the heterotrophic growth of the type M RFMs W6A and W3A1, both of which lack OGDH (Colby and Zatman, 1975a). Nor does the possession of OGDH stop some type II methanotrophs being obligate methylotrophs (Davey et al., 1972; Trotsenko, 1976). Taylor and Anthony (1976a) have suggested the effects of an OGDH lesion may also result from low expression of CAC enzymes. This may stop supply of CAC products at rates needed for heterotrophic growth. Most facultative organisms avoid this by increasing CAC enzyme activity (**1.10.2.1.**). An inability to do so may hinder heterotrophic growth. This may be so for RFMs where heterotrophic substrates cannot increase the activity of CAC enzymes (Colby and Zatman, 1975a). This may explain the restricted range of heterotrophic substrates the RFMs can grow on.

SECTION B

THE CITRIC ACID CYCLE ENZYMES STUDIED IN THIS THESIS

The CAC enzymes to which a large part of this thesis is devoted are CS and IDH and they are introduced here. Most information about these enzymes is from heterotrophic sources and this is introduced prior to the available information about the methylotrophic enzymes.

1.12. Bacterial Citrate Synthase

1.12.1. CS: Reaction, Catalysis and Role

CS allows entry of carbon as acetyl-CoA into the CAC from a

variety of metabolic sources. CS is central to the metabolism of most living things, as attested to by its wide occurrence (Weitzman and Danson, 1976). CS catalyses the condensation of OAA and acetyl-CoA:

$$\text{Acetyl-CoA} + \text{OAA} + H_2O \longrightarrow \text{CoA} + \text{Citrate} + H^+$$

For most CSs, enol acetyl-CoA attacks the si face of the keto tautomer of OAA. The reaction inverts the configuration of hydrogen atoms on the C-1 position of acetyl-CoA (Srere, 1967; Eggerer et al., 1970; Srere, 1972). The result of this is probably S-citryl-CoA which is hydrolysed to form citrate and CoA (Eggerer and Remberger, 1963; Bukel and Eggerer, 1969). Thus, it is clear that CS can support three separate activities that make up the overall condensation reaction:

a) Enolase (D = deuterium):

$$CH_3CO.SCoA + D_2O \longrightarrow DCH_2CO.SCoA + HDO$$

b) Ligase:

$$CH_3CO.SCoA + OAA \longrightarrow \text{Citryl-CoA}$$

c) Hydrolase:

$$\text{Citryl-CoA} + H_2O \longrightarrow \text{Citrate} + \text{CoA}$$

An exception to the above are CSs from the obligately anaerobic Clostridia and Desulphovibrio vulgaris that direct the attack of acetyl-CoA to the re face of OAA presumably to generate R-citryl-CoA as an intermediate (Gottschalk and Barker, 1966, 1967).

The crystal structure of the porcine heart enzyme has been solved (Weigand and Remington, 1986). As a result, it has been possible to correlate structure and function. Porcine CS can be crystallised with and without substrates, products or analogues thereof in a single open and two closed conformations. The open and closed conformations differ by the relative position of two domains in the enzyme's structure. It

has been proposed that the closed conformations are those that CS adopts to catalyse the ligase (closed state A) and hydrolase (closed state B) reactions. The open state is that which allows substrate binding and product release (Weigand and Remington, 1986). There is one substrate binding site per subunit, located between the large and small domains. Residues that make up this site are contributed by both subunits. It appears that OAA binds to the unliganded CS (Srere, 1972, 1975; Johansson and Petersson, 1974a, b). So, it has been proposed that, for the eukaryotic CS, OAA binding changes the conformation to closed state A which then can bind acetyl-CoA. The methyl group of the latter is deprotonated by His 274. The enol-acetyl-CoA then attacks OAA on its si face. This is aided by polarisation and protonation of the OAA carbonyl by His 320. Once citryl-CoA has been produced, closed state B (observed with analogues of citryl-CoA) hydrolyses citryl-CoA to CoA and citrate. The use of two closed states prevents premature hydrolysis of acetyl-CoA (Weigand and Remington, 1986).

Although E. coli CS has been crystallised (Srere, 1972; Rubin et al., 1983), no information has been derived from this. However, there are reasons to suggest that the eukaryotic CS may be a reasonable model for certain aspects of the prokaryotic CSs. Indeed experiments based on this premise have vindicated this proposal (see below).

1.12.2. Structure of Bacterial CSs

Weitzman and Dunmore (1969a) studied the gel filtration behaviour of a variety of CSs: CSs from Gram-negative bacteria eluted with an M_r of around 240,000 wheras elution of CSs from Gram-positive bacteria and eukaryotes indicated an M_r of around 100,000. This division of 'large' and 'small' M_r values extends throughout a wide range of organisms. Gram-negative bacteria have large CSs with M_r values of about 250,000, while the Gram-positive CSs contain a small CS with M_r values of about 100,000 (Weitzman and Danson, 1976; Weitzman, 1981).

1.12.3. Quaternary Structure

All CSs are oligomers of identical subunits with similar M_r

values of between 40,000 and 50,000. The CSs from Gram-negative sources are generally hexameric, while the enzyme from Gram-positive and eukaryotic sources is dimeric. The findings that justify these generalisations are as follows.

The most studied prokaryotic CS with regard to its structure is the large E. coli CS. Tong and Duckworth (1975) estimated the subunit Mr value for this enzyme to be 47,000. This agrees with the results of Robinson et al. (1983a) and is consistent with the M_r inferred from the amino acid sequence derived from both protein (Bhayana and Duckworth, 1984) and gene (Ner et al., 1983). A variety of techniques show that E. coli CS is composed of one type of polypeptide (Wright et al., 1971; Tong and Duckworth, 1975; Duckworth and Bell, 1982). Other large CSs are also composed of subunits of M_r values of between 47,000 and 53,000 (Morse and Duckworth, 1980; Higa et al., 1978; Mitchell and Weitzman, 1983a). Likewise, where investigated, these enzymes only possess one subunit species (Donald and Duckworth, 1986). Fewer Gram-positive organisms have been studied. Only the CS from Bacillus megaterium has been purified to homogeneity, and it is composed of two subunits of identical M_r (40,300) - just under the M_r of the Gram-negative CS subunit (Robinson et al., 1983b). The eukaryotic enzymes have one type of subunit of comparable size to the prokaryotic CSs (Singh et al., 1970; Wu and Yang, 1970; Moriyama and Srere, 1971).

The subunit stoichiometry of some large CSs is sensitive to conditions of analysis. Native PAGE and high speed sedimentation equilibrium centrifugation studies indicate the E. coli CS exists as an equilibrium of a number of aggregates of up to fifteen subunits (Wright and Sanwal, 1971; Danson and Weitzman, 1973; Tong and Duckworth, 1975; Morse and Duckworth, 1980). It appears that CS from other enteric bacteria also exist in a variety of polymeric states (Weitzman and Danson, 1976). The association-dissociation equilibria shown by E. coli CS are simplified in conditions that are optimal for activity (Tong and Duckworth, 1975; Weitzman and Danson, 1976; Robinson et al., 1983a). At pH 7.8, in 0.05 M-KCl, CS is dimeric or hexameric, but in 0.1 M-KCl, CS is totally hexameric.

Unlike E. coli CS, heterogeneity of subunit aggregation is not shown by CSs from Gram-negative aerobes, e.g. Acinetobacter anitratum

CS. Here again, the quaternary structure is hexameric (Johnson and Hanson, 1974; Morse and Duckworth, 1980). There is also evidence that CSs from Acinetobacter calcoaceticus, Azotobacter vinelandii and a marine Pseudomonas sp. are similarly hexameric proteins (Srere, 1972; Higa et al., 1978; Mitchell and Weitzman, 1983b).

The 'small' Gram-positive CS has a simpler quaternary structure than its Gram-negative counterparts. The Bacillus megaterium CS is dimeric (Robinson et al., 1983b). In this respect the Gram-positive CS probably resembles the eukaryotic enzymes. The porcine heart, rat heart and rat liver enzymes are also dimeric proteins (Singh et al., 1970; Wu and Yang, 1970; Moriyama and Srere, 1971).

1.12.4. Subunit Analysis

In addition to the E. coli CS, the amino acid sequence of two other CSs from Gram-negative sources are now known. The Acinetobacter anitratum CS subunit shares a high degree of sequence homology (70%) with the E. coli CS sequence (Morse and Duckworth, 1980; Donald and Duckworth, 1986, 1987). The second example is the Rickettsia prowazekii CS sequence (although see **1.12.7.3.**). Again there is a reasonable degree of homology (59%) between this sequence and the E. coli CS sequence (Wood et al., 1987). Eukaryotic (small) CSs also share strong sequence homology (55-75%) between themselves (Bloxham et al., 1981; Rozenkrantz et al., 1986; Evans et al., 1988). However, as suggested by the E. coli sequence, there is far less homology (20-30%) between the Gram-negative CSs and the eukaryotic enzymes (Ner et al., 1983; Rozenkrantz et al., 1986).

Despite the lack of overall homology between the CSs from prokaryotes and eukaryotes, the active site amino acids in the pig heart CS have been highly conserved in CS sequences from both Gram-negative and eukaryotic sources (Bloxham et al., 1981; Ner et al., 1983; Bhayana et al., 1984; Rosenkrantz et al., 1986; Weigand and Remington, 1986; Donald and Duckworth, 1987; Wood et al., 1987; Evans et al., 1988). This has been shown by active-site directed mutagenesis of histidyl and arginyl residues in the E. coli CS sequence that reference to the porcine heart CS structure would suggest are critical

to polarisation of the carbonyl group of OAA during formation of the transition state. The resulting mutant proteins have drastically reduced turnover numbers but the effects on the affinity for OAA are far less marked relative to the wild-type. This shows that the E. coli residues have similar functions to those ascribed to the homologous residues in the porcine CS (Anderson and Duckworth, 1989). Further, it has been concluded from comparison of the effects of subtilisin on the E. coli and pig heart CSs and the effect of an anti-pig heart CS antibody on the CSs from pig, pigeon, Bacillus megaterium and E. coli that both large and small enzymes may share some features of polypeptide-folding (Bell et al., 1983; Pullen et al., 1985).

1.12.5. The Minimal Subunit Requirements of CS Structure and Function

The pig heart CS subunit is inactive (Wu and Yang, 1970) as each of its two subunits contribute amino acid residues to the active site of the other subunit (Weigand and Remington, 1986). Thus it is likely that the dimeric CS is the minimum arrangement of subunits required for activity. This may also be so for the large Gram-negative CSs.

If the hexameric forms of E. coli or A. calcoaceticus CS were crosslinked, the subunit multimers that were most common were dimeric, tetrameric and hexameric species (Tong and Duckworth, 1975; Robinson et al., 1983a; Mitchell and Weitzman, 1983b). Thus the large CSs may consist of trimers of dimers. The tri-lobate structure of the A. calcoaceticus CS revealed by electron microscopy is also consistent with this suggestion (Rowe and Weitzman, 1969).

The dimeric unit of large CSs may also be the minimum catalytic unit. Else et al. (1988) have shown proteolysis of 3 of the 6 subunits of E. coli CS causes total inactivation. Similar conclusions can be reached from studies on E. coli or Acinetobacter CS mutants (**1.12.11.**) which have identical native M_r values to those of small CSs (Harford and Weitzman, 1978; Weitzman et al., 1978; Danson et al., 1979).

1.12.6. Regulation of CS

The regulation of CS activity has been studied in a wide range of

organisms. As will become clear, there is a diversity of regulatory mechanisms of CS activity. This diversity is a reflection of the multi-functional capability of the CAC which will be differentially exploited depending upon the organism's needs. It is interesting to reflect that despite the diversity of regulation 'tacked' onto it, the catalytic site of CS has probably remained essentially unchanged.

There are reasons why *a priori* CS might be expected to be regulated. First, there is its position in metabolism. CS allows entry of carbon into the CAC as acetyl-CoA. In its oxidative capacity, CS is the 'gateway' to energy generation from oxidation (*via* acetyl-CoA) of all the major groups of cell components. As it is often the first committed enzyme in a pathway that is regulated, so CS is likely to be regulated. Even where the CAC is incomplete and has a biosynthetic role, CS is still the initiating enzyme in a pathway that produces vital precursors such as C_5 skeletons and thus it may be expected that the regulation of CS activity would be a crucial biosynthetic control.

Secondly, the equilibrium of the CS-catalysed reaction lies very much in the direction of citrate production and consequently proceeds in that direction with a large decrease in free energy. Garland (1968) has noted that these features make the CS reaction a logical point of control. However, Srere (1975) has noted that in the complete CAC, the malate dehydrogenase reaction greatly favours OAA reduction, and so the irreversibility of CS *in vitro* may differ from that *in vivo*.

Thirdly, CS can be rate-limiting in the CAC of *E. coli* (Walsh *et al.* 1987). These workers constructed plasmids that allowed controllable expression of the *E. coli* CS gene. These were inserted into a CS^- mutant of *E. coli*. The intracellular level of CS could be controlled with a non-metabolisable inducer in the growth medium. Walsh *et al.* (1987) measured the effects of changes in the level of CS on flux through the CAC. Relative to wild-type, under-production of CS caused a decrease of flux. This indicates the potential for rate-limitation of the CAC that CS can exert and supports the contention that CS is a logical target for regulation.

1.12.6.1. Regulatory Properties of CS Concerned with Catabolism. It can be argued that as one of the functions of a complete CAC is to

oxidise acetyl-CoA to produce NADH and, from this, ATP by oxidative phosphorylation, both NADH and ATP are CAC end products. It has thus seemed logical to examine their effects on CS.

1.12.6.1.1. Non-Specific Adenine Nucleotide Inhibition. Inhibition of CS by ATP was first shown in baker's yeast and pig heart by Hathaway and Atkinson (1965). ADP and AMP also inhibited CS and the order of potency of the adenylates was ATP> ADP> AMP. ATP-inhibition was competitive with acetyl-CoA. It was suggested that relative rather than absolute concentrations of adenine nucleotides were responsible for the inhibitory effects *in vivo*, the higher the level of ATP relative to AMP and ADP, the less active CS would be. This idea developed into the energy charge concept (Atkinson, 1968) which places importance on the amount of high energy phosphodiester bonds available relative to the total amount of adenine nucleotides. Indeed, increasing energy charge inhibited the yeast CS (Atkinson, 1968).

ATP-inhibition of CS has subsequently been observed in a wide range of organisms (Weitzman and Danson, 1976; Weitzman, 1981), including a wide variety of bacteria. As in eukaryotes, ATP-inhibition of the bacterial CSs is competitive with acetyl-CoA, e.g. the CSs from *Rhodospirillum rubrum*, *Acetobacter xylinum*, *Nostoc* sp.E, *E. coli*, *Bacillus megaterium*, *Pseudomonas aeruginosa*, *Azotobacter vinelandii*, *Pseudomonas fluorescens*, *Bacillus polymyxa*, *Bacillus macerans*, *Bacillus subtilis* and *Rickettsia prowazekii* (Srere, 1968; Flechtner and Hanson, 1969, 1970; Tanaka and Hanson, 1975; Harford and Weitzman, 1975; Massarini *et al.*, 1976; Swissa and Benziman, 1976; Lucas and Weitzman, 1977; Robinson, 1984; Phibbs and Winkler, 1982).

Some CSs that are inhibited by ATP are weakly inhibited by nicotinamide nucleotides, e.g. the *Bacillus megaterium* CS (Robinson *et al.*, 1983) and the eukaryotic enzymes from pig heart and rat kidney (Lee and Kosicki, 1967; Srere *et al.*, 1973). Inhibition is exerted non-specifically by (in order of potency) NADPH, NADH, $NADP^+$ and NAD^+ (Lee and Kosicki, 1967; Srere *et al.*, 1973; Weitzman and Danson, 1976 Robinson *et al.*, 1983b). Again, as with ATP-inhibition, in these cases nicotinamide nucleotide inhibition was competitive with acetyl-CoA.

Thus, nucleotide inhibition probably results from interaction of

the inhibitory nucleotide at the acetyl-CoA binding site. This is predictable because CoA and the nucleotides considered here share the 5'AMP moiety. That acetyl-CoA and ATP did interact at the same site was confirmed by multiple inhibition studies using ATP and the coenzyme analogue bromoacetyl-CoA on eukaryotic and prokaryotic CSs (Harford and Weitzman, 1975) and by the positive linear relationship between acetyl-CoA K_m values for a wide range of CSs and their K_i values for ATP (Weitzman, 1981).

It is apparent from the above that the more negatively charged the nucleotide, the greater is its inhibitory potency. Thus Weitzman (1981) has suggested that electrostatic interactions may be important in nucleotide binding. Indeed, the three phosphates of CoA each form salt bridges with an arginine residue (Weigand and Remington, 1986).

Although ATP-inhibition of CS is a plausible negative feedback control, its physiological relevance is debatable. As ATP is biochemically active as its chelate with a divalent cation, the finding that CSs from pig heart and Bacillus sp. are desensitised to ATP by Mg^{2+} weakens the proposal that ATP is a physiological regulator of CS (Kosicki and Lee, 1966; Lee and Kosicki 1967; Flechtner and Hanson, 1969; Tanaka and Hanson, 1975; Robinson, 1984). It is believed that chelation of the pyrophosphate of ATP reduces its charge and prevents it from interacting with the acetyl-CoA binding site of CS (Kosicki and Lee, 1966; Lee and Kosicki, 1967).

Further, the behaviour of CS at in vivo concentrations in toluenised cells contradicts the hypothesis that CS is regulated by ATP. It has been shown that yeast cells and rat liver mitochondria can be permeabilised to CS substrates by treatment by toluene. Under these circumstances, CS in situ in both yeast and mitochondria was markedly less sensitive to ATP-inhibition than either CS at much lower concentrations in vitro (Weitzman and Hewson, 1973; Matlib et al., 1978). This suggests that, as physiological conditions are approached, the in vivo role of ATP-inhibition of CS becomes uncertain.

A final objection to ATP-inhibition of CS is its ubiquity. Even organisms that use the CAC for anabolic purposes alone, e.g. obligate autotrophs and anaerobic bacilli, contain CSs that are subject to ATP-inhibition (Taylor, 1970, 1973; Tanaka and Hanson, 1975; Lucas

and Weitzman, 1975, 1977).

1.12.6.1.2. Specific Inhibition by NADH. Under the conditions employed, Weitzman (1966a) could not detect any ATP-inhibition of CS from the facultative anaerobe E. coli but it was found that the other major catabolic end product of the CAC, NADH, was a powerful inhibitor of CS activity at low (up to 0.1 mM-NADH) concentrations. This effect was repeated with CSs from Azotobacter vinelandii and Pseudomonas ovalis but not with CSs from pig heart or yeast. Thus Weitzman (1966a) concluded that NADH-inhibition of CS was a prokaryotic phenomenon. Inhibition by NADH was found to be specific in that NADPH, $NADP^+$ and NAD^+ all failed to influence enzyme activity. This and the far more powerful inhibition by NADH on E. coli than eukaryotic CSs (E. coli CS is > 100-fold more sensitive to NADH-inhibition than pig heart or rat kidney CS) distinguishes the specific NADH-inhibition described here from non-specific nicotinamide nucleotide inhibition (**1.12.6.1.1.**; Lee and Kosicki, 1967; Srere et al., 1973; Weitzman and Danson, 1976).

When the effects of NADH were tested on the CS from the obligate aerobe A. calcoaceticus, NADH-inhibition was still observed, but it was reversed by AMP (Weitzman, 1967; Weitzman and Jones, 1968; Weitzman and Danson, 1976). This led to a survey of the effects of NADH and AMP on a range of bacteria (Weitzman and Jones, 1968) which showed that CSs from Gram-positive bacteria were insensitive to NADH and resembled the eukaryotic enzymes in this respect. However, CSs from Gram-negative bacteria were inhibited by NADH. There were two groups of NADH-sensitive organisms, those for which NADH-inhibition of CS was reversed by AMP and those for which NADH-inhibition of CS was unaffected by AMP. Weitzman and Jones (1968) found AMP reactivation of NADH-inhibited CSs was restricted to obligate aerobes, while NADH-inhibition of CSs of facultative anaerobes was not AMP-reversible.

On the basis of these findings, Weitzman and Jones (1968) proposed the following rationale. Facultative anaerobes, like the enteric bacteria, use the glycolytic Embden-Meyerhof pathway which contains enzymes like phosphofructokinase and pyruvate kinase that respond to low-energy indicators such as AMP. Thus, such organisms would not need the activating effect of AMP further 'down stream'.

However, the glucose dissimilation pathway probably used by the obligate aerobes (the Entner-Doudoroff pathway) does not have AMP-responsive enzymes and so would require a regulatory site in the CAC that would respond to raised levels of AMP.

However, during growth on, e.g. acetate, the regulatory sites that are responsive to AMP during glucose-growth may not be available to facultative anaerobes. Although this would seem to question the rationale proposed by Weitzman and Jones (1968), there are other CAC enzymes whose activity is activated by AMP. Thus, AMP stimulates dephosphorylation and activation of E. coli NADP-IDH (**1.13.8.3.**), thus obviating the need for AMP-reversal of inhibition of CS by NADH.

The coincidence of the large Gram-negative quaternary structure with NADH-inhibition and the small Gram-positive quaternary structure with NADH insensitivity is striking. It seems that NADH-inhibition is a result of heterotropic, co-operative effects within the large CS, that may not be allowed in the simpler structure of the small CSs (see **1.12.11.**). Although the regulatory and structural properties of CSs from prokaryotic organisms are so marked as to have found use in taxonomy (Weitzman and Jones, 1975), it remains a mystery as to why NADH-inhibition of CS is restricted solely to Gram-negative organisms.

1.12.6.1.3. AMP Activation. Although far less common than **AMP-reactivation** of NADH-inhibition, AMP-activation in the absence of NADH occurs with CSs from Azotobacter vinelandii, a mutant strain of Pseudomonas aeruginosa and a marine pseudomonad. In all cases, the enzymes are also subject to AMP-reversible NADH-inhibition, consistent with the obligately aerobic nature and Gram stain of the organisms (Flechtner and Hanson, 1970; Massarini and Cazzulo, 1974; Higa et al., 1978; Solomon and Weitzman, 1983). AMP activation of CS is consistent with the energy-generating role of the CAC.

1.12.6.2. Regulatory Properties of CS Associated with Anabolism

1.12.6.2.1. Inhibition of CS by 2-OG. The CAC is a vital source of C_5 skeletons. When cellular energy generation is not a function of the CAC, OGDH is absent or much repressed (Chapter 1; **1.4.2.**). Thus, now,

CS is the first committed step in a pathway whose product is the C_5 skeleton 2-OG (Figure 3). This occurs in, for example, anaerobically or glucose-grown E. coli (Amarasingham and Davis, 1965).

With this in mind, Wright et al. (1967) found that E. coli CS was inhibited by 2-OG. Weitzman and Dunmore (1969b) repeated this result with CSs from other Gram-negative facultative anaerobes and it was later shown that CSs from Gram-positive facultatively anaerobic bacilli, the Gram-positive obligate anaerobe Clostridium acidi-urici and the Gram-negative obligately aerobic autotrophic thiobacilli were inhibited by 2-OG (Gottschalk and Dittbrenner, 1970; Taylor, 1970; Tanaka and Hanson, 1975). All these organisms have OGDH lesions, and thus it seems that 2-OG-inhibition of CS may be a feature of the enzyme from all organisms without OGDH, irrespective of Gram-stain .

1.12.6.2.2. Inhibition of CS by 2-OG and Succinyl-CoA. The cyanobacterial CAC operates so that, like 2-OG, succinyl-CoA is an end product of a pathway initiated by CS (**1.10.1.**; Figure 9). Taylor (1973) showed that, consistent with the CAC's biosynthetic role in the Gram-negative cyanobacterium Aphanocapsa F, its CS was not NADH-sensitive, but was inhibited by 2-OG. Lucas and Weitzman (1975, 1977) confirmed these findings in Anabaena flos-aquae, Anacystis nidulans, Aphanocapsa 6714 and Gloeocapsa alpicola and found that these CSs were also inhibited by succinyl-CoA. That this regulation was specific to the control of CS in this type of CAC was shown by the insensitivity of CSs from a complete CAC (A. calcoaceticus and pig heart) or a OGDH-deficient CAC without ICL (anerobic E. coli) to succinyl-CoA.

1.12.7. Extensions and Exceptions to the Enzyme Patterns

1.12.7.1. The Archaebacteria and other 'Extremophiles'. In addition to the two primary Kingdoms, the prokaryotes and the eukaryotes, a third Kingdom has recently been recognised - the archaebacteria. The organisms making up this kingdom are as related to the prokaryotes as they are to the eukaryotes (Danson, 1988) and so lie outside the taxonomic divisions delineated by the large and small CSs. Thus the properties of the archaebacterial CSs are extensions of the patterns

shown by the prokaryotic and eukaryotic CSs. The first CS from an archaebacterium to be studied was the Halobacterium cutirubrum enzyme (Cazzulo, 1973). The properties of this and other archaebacterial CSs are very similar to the small CSs in that they are NADH-insensitive dimeric molecules composed of identical subunits (Danson et al., 1985; Smith et al., 1987; Danson, 1988). Thermus aquaticus and Chloroflexus aurantiacus are not archaebacteria but are Gram-negative, possess small CSs and are thus true exceptions in this respect (Weitzman, 1978; Kelly, 1988; Danson, 1988). Both these organisms and the archaebacteria grow in conditions of extreme salinity, pH, and/or temperature (Danson, 1988). It has been argued that adaptation to such conditions is incompatible with the operation of allosteric inhibition by NADH because of the more simple and robust structure CS may adopt to survive these extreme conditions (Cazzulo, 1973; Weitzman, 1978).

1.12.7.2. Existence of Large and Small CSs in the Same Organism. Thus far, the discussion has considered organisms that contain either a large or a small CS. However, recently, it has been found that CS from a variety of Pseudomonas sp. could be fractionated into large and small species with their attendant regulatory features (Mitchell and Weitzman, 1986; Mitchell et al., 1986). These enzymes displayed no dissociation or association phenomena (c.f. CSs from E. coli and isolates of a marine pseudomonad; **1.12.11.**).

1.12.7.3. Other Exceptions. Mesophilic Gram-negative organisms that have only a small CS have been reported: Pseudomonas maltophilia, Pseudomonas saccharophila and Rickettsia prowazekii (Mitchell and Weitzman, 1986; Phibbs and Winkler, 1982). Further, large NADH-insensitive CSs from Gram-negative organisms with complete CACs have been reported, e.g. the CSs from Acetobacter xylinum, Pseudomonas testosteronii, Branhamella, Kingella, Moraxella and Neisseria spp. (Swissa and Benziman, 1976; Weitzman, 1981; Mitchell and Weitzman, 1986). CSs from obligately autotrophic thiobacilli are also NADH-insensitive, in agreement with the biosynthetic role of their OGDH-deficient CAC, but in contrast with their Gram-stain (Taylor, 1970).

No large CS has been isolated from a eukaryotic or Gram-positive

source. However, the abyssal fish Antimora rostrata has an apparently large CS of M_r 270,000 although, when the enzyme is subjected to the pressures that pertain in the habitat of the fish, it depolymerised into a more active small CS (Hochachka et al., 1975).

1.12.8. Kinetic Properties of CS

A manifestation of allosteric phenomena is the sigmoid substrate or effector dependences that may be caused by homotropic interactions between subunits. It appears that the large Gram-negative CSs show such characteristics. There is a kinetic distinction between those NADH-sensitive CSs that are insensitive to AMP and those CSs subject to AMP-reversible NADH-inhibition. The CS from the facultative anaerobe E. coli has sigmoidal dependences on substrates while NADH-inhibition is hyperbolically dependent on NADH. The obligately aerobic group, typified by A. calcoaceticus, have hyperbolic substrate dependences and sigmoid dependences of inhibition upon NADH. Most observations also suggest that AMP reactivation of the 'Acinetobacter-type' CS is also sigmoidal (Weitzman, 1966a; Weitzman, 1967; Faloona and Srere, 1969; Wright and Sanwal, 1971; Eidels and Preiss, 1970; Johnson and Hanson, 1974; Massarini et al., 1976; Higa et al., 1978; Morse and Duckworth, 1980). Thus it seems that the Gram-negative CSs behave like the "K" types of allosteric regulatory enzyme defined by Monod et al. (1965), where the equilibria between high affinity ("R") states and lower affinity ("T") states are displaced in favour of the T state in the case of E. coli CS and the R state in the case of Acinetobacter CS types.

The substrate dependences of small CSs are hyperbolic (Johnson and Hanson, 1974; Shiio et al., 1977; Robinson et al., 1984)

1.12.9. Allosteric Nature of NADH-Inhibition of CS

Kinetic evidence for the allosteric nature of NADH-inhibition has been obtained by multiple inhibition studies on CS from Pseudomonas aeruginosa CS which have demonstrated that the binding sites for acetyl-CoA and NADH are distinct (Harford and Weitzman, 1975).

Further evidence that NADH exerts its effects as a result of conformational change bought about by binding at an allosteric site has been provided by two lines of inquiry:

1.12.9.1. Desensitisation to NADH-Inhibition. Weitzman (1966b) showed that treatments such as raised pH or ionic strength desensitised E. coli CS to NADH without a similar effect on activity. Similar ionic strength effects have also been observed on CSs from other Gram-negative bacteria: A. calcoaceticus, Salmonella typhimurium, Rhodospirillum rubrum, Thiobacillus verstus and Rhodopseudomonas capsulata (Rowe and Weitzman, 1969; Eidels and Preiss, 1970; Flechtner and Hanson, 1970; Taylor, 1970; Massarini et al., 1976). It has also been noted that anti-A. calcoaceticus CS antibody abolishes NADH-inhibition prior to inactivation, thus emphasising the distinct nature of the enzyme's substrate and NADH-binding sites (Weitzman, 1981).

CS has also been desensitised to NADH by photochemical and chemical modification. Thus, the A. calcoaceticus and Pseudomonas aeruginosa CSs can be desensitised by modification of histidyl residues with diethylpyrocarbonate or by photo-oxidation in the presence of photosensitive dyes (Weitzman et al., 1974; Weitzman and Danson, 1976). E. coli CS can be desensitised to NADH by modification of thiol residues by photo-oxidation or thiol-directed reagents like 5,5'-dithiobis (2-nitrobenzoic acid; DTNB) (Danson and Weitzman, 1973, 1977; Talgoy and Duckworth, 1979). Indeed, DTNB has implicated thiol residues in NADH-inhibition of CSs from enteric bacteria, Azotobacter beijerinckii and some pseudomonads but not the CS from Acinetobacter or related organisms (Senior and Dawes, 1971; Weitzman and Danson, 1976; Danson and Weitzman, 1977; Morse and Duckworth, 1980).

Desensitisation studies have also been used to locate the NADH-binding site of the E. coli CS. Under limiting conditions, reaction of DTNB with a single thiol per subunit, desensitises this CS to NADH. The thiol is probably at the NADH-binding site because competitors of NADH binding prevent desensitisation of E. coli CS to NADH (Talgoy and Duckworth, 1979; Talgoy et al., 1979). Duckworth et al. (1987) extended these studies by modifying E. coli CS with a fluorescent thiol-alkylating reagent - mono-bromobimane. This had the same effect

on E. coli CS as DTNB, but the modification was stable enough for a peptide containing the alkylated cysteine to be isolated. It was deduced that this residue was cys 206. Duckworth et al. (1987) used the pig heart CS subunit structure as a model of the E. coli CS. This made a lot of assumptions about the 70% of the E. coli sequence that is not homologous with the pig heart CS. However, Duckworth et al. (1987) concluded that the distance of cys 206 from the active site would be large enough to show that NADH must interact with the active site of E. coli CS by a heterotropic mechanism. It is significant that the respective presence and absence of cys 206 in CSs from Pseudomonas aeruginosa and Acinetobacter anitratum reflects the ability of DTNB to desensitise only the former enzyme to NADH (Weitzman and Danson, 1976; Duckworth et al., 1987; Donald and Duckworth, 1987).

1.12.9.2. Observation and Restriction of Conformational Change. That the NADH-binding and active sites are separated makes it likely that NADH exerts its effects on CS via a conformational change. That this may occur on binding NADH has been concluded from thermal inactivation experiments. Consistent with the idea that ligand-induced protection of an enzyme against thermal inactivation is the result of a conformational change to a more stable structure (Citri, 1973), Weitzman (1981) has observed that NADH prevents thermal inactivation of CSs from Pseudomonas aeruginosa and A. calcoaceticus.

Rowe and Weitzman (1969) used electron microscopy to examine CS from A. calcoaceticus for NADH-induced conformational changes. NADH caused the enzyme's diameter to increase by 19-24%. The effect did not occur if AMP was also present. This is consistent with reversibility of NADH-inhibition by AMP. Further evidence that the NADH-swollen form of CS was that of the inhibited CS was the observation that no size change occurred if the enzyme was desensitised to NADH by 0.2-M KCl. Support for these results came from centrifugational studies; NADH caused an AMP reversible retardation of the sedimentation rate of CS.

NADH-induced conformational changes have been implicated by crosslinking studies. Mitchell and Weitzman (1983b) have shown that crosslinking of the subunits of A. calcoaceticus CS with a crosslinker that is cleavable by dithiothreitol (DTT) causes the sigmoidal

dependence of inhibition/reactivation on NADH/AMP to be converted to a hyperbolic one. Thus, crosslinking destroys homotropic interactions between NADH binding sites that cause sigmoid inhibition. Treatment of the crosslinked CS with DTT restored the co-operativity of NADH-inhibition, showing that conformational flexibility was important in the response to NADH. Further, Lloyd and Weitzman (1987) showed that crosslinking also prevents NADH-induced conformational changes of the A. calcoaceticus CS that effect thermal stabilisation. Again, the protective effect of NADH could be restored on cleavage of the crosslinks.

1.12.10. Mechanistic Features of the Biosynthetic Regulation of CS

That 2-OG-inhibition of E. coli CS was eliminated by raising the pH from 8 to 10 or by addition of 0.1 M-KCl suggested that 2-OG was an allosteric inhibitor of CS (Wright et al., 1967; Weitzman and Dunmore, 1969b). Similar findings were made with the Salmonella typhimurium, Thiobacillus denitrificans, and cyanobacterial CSs (Flechtner and Hanson, 1970; Taylor, 1970, 1973). However, Talgoy and Duckworth (1979) considered that 2-OG and OAA may compete for the same site and suggested that the apparent allosteric phenomena arose from an inability of the inactive ("T") state of E. coli CS to discriminate between 2-OG and OAA. Recent work suggests that this is more likely than the existence of a separate allosteric site for 2-OG. Duckworth et al. (1987) and Anderson et al. (1988) have changed residues by site-directed mutagenesis that are believed (from the porcine heart CS structure) to be involved in OAA binding or are in the vicinity of the OAA-binding site. Such substitutions (both His to Gln) desensitised CS to 2-OG, showing that 2-OG was likely to bind at the same site as OAA.

The inhibition of cyanobacterial CSs by succinyl-CoA has been studied by Lucas and Weitzman (1977). Inhibition is sigmoidally dependent on succinyl-CoA. Further, multiple-inhibition studies with bromoacetyl-CoA show the sites of interaction of succinyl-CoA and substrate are distinct. This led Lucas and Weitzman (1977) to suggest that cyanobacterial CSs are inhibited allosterically by succinyl-CoA.

1.12.11. Structural Aspects of the Allosteric Properties of CSs

CS subunits from all sources have a similar M_r value and yet allosteric properties are restricted to the large CS. This may indicate that such properties are related to differences in subunit arrangement instead of differences in subunits themselves. Evidence that this is the case has come from a number of quarters.

Tong and Duckworth (1975) found that at pH 9.0, the E. coli CS was dimeric. At this pH, E. coli CS had about 50% of the activity it had at the pH optimum, but the enzyme was desensitised to NADH (Weitzman, 1966b). Similarly, the marine pseudomonad studied by Higa et al. (1978) reversibly dissociates to an active dimeric form with a loss of NADH-inhibition. Upon reassociation, NADH sensitivity returns. Such observations suggest that NADH-inhibition of CS is a function of the way the dimers of the large CS associate to form the hexamer.

Weitzman and his colleagues have investigated the structure-function relationships of large CSs; spontaneous revertants (CS^+) of a CS deficient (CS^-) strain of E. coli were isolated. Two revertant CSs with altered properties were examined. One of these was a small CS and was identical to the small dimeric CSs. Similar mutant CSs have also been produced from CS^- strains of A. calcoaceticus (Weitzman et al., 1978; Harford and Weitzman, 1978; Danson et al., 1979). The E. coli CS revertant is analogous to the wild-type CS at pH 9.0 except that the mutant CS retains its small size under conditions where the native enzyme has a M_r typical of a large CS. The second mutant was a large NADH-insensitive CS resembling the Acetobacter xylinum CS (**1.12.7.3.**).

With regard to the size of CS and its regulation by NADH, it seems from the above that three CSs occur in Nature; large/NADH-sensitive (typical Gram-negative), large/NADH-insensitive (atypical Gram-negative Acetobacter-type) and small/NADH-insensitive (typical Gram-positive or eukaryotic). The fact that they are all accessible by mutation of one CS indicates a close relationship between the different types of CS. Further, the mutation work shows that the NADH-sensitivity of the large CS may result from dimer-dimer interaction. In this context, Duckworth and Tong (1976) have shown that E. coli CS maximally binds close to 0.5 mol-NADH per subunit.

1.12.12. CSs of Methylotrophs

1.12.12.1. Structural Properties. As no CS so far has ever been purified from a methylotroph, the quaternary structure of these CSs are unavailable. However, it seems that the taxonomic division obeyed by the large and small CSs can be extended to the methylotrophic CSs. Thus the Gram-negative methylotrophs Pseudomonas oleovorans, M. methylotrophus, Methylobacterium extorquens, and Hyphomicrobium X all have large CSs, while the Gram-positive methylotrophs Arthrobacter 2B2 and Organism S2A1 have small CSs (Anthony and Taylor, 1975; Cox and Quayle, 1976; Taylor, 1977; Babel and Müller-Kraft, 1979; Otto, 1986).

It was noted above that an extension to the general pattern of occurrence of bacterial CS characteristics in heterotrophs was the presence of both large and small CSs in the pseudomonads (**1.12.7.2.**). This also seems to be so for the methylotroph Pseudomonas oleovorans which, as well as a large isozyme, contains another CS of unknown M_r (Babel and Müller-Kraft, 1979). The variation of activities of these two CSs between methylotrophic and heterotrophic growth makes it unlikely that they result from reversible association or dissociation.

1.12.12.2. Regulatory and Kinetic Features of Methylotrophic CSs

1.12.12.2.1. Obligate Methylotrophs and Type M RFMs. In obligate methylotrophs and type M RFMs that use the RuMP cycle, as suggested by the OGDH lesion in these organisms (Table 3), the CAC is not involved in energy generation. This is a probable explanation of the observation that even though these organisms are Gram-negative (**1.6.**), their CSs are NADH-insensitive, e.g. M. methylotrophus, organisms 4B6, C2A1, W3A1 and the obligate type I methanotrophs Methylomonas P11 and Methylomonas albus BG8 (Colby and Zatman, 1975c; Taylor, 1977; Michalik et al., 1979; Aperghis, 1981; Otto, 1986).

Thus, M. methylotrophus deviates from the overall trends shown by bacterial CSs as it has a large CS that is not sensitive to NADH. This is reminiscent of the Acetobacter CS (**1.12.7.3.**). As M_r values for the 4B6, C2A1 and W3A1 CSs are unavailable, it is impossible to distinguish between the possibility that these enzymes are insensitive

to NADH because they are small or are large CSs that, like Acetobacter CS, are NADH-insensitive.

The type II methanotrophs use the serine pathway and are the only obligate methylotrophs with a complete CAC (**1.7.1.3., 1.8.1., 1.8.2., 1.8.3.**). Further, these methanotrophs may have to service the highest NADH requirements of all the methylotrophs. This led to speculation that the CAC may contribute to NADH synthesis in methanotrophic growth (**1.10.2.2.2.**). As the type II methanotrophs discussed here are Gram-negative, the foregoing would suggest that CS might be NADH-sensitive. However, CS from the obligate type II methanotroph Methylosinus trichosporium OB3B, has been found to be NADH-insensitive (Colby and Zatman 1975c).

The sensitivity of CSs from obligate methylotrophs to adenine nucleotides has also been studied. As with the other CSs, ATP is more inhibitory than ADP in obligate organisms Methylomonas albus BG8, 4B6, C2A1, Methylosinus trichosporium OB3B and M. methylotrophus (Colby and Zatman, 1975c; Aperghis, 1981). As discussed before (**1.12.6.1.1.**), it is unlikely that ATP would inhibit CSs from organisms with biosynthetically orientated OGDH-deficient CACs in vivo.

In view of the biosynthetic nature of the CAC of the RuMP cycle-utilising obligate methylotrophs and type M RFMs, the sensitivity of their CSs to 2-OG-inhibition was examined. The M. methylotrophus, 4B6, C2A1 and W3A1 CSs were all inhibited (up to 64%) by high concentrations (10 mM) of 2-OG. Perhaps significantly, the serine pathway utiliser Methylosinus trichosporium OB3B with its complete CAC was insensitive to 2-OG (Colby and Zatman, 1975c Aperghis, 1981). The significance of 2-OG-inhibition in vivo is unclear due to the large quantities of 2-OG required to give an effect (Colby and Zatman, 1975c). However, it should be noted that 2-OG-inhibition is likely to be competitive with OAA and so the physiological relevance of 2-OG-inhibition may depend partly upon OAA concentration.

1.12.12.2.2. Facultative Methylotrophs and Type L RFMs. Most CSs from these organisms obey the trends followed by their heterotrophic counterparts. Thus the obligately aerobic and Gram-negative organisms Pseudomonas 3A2, Hyphomicrobium X, Methylobacterium extorquens AM1 and

Organism 5B1 are all sensitive to AMP-reversible inhibition by NADH, while the Gram-positive organisms Arthrobacter 2B2, Bacillus PM6 and organism S2A1 are all NADH-insensitive (Anthony and Taylor, 1975; Colby and Zatman, 1975c; Otto, 1986; Paddon et al., 1985). An apparent exception to this trend is the facultative Pseudomonas J26 which has an NADH-insensitive CS (Michalik et al., 1979). The Methylobacterium extorquens AM1 CS has been studied in more detail than most (Anthony and Taylor, 1975). This CS has hyperbolic substrate dependences that become sigmoid in the presence of NADH. The allosteric nature of NADH-inhibition of this enzyme is shown by the observation that it is desensitised to NADH by 50 mM-KCl.

It is probable that in heterotrophic growth of facultative organisms, CS functions and is regulated in the same way as it is in typical heterotrophs. As the properties for the CSs from Pseudomonas 3A2, Hyphomicrobium X and organism 5B1 have been observed with CS from methylotrophically grown cells, NADH sensitivity is also a feature of CSs during methylotrophic growth. Zatman (1981) proposed that, as most C_1-grown facultative organisms have some OGDH activity, NADH-inhibition of CS is used to prevent wasteful carbon oxidation. It should, however, be noted that this proposal also applies to the obligate Methylosinus trichosporium OB3B CS (**1.12.12.2.1.**), which, contrary to what would be expected, is NADH insensitive.

A different situation occurs in Pseudomonas oleovorans. Like other pseudomonads, it is novel in that it has two CSs. During heterotrophic growth a large CS subject to NADH-inhibition is expressed. In C_1-growth, an NADH-insensitive CS is produced (Babel and Müller-Kraft, 1979). This counters the hypothesis of Zatman (1981) but he argues that as OGDH is totally suppressed in this organism during methylotrophy, an NADH-sensitive CS is not required.

1.13. Bacterial Isocitrate Dehydrogenases

1.13.1. The Discovery of IDH

Martius (1937, 1939) showed that biological citrate oxidation proceeded via D-isocitrate to 2-OG and CO_2 and suggested isocitrate

would be oxidised to the keto-acid oxalosuccinate, which was then assumed to undergo non-enzymic decarboxylation to 2-OG and CO_2. Alder et al. (1939) determined the stoichiometry of the reaction, showing the destination of electrons from isocitrate reduction to be $NADP^+$. Alder et al. (1939) also showed that the reaction required either Mg^{2+} or Mn^{2+}. Ochoa (1945) showed that the oxidation and decarboxylation reactions were reversible and enzymically catalysed by acetone powders of pig heart. Thus Ochoa (1945) concluded that isocitrate was oxidised to 2-OG and CO_2 in two reactions - the oxidation of a secondary alcohol (I) and the decarboxylation of a beta-keto acid (II):

(I) D_s-Isocitrate + $NADP^+$ ⟶ Oxalosuccinate + NADPH + H^+

(II) Oxalosuccinate + H^+ ⟶ 2-OG + CO_2

SUM: D_s-Isocitrate + $NADP^+$ $\xrightarrow{Mg^{2+}/Mn^{2+}}$ 2-OG + NADPH + CO_2

These were the first observations of net CO_2 fixation in heterotrophs.

Moyle and Dixon (1956) co-purified both activities from pig heart, finding them to be due to the same enzyme. This is known now as NADP-IDH (threo D_s-Isocitrate: $NADP^+$ Oxidoreductase (decarboxylating); EC 1.1.1.42). Moyle (1956) showed that the porcine enzyme catalysed the reverse reactions, i.e. the carboxylation of 2-OG and the NADPH-dependent reduction of oxalosuccinate. That the former reaction only took place if NADPH was present showed the close coupling of oxidation /reduction and decarboxylation/carboxylation and the intermediacy of oxalosuccinate in the conversion of isocitrate and 2-OG (Moyle, 1956).

That IDH could also use NAD^+ as an electron acceptor was shown when an NAD-linked IDH (threo D_s-Isocitrate: Oxidoreductase (decarboxylating); EC 1.1.1.41) was isolated from yeast (Kornberg and Pricer, 1951). This was followed by isolation of another NAD-IDH from mammalian mitochondria by Plaut and Sung (1954). Like the NADP-linked enzyme, the activity of both NAD-IDHs required a metal ion.

1.13.2. The Role of IDH

All bacteria that are not glutamate auxotrophs require IDH for

the same reason they require CS, that is, for the production of C_5 skeletons (2-OG). In addition, IDH has two other crucial roles in the CAC depending on the direction in which it turns. In the reductive CAC, IDH is crucial in CO_2 fixation and thus carbon assimilation. In the oxidative CAC, it functions catabolically in the oxidation of acetyl-CoA to CO_2. As will be discussed below, where IDH has the latter function during growth on C_2 compounds, it may compete for isocitrate with ICL, in which case regulation of one or other enzyme is required to divide isocitrate between the CAC or the glyoxylate cycle in the correct proportions to support growth (**1.13.8.**).

1.13.3. Coenzyme Specificity of Bacterial IDH

It is commonly held that bacterial IDHs are NADP-linked (Plaut, 1963). Indeed, a survey of the coenzyme usage of IDHs from 28 bacteria showed that, independent of Gram stain, in both obligate aerobes and facultative anaerobes, all but one (Xanthomonas pruni) used only $NADP^+$ (Ragland et al., 1966). However, it now appears that, although uncommon, the NAD-linked activity of IDH is more common amongst prokaryotes than was at first suspected.

There are two possible causes of dual coenzyme utilisation: 1) one enzyme uses NAD^+ and $NADP^+$; or 2) there are two IDHs, one NAD-specific and one NADP-specific. In a large number of cases it is not possible to distinguish between these two cases (**1.13.9.**)

1.13.3.1. Dual Coenzyme Utilisation by One (Dual-Specific) Enzyme. In contrast to early reports of Rhodopseudomonas spheroides and Azotobacter vinelandii NADP-IDHs being dual-specific (Chung and Franzen, 1969; Chung and Braginski, 1972), there have been three reports of dual coenzyme utilisation by bacterial IDHs where the purity of the $NADP^+$ and NAD^+ used was confirmed. The first report was made with the IDH from the archaebacterium Sulpholobus acidocaldarius. Although they did not purify the IDH to homogeneity, Danson and Wood (1984) showed the behaviour of NAD- and NADP-linked IDH during thermal inactivation, gel filtration, native PAGE and partial purification was identical. From this, and kinetic data, Danson and

Wood (1984) concluded that NAD-IDH and NADP-IDH activities were functions of the same protein. Using similar techniques, Leyland et al. (1989) came to a similar conclusion regarding the IDH from Rhodomicrobium vanielli. Finally, a homogeneous preparation of a dual-specific IDH was purified from the thermophilic bacterium Thermus thermophilus HB-8 by Eguchi et al. (1989). Thermus thermophilus, Sulpholobus acidocaldarius and Rhodomicrobium vanielli IDHs were 562, 87 and 89 times more specific for $NADP^+$ than NAD^+ (re-calculated from Danson and Wood, 1984; Leyland et al., 1989; Eguchi et al., 1989).

As K_m values for $NADP^+$ ranged from 6.3-30 μM, and K_m values for NAD^+ ranged from 0.75-3.4 mM (Danson and Wood et al. 1984; Leyland et al., 1989; Eguchi et al., 1989), it is likely that the NAD-linked activity of dual-specific IDHs is physiologically irrelevant. As of yet, no dual-specific IDH has been found that is predominantly NAD-linked or can use both coenzymes with equal efficiency.

1.13.4. Quaternary Structures of Bacterial NADP and Dual-Specific IDH

The native M_r values of bacterial NADP-IDHs fall into two groups. 'Group A' are enzymes with a native Mr of up to 120,000; 'Group B' have a native M_r of >300,000. Table 4 lists the IDHs in Group A. The enzymes in this group are from diverse sources, including Gram-negative, Gram-positive, archaebacterial, obligately aerobic and facultatively anaerobic organisms. The list includes psychrophilic, mesophilic and thermophilic organisms, acidophiles and alkalophiles.

Group B NADP-IDHs are isozymes, co-occurring with a Group A enzyme. Group B IDHs have been found in A. calcoaceticus (lwoffi), Bordetella bronchiseptica, Brevibacterium leucinophagum, Chromobacterium violaceum and Xanthomonas hyacinthi (Self and Weitzman, 1972; Kleber and Aurich, 1976; Weitzman, 1981). All are obligately aerobic Gram-negative organisms.

An exception to the above are the IDHs from the halophilic Halobacteriace (Hubbard and Miller, 1970; Aitken and Brown, 1972). The sizes of these IDHs are very dependent on KCl or NaCl concentrations.

The subunit values of Group A IDHs in Table 4 suggest that in Group A IDHs there are two types of subunit arrangement. Most Group A

Table 4

Native and Subunit M_r Values of Group A NADP-IDHs

Organism	Native M_r	Subunit M_r	Quaternary Structure	References
Salmonella typhimurium	102,000	46,000	Dimeric	Marr and Weber (1973) and Wang and Koshland (1982)
Escherichia coli	80,000; 97,000	42,000; 44,000	Dimeric	Reeves et al. (1972); Burke et al. (1974) and Borthwick et al. (1984)
Rhizobium meliloti	60,000-65,000	-	-	Chandrasekharan-Nambiar and Shethna (1976)
Halobacterium cutirubrum	75,000	-	-	Hubbard and Miller (1970)
Thermus aquaticus	60,000-70,000	-	-	Ramaley and Hudock (1973)
Bacillus subtilis	75,000-80,000	-	-	Ramaley and Hudock (1973)
Bacillus KSM-1050	90,000	44,000	Dimeric	Shikata et al. (1988)
Bacillus stearothermophilus	92,500	45,000	Dimeric	Howard and Becker (1970)
Azotobacter vinelandii	78,000-81,000	81,000	Monomeric	Chung and Franzen (1969)
Vibrio ABE-1				
Isoenzyme 1	88,100	49,100	Dimeric	Ishii et al. (1987)
Isoenzyme 2	80,500	79,500	Monomeric	Ishii et al. (1987)
Acinetobacter calcoaceticus				
Isoenzyme I	100,000	-	-	Self and Weitzman (1972)
Rhodopseudomonas spheroides	105,000	50,000	Dimeric	Chung and Braginsky (1972)
Sulpholobus acidocaldarius D*	96,000	-	-	Danson and Wood (1984)
Thermus thermophilus HB8 D*	95,000-120,000	57,500	Dimeric	Eguchi et al. (1989)

*: D signifies those enzymes that exhibit dual coenzyme specificity.

enzymes are dimers ('**Group A1**'), but the Azotobacter vinelandii enzyme and the Vibrio ABE-1 isoenzyme 2 are both monomeric ('**Group A2**').

The dimeric structure of Group A1 IDHs has been confirmed for the E. coli enzyme by covalent crosslinking (Burke et al., 1974). Where studied (E. coli and Vibrio ABE-1 isoenzyme 1), the Group A1 NADP-IDHs have identical subunits (Burke et al., 1974; Borthwick et al., 1984; Ishii et al., 1987; Thorness and Koshland, 1987).

The dual-specific IDHs in **1.13.3.1.** have quaternary structures identical to those of Group A1 (Table 4).

1.13.5. Substrate, Stereochemical and Metal Specificity of NADP-IDH

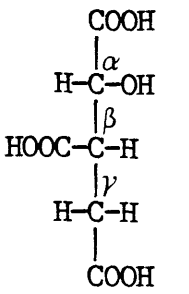
The prokaryotic NADP-IDHs catalyse: a) oxidation of the alpha carbon hydroxyl of isocitrate (Figure 11) to a ketone (oxalosuccinate; Figure 11); b) the transfer of reducing equivalents resulting from this reaction to the A side of the nicotinamide ring of $NADP^+$; c) the decarboxylation of oxalosuccinate in the beta-position (Figure 11) and d) the replacement of the departing CO_2 by a proton from the solvent such that its configuration in the product (2-OG; Figure 11) is identical to that of the carboxyl moiety it replaced (Chung and Franzen, 1970; Reeves et al., 1972).

Of the four isocitrate diastereoisomers (Figure 11; Vickery, 1962), the Salmonella typhimurium and Bacillus KSM-1050 NADP-IDHs are stereo-specific for the threo-D_s and inactive with the threo-L_s diastereoisomer (Marr and Weber, 1968; Shikata et al., 1988). As well as isocitrate, the Salmonella NADP-IDH decarboxylates oxalosuccinate (Marr and Weber, 1973). This shows the intermediacy of oxalosuccinate in the decarboxylation of isocitrate by bacterial NADP-IDHs.

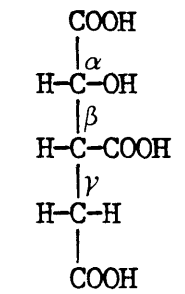
All IDHs require a divalent metal ion for activity. Generally, the prokaryotic NADP-linked IDH has a preference for Mn^{2+}. Thus the enzymes from Brevibacterium flavum and E. coli have much lower Kms for Mn^{2+} than Mg^{2+}, (Reeves et al., 1972; Ozaki and Shiio, 1968). Similar conclusions have been reached for NADP-IDHs from Bacillus KSM-1050, Mycobacterium phlei, A. calcoaceticus, Thermus aquaticus, Bacillus stearothermophilus, Bacillus subtilis, Thiobacillus novellus, Salmonella typhimurium and Rhizobium meliloti (Chandrasekharan-Nambiar

Figure 11

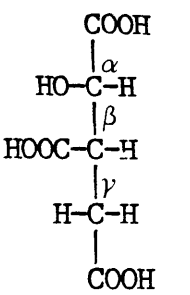
Substrate Diastereoisomers, Intermediates and Products of IDH



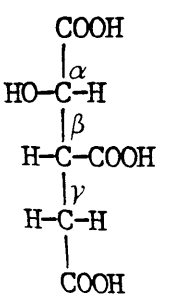
threo-D_s Isocitrate



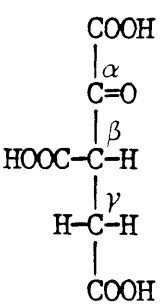
erythro-D_s Isocitrate



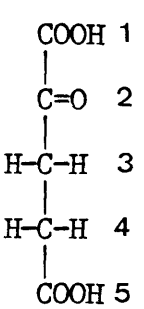
erythro-L_s Isocitrate



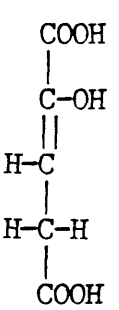
threo-L_s Isocitrate



Oxalosuccinate



2-OG



enol-2-OG

and Shethna, 1976; Dharwinal and Venkitasubramanian, 1987; Self and Weitzman, 1972; Howard and Becker, 1970; Charles, 1970; Shikata et al., 1988; Ramaley and Hudock, 1973; Marr and Weber, 1968). Further, the dual-specific IDH of Thermus thermophilus follows this trend (Eguchi et al., 1989). In contrast, Azotobacter vinelandii NADP-IDH is as active with Mg^{2+} as it is with Mn^{2+} (Chung and Franzen, 1969).

Other di-cations can partially replace Mn^{2+} or Mg^{2+}; [di-cation preference] Mycobacterium phlei [Mn>Mg>Zn], Thermus thermophilus [Mn>Mg>Ca>Sr>Co], Thiobacillus novellus [Mn>Co>Zn>Mg>Ni>Pb>Ca] and Salmonella typhimurium [Mn>Mg>Co] (Charles, 1970; Marr and Weber, 1968; Dharwinal and Venkitasubramanian, 1987; Eguchi et al., 1989). Apart from the preference for Mn^{2+}, the order of reactivity of ions is variable. This order may be determined by the relative stability of metal:isocitrate chelate or the affinity of NADP-IDH for a chelate.

In the pH range expected in vivo, isocitrate (pKa 5.75) is in its di- and tri-basic forms and (with M^{2+}) metal chelates thereof. Thus, a question arises about the actual substrate of NADP-IDH. For Salmonella typhimurium and Azotobacter vinelandii NADP-IDHs this is the isocitrate:metal chelate (Wicken et al., 1972; Marr and Weber, 1973).

1.13.6. Substrate Binding and Analysis of the Active Site of NADP-IDH

Ligand binding and kinetic studies show that NADP-IDHs are saturated hyperbolically with isocitrate and $NADP^+$ and obey sequential mechanisms with ordered (E. coli, Rhodopseudomonas spheroides; $NADP^+$ first) or random (Azotobacter vinelandii, Salmonella typhimurium) addition of isocitrate and $NADP^+$ (Wicken et al., 1972; Marr and Weber, 1973; Buzdygon et al., 1973; Nimmo, 1986). E. coli NADP-IDH and Azotobacter vinelandii NADP-IDH have one binding site per subunit for $NADP^+$ and isocitrate (Wicken et al., 1972; Garland and Nimmo, 1984). However, the dimeric Rhodopseudomonas spheroides enzyme binds one $NADP^+$ per dimer (Buzdygon et al., 1973), but if this due to mutually exclusive binding at two sites is unknown.

1.13.6.1. Amino Acid Residues Involved in Catalysis

(i) Cysteine. All enzymes tested are inactivated by DTNB, iodoacetate

(IAA), N-ethyl maleimide (NEM) or para-chloromercuribenzoate (PCMB) (see Table 5). It should be noted that sometimes the specificity of IAA is uncertain (see (ii) below). Protection of activity by isocitrate is enhanced by or requires Mn^{2+} or Mg^{2+}, e.g. the NADP-IDHs from Mycobacterium phlei and Bacillus stearothermophilus (Howard and Becker, 1970; Nagaoka et al., 1977; Dhariwal and Venkitasubramanian, 1987). This is consistent with the preferential binding of the metal: isocitrate chelate to the enzyme (**1.13.5.**) and shows that thiol(s) may be involved in isocitrate binding. KCN can displace thionitrobenzoate (TNB) from DTNB-inactivated Azotobacter IDH with incorporation of cyanide (Chung et al., 1971) and recovery of activity. This shows the thiol residue is in the vicinity of the active site but not crucial to its operation.

(ii) Methionine. When Azotobacter NADP-IDH was inactivated with [2-^{14}C]IAA, carboxymethylcysteine was not labelled but a product of carboxymethylmethionine degradation - carboxymethylhomocysteine was (Edwards et al., 1974). Thus, methionine may be associated with the active site of Azotobacter NADP-IDH in some way.

(iii) Arginine. Modification of an arginyl residue by phenylglyoxal inactivates the E. coli enzyme. This effect is slowed only by $NADP^+$. It has been suggested this group may neutralise the negative charges of the $NADP^+$ phosphates (McKee and Nimmo, 1989).

1.13.7. Isoenzymes

The first bacterium found with more than one NADP-IDH was glucose-grown E. coli. The two active forms differed in elution from CM-Sephadex and stability in 2M-urea and were separable by isoelectric focussing (Reeves et al., 1968; Vasquez and Reeves, 1981). Both forms had an identical native M_r. As E. coli NADP-IDH has identical subunits (**1.13.4.**), a possible explanation of the data of Vasquez and Reeves (1981) may be that the isoenzymes may arise from more than one set of intra-subunit interactions, leading to differing exposure of charged amino acids on the enzyme. The role of these isenozymes is unclear.

A. calcoaceticus has two isoenzymes (Reeves et al., 1986, Self and Weitzman, 1970, 1972; O'Neil and Weitzman, 1988). The isoenzymes

Table 5

Inactivation of Group A NADP-IDHs by Chemical Modification of Thiol Residues

Organism	Reagent	Prevention of Inactivation by:		References
		Isocitrate	NADP	
Bacillus stearothermophilus	PCMB	Yes	No	Howard and Becker (1970)
	DTNB	Yes	No	Nagaoka et al. (1977)
Azotobacter vinelandii	DTNB, PCMB, NEM, IAA	Yes	Yes	Chung and Franzen (1969)
Rhizobium meliloti	DTNB, PCMB, NEM, IAA	Yes	Yes	Chandrasaekharan-Nambiar and Shethna (1976)
Bacillus KSM-1050	NEM, PCMB	?	?	Shikata et al. (1988)
Thermus thermophilus	PCMB	?	?	Eguchi et al. (1989)
Thiobacillus novellus	PCMB, NEM	Yes	Yes	Charles et al. (1970)
Rhodopseudomonas spheroides	DTNB, PCMB, NEM	?	?	Chung and Braginski (1972)
Mycobacterium phlei	PCMB	Yes	Yes	Dhariwal and Venkitasubramanian (1987)

differ in M_r (**1.13.4.**, Table 4) and regulation (**1.13.8.**). Isoenzyme II is a Group B IDH and Isoenzyme I is a Group A IDH. The isoenzymes have different pH optima and sensitivities to inactivation by urea and heat (Self and Weitzman, 1972). Isoenzyme II has regulatory properties that may make it responsive to the operation of the glyoxylate cycle (see **1.13.8.**). In accord with this, growth on acetate increases the isoenzyme II:I ratio (Reeves et al., 1986).

The psychrophile Vibrio ABE-1 contains two isoenzymes that are separable by hydrophobic interaction chromatography. Isoenzyme 1 is a Group A1 dimer, Isoenzyme 2 is a Group A2 monomer (Ishii et al., 1987). Although amino acid compositions are similar, the IDHs differ in their pH optima, temperature optima and immunology (Ochiai et al., 1979; Ishii et al., 1987). The isoenzyme 1 activity is increased by acetate (c.f. Acinetobacter IDH isoenzymes; Ishii et al., 1987), although why this isoenzyme is more heavily expressed is unclear.

1.13.8. Regulation of Bacterial NADP-IDH

1.13.8.1. ATP-Inhibition. If the CAC is involved in energy production then it may be expected that IDH may be inhibited by ATP. This seems to be so for a variety of organisms (see Table 6). In some cases e.g. Bacillus KSM-1050 (Shikata et al., 1988), Azotobacter vinelandii (Chung and Franzen, 1969), Bacillus stearothermophilus (Hibino et al., 1974) and Rhizobium meliloti (Chandrasekharan-Nambiar and Shethna, 1976), the enzymes are not very sensitive to ATP and it seems unlikely that they are affected by ATP in vivo. Except for the Alcaligenes and Acinetobacter enzymes, the IDHs in Table 6, are also inhibited by ADP and AMP (ADP> AMP) to a lesser extent than ATP.

As suggested by the structrual similarity between ATP and $NADP^+$, ATP is a competitive inhibitor with respect to $NADP^+$. This inhibition is also competitive with respect to isocitrate (Table 6). This latter effect in the Salmonella typhimurium NADP-IDH may be due to chelation of the metal ion by ATP which may reduce the concentration of the true substrate. Further, the pyrophosphate of ATP may also act as a conventional competitive inhibitor with respect to isocitrate (Marr and Weber, 1968). The combination of these effects may cause non-

Table 6

Inhibition of Prokaryotic NADP-IDH by ATP

Organism	Type of Inhibition With Respect to:		References
	NADP	Isocitrate	
Bacillus stearothermophilus	N/D*	N/D	Hibino et al. (1974)
Azotobacter beijerinkii	N/D	N/D	Senior and Dawes (1971)
Acinetobacter calcoaceticus	Competitive	Competitive	Kleber and Aurich (1976)
Alcaligenes eutropha	N/D	Competitive	Glaeser and Schlegel (1972a)
Rhizobium meliloti	N/D	N/D	Chandrasekharan-Nambiar and Shethna (1976)
Azotobacter vinelandii	N/D	N/D	Chung and Franzen (1969)
Thiobacillus novellus	N/D	Competitive	Charles (1970)
Bacillus KSM-1050	Competitive	Competitive	Shikata et al. (1988)
Salmonella typhimurium	Competitive	Competitive	Marr and Weber (1968)
Pseudomonas flourescens	N/D	Competitive	Hampton and Hanson (1969)

*: Not Determined.

linearity of secondary plots of ATP-inhibition at varying isocitrate (Marr and Weber, 1968). This has also been observed with the A. calcoaceticus IDH isoenzyme II (Kleber and Aurich, 1976).

In connection with the effect of ATP on Acinetobacter IDH isoenzyme II, Parker and Weitzman (1970) found this enzyme to be inhibited by high energy charge. However, in view of the effects of adenine nucleotides on M^{2+} concentrations, this inhibition may not solely be due to increasing energy charge.

1.13.8.2. Concerted Inhibition by Glyoxylate and OAA. Shiio and Ozaki (1968) recognised the need for a control to correctly apportion acetate into the CAC and glyoxylate cycle in accord with growth requirements. It was found that the NADP-IDH from Brevibacterium flavum was subject to concerted inhibition by OAA and glyoxylate. As the organism's ICL was inhibited by mixtures of CAC carboxylates, Ozaki and Shiio (1968) suggested in vivo, ICL-generated glyoxylate and OAA would inhibit IDH and allow the glyoxylate cycle to function until enough CAC intermediates were formed to inhibit ICL and so terminate inhibition of IDH. However, all IDHs tested were inhibited by OAA and glyoxylate (Table 7). This has provided a strong case against the operation of this effect. Mammalian tissues have no glyoxylate cycle, yet porcine NADP-IDH is inhibited by OAA and glyoxylate. Inhibition by OAA and glyoxylate is competitive with isocitrate (Shiio and Ozaki, 1968; Chandrasekharan-Nambiar and Shethna, 1976; Johanson and Reeves, 1977; Nimmo, 1986). As the ratio ([OAA]+[glyoxylate]):[isocitrate] in vivo in acetate-grown E. coli may be very small (Nimmo, 1986), OAA and glyoxylate probably do not regulate IDH.

1.13.8.3. Nucleotide Activation. AMP and to a lesser extent ADP activates the Group B NADP-IDH (isoenzyme II) from A. calcoaceticus up to thirty-fold (Parker and Weitzman, 1970; O'Neil and Weitzman, 1988). The dependence of activation on AMP or ADP is hyperbolic, a half-maximal effect is attained by about 0.18 mM-AMP (Parker and Weitzman, 1970). AMP mainly affects Vmax. NADP-IDH from Alcaligenes eutropha H16 is also activated by AMP. However, here, activation is due to a reduction in the Km for $NADP^+$ (Glaeser and Schlegel, 1972a). Such

Table 7

The Occurance of Concerted Inhibition of NADP-IDH by OAA + Glyoxylate.

Organism/Source	Reference
Bacillus KSM 1050	Shikata et al. (1988)
Bacillus stearothermophilus	Hibino et al. (1974)
Rhizobium meliloti	Chandrasekharan-Nambiar and Shethna (1976)
Brevibacterium flavum	Shiio and Ozaki (1968); Ozaki and Shiio (1968)
Pseudomonas flourescens	Hampton and Hanson (1969)
Acinetobacter calcoaceticus	
Isoenzymes I and II	Self et al. (1973)
Mycobacterium phlei	Dharwinal and Venkitsubarmanian (1987)
E. coli	Johanson and Reeves (1977); Nimmo (1986)
Salmonella typhimurium	Marr and Weber (1968)
Alcaligenes eutropha	Glaeser and Schlegel (1972a)
Thiobacillus novellus	Charles (1970)
Vibrio ABE-1	
Isoenzymes 1 and 2	Ochiai et al. (1979)
Bacillus subtilis	Ramaley and Hudock (1973)
Thermus aquaticus	Ramaley Hudock (1973)
Halobacterium cutirubrum	Hubbard and Miller (1970)
Porcine Heart	Shiio and Ozaki (1968)

stimulatory effects by AMP (an indicator of low energy status in the cell) on the CAC would suggest that these IDHs are involved with and regulate the catabolic function of the CAC.

How AMP stimulates Acinetobacter IDH is unknown; however, it is subject to marked hysteresis which is abolished by AMP (O'Neil and Weitzman, 1988), suggesting that AMP promotes an active conformation of the enzyme. AMP-induced conformational changes may also be evidenced from the ability of AMP to modify the thermal stability of the enzyme (Parker and Weitzman, 1970). The ability of urea to desensitise the IDH to AMP (Self et al., 1973) suggests the site of interaction of AMP with the enzyme is distinct from its active site.

1.13.8.4. Pyruvate and Glyoxylate Activation. In addition to AMP, glyoxyate and pyruvate stimulate Acinetobacter isoenzyme II. The dependence of activation upon pyruvate and glyoxylate is hyperbolic, half-maximal activation occurring at 15 μM for both activators (Self et al., 1973). In contrast to AMP, glyoxylate and pyruvate affect both Vmax and Km for isocitrate and $NADP^+$. Again, as for AMP, pyruvate and glyoxylate may not bind at the active site (Self et al., 1973). It is also probable that glyoxylate and pyruvate bind at a site distinct from the AMP-binding site (Self et al., 1973). The properties of Acinetobacter isoenzyme (II) may allow it to respond both to energy demands made on the CAC (AMP-activation) and the glyoxylate cycle. In the latter case a rise in levels of glyoxylate and (perhaps more importantly) pyruvate show the glyoxylate cycle is actively synthesising C_4-precursors. Thus, flux is diverted back through the CAC to provide energy for biosynthesis.

1.13.8.5 Covalent Control of NADP-IDH. Addition of acetate to glucose-grown E. coli causes a fall in NADP-IDH activity that is reversed on exhaustion of the acetate (Bennett and Holms, 1975). This fall and rise in activity coincides with phosphorylation and dephosphorylation of IDH (Garnak and Reeves (1979a,b; Wang and Koshland, 1982). The kinase and phosphatase (IDH-K/P) that does this was purified from E. coli (LaPorte and Koshland, 1982). The same protein and active site catalyse kinase and phosphatase reactions (LaPorte and Chung, 1985;

Varela and Nimmo, 1988; Stueland et al., 1989). IDH-K/P transfers a gamma-phosphoryl group of ATP to a serine residue in the $NADP^+$ binding site and stops $NADP^+$ binding. The phospho-enzyme is inactive (Nimmo et al., 1984; LaPorte and Koshland, 1983; Nimmo and Craig, 1989).

The significance of these observations is related to the need to correctly apportion carbon between the CAC and the glyoxylate cycle during growth on acetate. The K_m values for isocitrate of ICL and IDH would indicate that most acetate would enter the CAC. However, inactivation of IDH by phosphorylation allows isocitrate to enter the glyoxylate cycle (Nimmo, 1987; Nimmo et al., 1987). As the glyoxylate cycle and the CAC are needed during growth on acetate, it is likely the IDP-K/P is regulated. Thus compounds that result from glyoxylate cycle activity ($C_{>2}$ compounds) and signals of a low cellular energy status (ADP and AMP) activate IDH by phosphatase activation and kinase inhibition of IDH-K/P (Nimmo and Nimmo, 1984). Of these metabolites, in vivo, isocitrate and pyruvate may be important (El-Mansi et al., 1985, 1986). It is of interest that similar effectors may control the activities of both Acinetobacter and E. coli IDH.

1.13.9. Bacterial NAD-Linked IDHs

The scope of the topic discussed here is limited to reports of NAD-IDH not specifically known to arise from the dual specificity of one IDH (Table 8). Most of the organisms in Table 8 are C_1-utilisers. Only one NAD-IDH has a Gram-positive source. The C_1-utilisers are all obligate methylotrophs or obligate and facultative autotrophs.

Most organisms have a NADP-IDH as well as the NAD-enzyme but, for most of these, it is impossible to tell if this is due to one dual-specific or two mono-specific IDHs. However, NADP-IDH and NAD-IDHs from the obligate methylotroph organism W6 (Hofmann and Babel, 1980), the facultative autotroph Alcaligenes H16 and the heterotroph Acetobacter peroxydans (Glaeser and Schlegel, 1972a; Hathaway and Atkinson, 1963) have been separated from each other. The former example will be dealt with in **1.13.10.** That the use of NAD^+ and $NADP^+$ by IDH in Alcaligenes H16 and other facultative autotrophs must be due to separate enzymes was also evident from the differential levels of

Table 8

The Occurrence of NAD-Linked IDH

Organism	Gram Stain	Co-occurrence of NADP-IDH	Evidence of Separate NAD-IDH and NADP-IDH	Nutritional Features	References
C2A1, W3A1, W6A	A11 -	Yes	Unknown	Methylotroph	Colby and Zatman (1975a)
4B6	-	Yes	Unknown	Methylotroph	Colby and Zatman (1972)
Methylomonas methanica	-	Yes	Unknown	Methylotroph	Davey *et al.* (1972)
Methylomonas albus	-	Yes	Unknown	Methylotroph	Davey *et al.* (1972)
Methylococcus minimus	-	Yes	Unknown	Methylotroph	Davey *et al.* (1972)
Methylococcus capsulatus [Bath]	-	No	Not Applicable	Methylotroph	Davey *et al.* (1972)
Methylococcus capsulatus [Texas]	-	No	Not Applicable	Methylotroph	Patel *et al.* (1975)
M. methylotrophus	-	Yes	Unknown	Methylotroph	This Thesis (Chapter 3)
Pseudomonas W6	-	Yes	Yes	Methylotroph	Hofmann and Babel (1980)
Methylobacter bovis	-	Yes	Unknown	Methylotroph	Trotsenko (1976)
Methylobacter chroococum	-	Yes	Unknown	Methylotroph	Trotsenko (1976)
Methylobacter vinelandii	-	Yes	Unknown	Methylotroph	Trotsenko (1976)
Thiobacillus thio-oxidans	-	No	Not Applicable	Autotroph	Matin and Rittenberg (1971)
Thiobacillus neapolitanus	-	Yes	Unknown	Autotroph	Matin and Rittenberg (1971)
Thiobacillus thioparus	-	Yes	Unknown	Autotroph	Matin and Rittenberg (1971)
Thiobacillus novellus	-	Yes	Yes	Autotroph	Matin and Rittenberg (1971)
Thiobacillus perometabolis	-	Yes	Unknown	Autotroph	Matin and Rittenberg (1971)
Thiobacillus intermedius	-	Yes	Yes	Autotroph	Matin and Rittenberg (1970)
Alcaligenes eutropha H16	-	Yes	Yes	Autotroph	Glaeser and Schlegel (1972a)
Thiobacillus ferro-oxidans	-	Yes	Yes	Autotroph	Tabita and Lundgren (1971)
Acetobacter peroxydans	-	Yes	Yes	Heterotroph	Hathaway and Atkinson (1963)
Acetobacter suboxydans	-	No	Not Applicable	Heterotroph	Greenfield and Claus (1969)
Acetobacter aceti	-	Yes	Unknown	Heterotroph	Greenfield and Claus (1969)
Streptococcus bovis	+	No	Not Applicable	Heterotroph	Burchall *et al.* (1964)
Xanthomonas pruni	-	Yes	Unknown	Heterotroph	Ragland *et al.* (1966)

Table 8 (Continued)

Organism	Gram Stain	Co-occurrence of NADP-IDH	Evidence of Separate NAD-IDH and NADP-IDH	Nutritional Features	References
Rhizobium species	-	Untested	-	Heterotroph	Moustafa and Leong (1975)
Desulphovibrio vulgaris	-	Untested	-	Heterotroph	Lewis and Miller (1975)
Desulphovibrio desulphuricans	-	Untested	-	Heterotroph	Lewis and Miller (1975)

their activities in autotrophy and heterotrophy (Glaeser and Schlegel, 1972b; Tabita and Lundgren, 1971; Matin and Rittenburg, 1970).

1.13.9.1. Physiological Significance of NAD-IDH. NAD-IDH is uncommon amongst the prokaryotes (**1.13.3.**). Its presence may indicate an excessive requirement for NADH. Suggestions of factors that may put a premium upon NADH-generating reactions can be made for most organisms in Table 8. This includes the methylotrophs (**1.13.10**).

All the autotrophs in Table 8 are OGDH-deficient or have very low levels of OGDH in C_1 growth (Smith et al. 1967; Matin and Rittenberg, 1970; Kelly, 1971; Tabita and Lundgren, 1971; Charles, 1971; Smith and Hoare, 1977). This could reduce the level of NADH, but may not be a sole cause for a requirement of a NAD-IDH as the cyanobacteria (Pearce and Carr, 1967) and anaerobically grown facultative anaerobes (e.g. E. coli) all have OGDH lesions but only have NADP-IDHs.

There are other aspects of autotrophic metabolism that may benefit from NAD-IDH. First, NAD-IDH in, e.g. the Thiobacilli may spare ATP used to make NADH by reversed electron transport (Friedrich, 1985; Jones, 1985). Secondly, the RubP cycle may have a high demand for NADH (**1.7.1.**; Anthony, 1982). Whatever the role of NAD-IDH in autotrophs, the rise in NAD-IDH and fall in NADP-IDH that accompany transitions between autotrophy and heterotrophy in some autotrophs (**1.13.9.**) suggest that this role is relevant to autotrophic growth.

It should be noted that as NAD-IDH occurs in essentially OGDH deficient CACs, its use to generate NADH will lead to stoichiometric and wasteful accumulation of 2-OG. Thus, it is unlikely that NAD-IDH alone would completely supply the demands of an organism for NADH, but it may be part of the capacity of the organism to generate NADH.

1.13.9.2. Characteristics of Bacterial NAD-IDHs. As well as the obligate methylotroph W6 (**1.13.10.**), NAD-IDHs from the obligate autotroph Thiobacillus thio-oxidans, the facultative autotroph Alcaligenes eutropha, and heterotrophs Rhizobium meliloti and Acetobacter peroxydans have been studied (Hampton and Hanson, 1968; Glaeser and Schlegel, 1972a; Hathaway and Atkinson, 1963; Moustafa and Leong, 1975). All these NAD-IDHs had hyperbolic dependences on

isocitrate and NAD^+. Acetobacter NAD-IDH fulfils its metal ion requirement with Mn^{2+} and/or Mg^{2+}. Co^{2+} and Zn^{2+} are also used to a lesser extent. This IDH is unable to decarboxylate oxalosuccinate .

Apart from the Acetobacter peroxydans enzyme, all NAD-IDHs above and Acetobacter suboxydans IDH (Greenfield and Claus, 1969) are inhibited by adenine nucleotides (Hathaway and Atkinson, 1963; Hampton and Hanson, 1968; Glaeser and Schlegel, 1972a; Moustafa and Leong, 1975). In all cases, ATP is a competitive inhibitor with respect to NAD^+ and, like NADP-IDH, Alcaligenes and Thiobacillus IDHs are inhibited competitively with respect to isocitrate (Hampton and Hanson, 1968; Glaeser and Schlegel, 1972a; Hathaway and Atkinson, 1963; Moustafa and Leong, 1975). As the CAC may be biosynthetic here, the relevance of ATP-inhibition is unclear. In this context, if low-energy conditions prevail (raised [ADP]), biosynthetic pathways may be inhibited. That this may be so for the CAC of Thiobacillus thio-oxidans is shown by the fact that its NAD-IDH has a 10-fold lower K_i for ADP than it does for ATP (Hampton and Hanson, 1969).

Both the Alcaligenes and Thiobacillus NAD-IDHs are inhibited by OAA and glyoxylate. This behaviour resembles that displayed by NADP-IDHs (Table 7). Again the physiological relevance of OAA+glyoxylate-inhibition is uncertain, as labelling studies (Smith et al., 1967) suggest that Thiobacillus thio-oxidans has no glyoxylate cycle.

1.13.10. IDH From Methylotrophs

All methylotrophs except Methylococcus capsulatus have an NADP-IDH (Table 9). In agreement with the foregoing (**1.13.9.1.**), NAD-IDH occurs only in methylotrophs with a OGDH lesion. Thus, NAD-IDH is found in those obligate methylotrophs and type M RFMs that use the RuMP cycle of C_1-assimilation. The IDHs of serine pathway and facultative RubP-cycle utilisers are solely NADP-linked (Table 9). This trend extends to the obligately methylotrophic type II methanotrophs which have a complete CAC and contain solely NADP-linked activities (Table 9). An exception to this is Pseudomonas oleovorans although, unlike other facultative organisms, it has no OGDH during methylotrophic growth (Anthony, 1982; Table 3)

Table 9

The Occurrence of NADP and NAD-linked IDH Amongst Methylotrophs

Organism	Type of Methylotroph	NAD-IDH	NADP-IDH	Presence of OGDH	C_1-Assimilation Cycle/Pathway	References
Methylomonas methanica	Obligate (I)*	+*	+	−*	RuMP	Davey et al. (1972)
Methylomonas albus	Obligate (I)	+	+	−	RuMP	Davey et al. (1972)
Methylomonas minimus	Obligate (I)	+	+	−	RuMP	Davey et al. (1972)
Methylococcus capsulatus [Bath]	Obligate (I)	+	−	−	RuMP	Davey et al. (1972)
Methylococcus capsulatus [Texas]	Obligate (I)	+	−	−	RuMP	Patel et al. (1975)
Methylobacter bovis	Obligate (I)	+	+	−	RuMP	Trotsenko (1976)
Methylobacter chroococum	Obligate (I)	+	+	−	RuMP	Trotsenko (1976)
Methylobacter vinelandii	Obligate (I)	+	+	−	RuMP	Trotsenko (1976)
Organism W6	Obligate	+	+	−	RuMP	Hoffman and Babel (1980)
C2A1 and 4B6	Obligate	+	+	−	RuMP	Colby and Zatman (1975a)
M. methylotrophus	Obligate	+	+	−	RuMP	This thesis
W3A1 and W6A	Type M RFM	+	+	−	RuMP	Colby and Zatman (1975a)
S2A1 and PM6	Type L RFM	−	+	+	RuMP	Colby and Zatman (1975a)
Arthrobacter 2B2	Facultative	−	+	+	RuMP	Paddon et al. (1985)
Methylobacterium extorquens AM1	Facultative	−	+	+	Serine	Taylor and Anthony (1976a)
Pseudomonas 3A2	Facultative	−	+	+	Serine	Colby and Zatman (1972)
Pseudomonas 5B1	Facultative	−	+	+	Serine	Colby and Zatman (1972)
Pseudomonas MA	Facultative	−	+	+	Serine	Colby and Zatman (1972)
Hyphomicrobium X	Facultative	−	+	+	Serine	Attwood and Harder (1974)
Methylosinus trichosporium OB3B	Obligate (II)*	−	+	+	Serine	Davey et al. (1972)
Methylosinus trichosporium PG	Obligate (II)	−	+	+	Serine	Davey et al. (1972)
Methylosinus sporium	Obligate (II)	−	+	+	Serine	Davey et al. (1972)
Methylocystis parvus	Obligate (II)	−	+	+	Serine	Davey et al. (1972)
Achromobacter 1L	Facultative	−	+	+	RubP	Loginova and Trotsenko (1979a)

Table 9 Continued

Organism	Type of Methylotroph	NAD-IDH	NADP-IDH	Presence of OGDH	C_1-Assimilation Cycle/Pathway	References
Pseudomonas 8	Facultative	-	+	+	RubP	Loginova and Trotsenko (1979a)
Mycobacterium 50	Facultative	-	+	+	RubP	Loginova and Trotsenko (1979a)

*: +=Presence of Enzyme; -=Absense of enzyme; (I)=Type I Methanotroph; (II)=Type II Methanotroph

It has previously been argued that, as prokaryotic NAD-IDHs are rare, there may be aspect(s) of the organism's metabolism that would benefit from NAD-IDH activity (**1.13.9.1.**). This may also be so for the methylotrophs. For the type I methanotrophs the drop in availability of NADH caused by an OGDH-deficient CAC and the high demand of NADH for methane hydroxylation (**1.7.2.1.**) may be partly ameliorated by NAD-IDH. As type II methanotrophs have a complete CAC that may function to supply methane hydroxylation with NADH (**1.10.2.2.2.**), NAD-IDH may not be needed and thus is absent from these organisms. For other RuMP cycle-utilising obligate methylotrophs and type M RFMs in Table 9 (M. methylotrophus, organisms W6, C2A1, W3A1 and W6A) there is not such an obvious reason why NAD-IDH activity is present.

For most organisms with NAD- and NADP-IDHs it remains to be seen whether the use of both coenzymes is due to one dual- or two mono-specific enzymes. The exception is organism W6 (Babel and Hofmann, 1980) where the two enzymes have been purified from each other.

1.13.10.1. Regulatory Properties of Methylotrophic IDHs. Two methylotrophs have been studied with regard to their regulation:

The facultative serine pathway utiliser Methylobacterium extorquens AM1 has an NADP-IDH that like other NADP-IDHs, is inhibited by OAA and glyoxylate (Anthony, 1982). As with other organisms (**1.13.8.2.**), the in vivo relevance of this effect is unclear. These observations do raise an interesting point. In methylotrophic growth, use of the icl^+ variant of the serine pathway (**1.7.1.3.**) depends on ICL in order to regenerate the C_1-acceptor. Thus the branch-point between ICL and IDH that this type of methylotroph has suggests that it shares problems similar to those of the enteric bacteria during growth on acetate (**1.13.8.5.**). However, how flux between ICL and IDH is controlled in methylotrophs is unclear.

Organism W6 has an NAD- and an NADP-IDH. The former, like other NAD-IDHs inhibited by adenine nucleotides. The NADP-IDH is inhibited by 2-OG and NADPH and is insensitive to adenine nucleotides. This led Hofmann and Babel (1980) to conclude that the CAC in W6 is amphibolic (even though it has an OGDH lesion), the NAD-IDH contributing to energy generation and the NADP-IDH being biosynthetic. If this is the

case or not is debatable. Analogy with similar obligate methylotrophs, e.g. M. methylotrophus, suggest that, compared with the enzymes of the dissimilatory version of the RuMP cycle (glucose 6-phosphate dehydrogenase and 6-phospho-gluconate dehydrogenase), NAD-IDH is an insignificant source of NADH (Large and Haywood, 1981; Beardsmore et al., 1982). Further, obligate methylotrophs oxidise NADH and NADPH to generate ATP (Anthony, 1982); thus here, the separation of function of the two IDHs by Babel and Hofmann (1980) may not be valid.

SECTION C

AIMS

The organism whose CAC is the subject of this thesis is Methylophilus methylotrophus strain AS1. This is an obligately aerobic, Gram-negative (virtually) obligate methylotroph that uses the RuMP cycle of C_1 assimilation and the cyclic derivative of the latter for carbon dissimilation (Large and Haywood, 1981; Beardsmore et al., 1982; Jenkins et al., 1987).

Imperial Chemical Industries plc has shown a great deal of interest in the large-scale growth of this organism on methanol because this provides an efficient system for large-scale production of crude cell protein (Senior and Windass, 1980; Smith, 1981).

The growth of methylotrophs that use the RuMP cycle on methanol is ATP-limited (Anthony, 1978, 1982). Thus, it was considered that replacement of steps in the organism's metabolism with those that use less or no ATP may increase methanol conversion to cell material. M. methylotrophus assimilates nitogen from ammonia via glutamine synthetase (GS; i) and glutamate synthase (GOGAT; ii);

(i) $\text{Glutamate} + NH_3 + \text{ATP} \longrightarrow \text{Glutamine} + \text{ADP} + \text{Pi}$

(ii) $\text{Glutamine} + \text{2-OG} + \text{NAD(P)H} + H^+ \longrightarrow 2\ \text{Glutamate} + \text{NAD(P)}^+$

Sum: $\text{2-OG} + NH_3 + \text{ATP} + \text{NAD(P)H} + H^+ \longrightarrow \text{Glutamate} + \text{ADP} + \text{Pi} + \text{NAD(P)}^+$

However, glutamate dehydrogenase (GDH) catalyses the same overall reaction without the expenditure of ATP:

$$2\text{-OG} + NH_3 + NAD(P)H + H^+ \longrightarrow \text{Glutamate} + NAD(P)^+$$

Thus, the E. coli GDH gene was cloned into a M. methylotrophus strain that was mutated so that it could not express GOGAT at the normal growth temperature (37°C) of the organism. M. methylotrophus was now reliant on GDH for nitrogen assimilation (Windass et al., 1980).

These experiments directed attention to the basis of this project, for whatever pathway was used to fix ammonia, the CAC provided the carbon skeleton for this purpose. In other words, the CAC is crucial to the supply of 2-OG for nitrogen assimilation. Thus, a characterisation of the CAC in this methylotroph could be useful. A little is known already about the CAC in M. methylotrophus. Its CAC is OGDH-deficient (Taylor, 1977; Large and Haywood, 1981). The CS and IDH from this organism have also been subject to preliminary examination. The CS is a large, and apparently NADH-insensitive enzyme (Taylor, 1977; Aperghis, 1981; Otto, 1986). The IDH is linked to NAD^+ and may be linked to $NADP^+$ although this latter observation is controversial (Taylor, 1977; Large and Haywood, 1981). These features are uncommon amongst the prokaryotic enzymes and the fact that they both occur in the same organism is interesting. Both the large NADH-insensitive CS and the bacterial NAD-IDH have had little attention relative to the more common large, NADH-sensitive CS and NADP-IDH and so their study would be a new contribution to the field of CAC enzymology.

It was therefore decided to conduct an enzymological study of the state of the CAC in M. methylotrophus and attempt purification and characterisation of CS and IDH from this organism. It was hoped that such a study would reveal properties of the enzymes not only of relevance to the production of 2-OG and thus nitrogen assimilation in M. methylotrophus, but also of relevance to the study of the CAC in other organisms.

CHAPTER 2

Materials and Methods

SECTION A

MATERIALS

Organisms and Tissue Sources

Methylophilus methylotrophus (Pseudomonas methylotrophica NCIB10515 strain AS1) was supplied by Dr. D. Byrom, Biological Products Business, Imperial Chemical Industries plc, Billingham, U.K., as a viable culture and a frozen cell paste. A. calcoaceticus strain 4B, Bacillus megaterium D101, Pseudomonas aeruginosa and E. coli K12D500 were from the culture collection of Prof. P.D.J. Weitzman (South Glamorgan Institute of Higher Education, Cardiff.U.K.). Sulpholobus acidocaldarius DSM639 was obtained as a frozen cell paste from the culture collection of Dr. M.J. Danson (University of Bath, U.K.).

Bovine heart was obtained from the local slaughter house.

Chemicals and Enzymes

Matrex Red Gel A (immobilised Red HE-3B) was from Amicon Ltd. Woking, Surrey, U.K.

The following chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset. U.K.: ammonium chloride, ammonium sulphate, bovine serum albumin, BRIJ-35, bovine erythrocyte carbonic anhydrase, Ches (2-[N-cyclohexyl] ethane suphonic acid), citric acid, equine cytochrome c, disodium succinate, disodium tetraborate, glacial acetic acid, glycerol, glycine, "Aristar" hydrochloric acid, iodoacetic acid, iron III chloride, 2-mercaptoethanol, methylviolet, 2-methoxyethanol, Mops (4-morpholinepropane sulphonic acid), equine myoglobin, Nessler's reagent, ninhydrin, neutral red, hen ovalbumin, hen ovotransferrin, potassium arsenite, potassium cyanide, propan-2-ol, sodium acetate, SDS (sodium dodecyl suphate), sucrose, TEMED (N,N,N',N'-tetra methylethylenediamine), thiodiglycol, trichloroacetic acid and urea.

Bio-Rad protein determination reagent was from Bio-Rad Laboratories Ltd., Watford, Herts. U.K.

The following chemicals were from Boehringer-Mannheim Corporation (London Ltd., Lewes, East Sussex. U.K.: rabbit muscle aldolase, bovine serum albumin, bovine liver catalase, chymotrypsinogen A, CoA (trilithium salt), equine heart cytochrome c, equine spleen ferritin, rabbit muscle lactate dehydrogenase, porcine heart malate dehydrogenase, $NADP^+$ (disodium salt), NADPH (tetrasodium salt), OAA and Tris (2-amino-2-hydroxymethyl propan-1,3-diol hydrochloride).

Amino acid standards were purchased from Calbiochem Brand Biochemicals, California. U.S.A.

Purified agar was from Difco, Michigan. U.S.A.

The following chemicals were from Fisons Scientific Apparatus Ltd., Loughborough, Leics. U.K.: acetamide, acetic anhydride, bromophenol blue, disodium EDTA (ethylenediamine tetra acetate), magnesium chloride hexahydrate, methanol and trisodium citrate.

Salmon roe protamine sulphate was from Koch-Light Laboratories, Berkshire. U.K.

Dialysis tubing was from Medicell International, London. U.K.

The following media were from Oxoid Ltd., Basingstoke, Herts. U.K.: nutrient agar, nutrient broth, peptone and yeast extract.

Mercaptoethane sulphonic acid was from Pierce Chemical Company, Illinois. U.S.A.

The following chromatographic media were from Pharmacia-LKB Biotechnology, Uppsala. Sweden: Blue dextran 2000, DEAE-Sephacel, Sephacryl S-200 (superfine), Sephadex G-25 (medium; either free or prepacked into 10 ml columns ("PD-10s") and Sepharose 4B.

The following chemicals were from Sigma Chemical Company Ltd., Poole, Dorset. U.K.: acetoacetyl-CoA (sodium salt), acetyl-phosphate (lithium potassium salt), ADP (sodium salt), AMP (free acid), ATP (disodium salt), ammonium phosphate, bis-acrylamide, bis-tris (bis(2-hydroxyethyl) aminotris-(hydroxymethylmethane), porcine heart CS, Coomassie Brilliant Blue R, cysteine hydrochloride, DCPIP (2,6-dichlorophenolindophenol; sodium salt), DTNB, DTNP (2,2'-dithiobis (5-nitropyridine), DTT, Freund's complete adjuvant, Freund's incomplete adjuvant, fumaric acid, glutamine, GDP (disodium salt), Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid), hydroxylamine hydrochloride, imidazole, IDP (sodium salt), iodoacetamide, trisodium

D,L-isocitrate, porcine heart NADP-IDH, malic acid, NAD^+, NADH (dipotassium salt), monosodium 2-OG, N-methylphenazine methosulphate, phenylhydrazine hydrochloride, trisodium PEP, sodium pyruvate, rabbit muscle pyruvate kinase, TPP.Cl (thiamin pyrophosphate), bovine thyroglobulin and triethanolamine hydrochloride.

The triazine dyes Blue HE-RD, Blue MX-3G, Blue PGR, Brown MX5BR, Brown H2G, Green HE-4BD, Orange MX-2R, Red HE-3B, Red R3BN, Ramazol Red, Red MX-50, Scarlet MXG, Turquoise H-A, Turquoise H-7G and Yellow MX-G8 were generously donated by Dr S. Syed (Department of Biochemistry, University of Bath, U.K.).

All other chemicals were of the highest available grade.

Water

Apart from growth media and HPLC solvents, all water used was double-distilled before use. Water for use in HPLC solvents was produced by deionisation of single distilled water using a Milli Q water purification system (Millipore S.A. Molsheim, France) such that the resulting solvent was distilled and deionised with a resistance of at least 18 MΩ.

Special Chemical Preparations

CoA thioesters were prepared by acylation of CoA with the appropriate anhydride:

(a) Acetyl-CoA. The procedure of Stadtman (1957) was used. 10 mg of CoA were dissolved in 1 ml of water. A 0.2 ml-aliquot of 1 M-$KHCO_3$ was added to neutralise the solution. 5 µl of acetic anhydride were then added and the mixture was vortexed and left on ice for 10 min prior to use.

(b) Succinyl-CoA. The method of Simon and Shemin (1953) was used. The procedure was identical to that used to prepare acetyl-CoA except that acetic anhydride was replaced by approximately 5 mg of succinic anhydride.

SECTION B

METHODS

2.1. Growth and Maintenance of Micro-organisms

All media for culturing of micro-organisms were sterilised by autoclaving at 121°C and 1.06 $kg.cm^{-2}$ pressure for 20 min. Apart from media such as nutrient broth or nutrient agar, carbon sources were added after autoclaving and were sterilised by passage through a 0.2 µm pore size filter (Gelman Sciences, U.K. or Sera-Lab Ltd., U.K.).

2.1.1. Growth and Maintenance of M. methylotrophus

2.1.1.1. Growth Media. M. methylotrophus was grown in a liquid medium (AS1 medium) of the following composition: K_2HPO_4 (1.9 $g.l^{-1}$), NaH_2PO_4 (1.56 $g.l^{-1}$), $(NH_4)_2SO_4$ (1.8 $g.l^{-1}$), $MgSO_4.7H_2O$ (0.2 $g.l^{-1}$), $FeCl_3$ (0.972 $mg.l^{-1}$), $CaSO_4$ (0.72 $mg.l^{-1}$), $MnSO_4$ (23 $\mu g.l^{-1}$), $ZnSO_4$ (23 $\mu g.l^{-1}$) and $CuSO_4$ (5 $\mu g.l^{-1}$). The pH of the medium was adjusted to 7.2 and then autoclaved. On cooling, methanol (for routine culturing and maintenance) was added to a final concentration of 0.5% (v/v). At this point, other carbon sources were added if desired.

M. methylotrophus could be cultivated on AS1 medium and carbon source that was solidified by addition of 1.5% (w/v) purified agar as follows: A four-times concentrated stock of $FeCl_3$-deficient AS1 medium and 2% (w/v) agar were autoclaved. The two solutions were cooled to 60°C and mixed in the ratio 1 part four-times concentrated stock of $FeCl_3$-deficient AS1 medium : 3 parts 2% (w/v) agar. $FeCl_3$ and carbon source(s) were then added through a 0.2 µm disposable filter (Gelman). The complete medium was then poured into plates which were stored at 4°C prior to use.

2.1.1.2. Maintenance. M. methylotrophus was routinely maintained on plates of solidified AS1 medium with methanol as the carbon source. Sub-culturing was performed weekly by inoculation of a fresh plate and incubation of the latter for 48 h at 37°C. Cultures were then stored

at 4°C. When experiments were carried out to study the consequences of a change in, e.g. carbon and/or nitrogen source on M. methylotrophus, the organism was pre-adapted by sub-culturing onto fresh AS1 medium plates supplemented with the new carbon and/or nitrogen source at least five times prior to commencement of the experiment.

2.1.1.3. Growth and Measurement of Growth of M. methylotrophus. M. methylotrophus was grown discontinuously at 37°C in shake flasks of twice the culture volume in a New Brunswick shaking incubator (New Brunswick Scientific, New Jersey, U.S.A.) at 250 rpm.

Samples were removed aseptically at various time intervals using sterile plugged Pasteur pipettes. The absorbance at 680 nm (A_{680}) was immediately measured in a single beam Pye-Unicam SP6 UV/Vis spectrophotometer (Pye-Unicam, Ltd., Cambridge, U.K.).

2.1.1.4. Harvesting of M. methylotrophus. Cultures were harvested in late exponential phase by centrifugation in a Sorvall RC 5B centrifuge (Dupont Company, Connecticut, U.S.A.) for 20 min at 4°C and 13.3 X 10^3g. The cell pastes were either immediately used or stored at -20°C.

2.1.1.5. Assessment of Culture Purity. Culture purity was assessed using a) nutritional characteristics and b) Gram's stain:

a) Nutritional Requirement: As M. methylotrophus is an obligate methylotroph, it cannot grow on multi carbon-carbon bond substrates or complex media. Thus nutrient agar plates or plates of solidified yeast extract (yeast extract agar: 0.3% (w/v) yeast extract, 0.5% (w/v) peptone and 1.5% (w/v) purified agar) were inoculated with culture samples. If growth was observed, the culture was disposed of.

b) Gram's Stain: A loopful of bacteria was spread over a microscope slide in a drop of sterile water. The slide was air-dried and heat-fixed by passing it through a bunsen flame. The slide was flooded with 0.5% (w/v) methyl violet, left for 20 min, gently washed with water and re-flooded with Gram's iodine (0.3% (w/v) iodine and 0.6% (w/v) potassium iodide). After a further 2 min the slide was washed with water, decolourised with acetone, immediately re-washed and counterstained for 2 min in neutral red (1% (w/v) in 1% (v/v)

glacial acetic acid). The slide was rinsed, dried and then studied. Pure cultures of M. methylotrophus only stained with neutral red.

2.1.2. Growth and Maintenance of Other Micro-organisms

Apart from Sulpholobus acidocaldarius, all other micro-organisms were maintained on nutrient agar plates. Sub-culturing was performed every six months by replating onto fresh media and incubating the plates at 37°C for 48 h. Cultures were then stored at 4°C.

Organisms were grown overnight in a shaking incubator at 250 rpm. Harvesting was carried out as described for M. methylotrophus.

2.2. Preparative Techniques

2.2.1. Crude Extract Preparation

Cell pastes were resuspended on ice in the ratio of 1 g cells: 2 ml of buffer. Cell disruption was carried out by sonication using an Ultrasonics 180 W sonicator (Ultrasonics Ltd, U.K.). For suspensions of 15 ml or less, a 5 mm diameter probe operating at 50 W was used. Volumes of cell suspensions from 15 to 60 ml were sonicated with a 20 mm-diameter probe operating at 100 W. All sonications were carried out in 15-sec bursts interspersed with cooling on ice for 30 sec. Unbroken cells and insoluble cell material was then removed by centrifugation at 12,000 X g at 4°C for 30 min in a Sorvall RC 5B centrifuge.

2.2.2. Permeabilisation of M. methylotrophus Cells

Cells were permeabilised according to Weitzman and Hewson (1973). Immediately after harvesting, cells were resuspended in 0.1 M-Tris, pH 8.0, and centrifuged for 20 min at 4°C and 13,300 X g. The pellet was resuspended to 100 $mg.ml^{-1}$ (wet weight) in the same buffer and warmed to 37°C. The mixture was then treated with 50 µl of toluene:ethanol (1:4) per ml of suspension to permeabilise the cells. The mixture was then vigorously agitated for 5 min at 37°C and then cooled on ice, after which it was centrifuged for 20 min at 4°C and 13,300 X g. Cells

were then washed in 0.1 M-Tris, pH 8.0, and recentrifuged as before. The cell pellet was then resuspended to 100 $mg.ml^{-1}$ for immediate use.

2.2.3. Preparation of Mitochondria

Fresh bovine heart muscle was stripped of fat and fibres and cut into cubes which were suspended in 50 mM-Tris, 1 mM-EDTA, 0.25 M-sucrose and 0.1% (w/v) bovine serum albumin adjusted to pH 7.5. The suspension was homogenised in a Waring blender for two 10-sec bursts. Insoluble material was removed by centrifugation at 4°C for 15 min at 1,600 X g. The pellet was discarded and the supernatant was filtered through a double cheese cloth and re-centrifuged at 14,500 X g for 30 min at 4°C to sediment the mitochondria. The pellet was resuspended in 50 mM-Tris, 1 mM-EDTA and 0.25 M-sucrose adjusted to pH 7.5. The mitochondria were then collected by centrifugation at 30,000 X g for 30 min at 4°C. The preparation was stored on ice for immediate use.

Mitochondria were disrupted according to Denton et al. (1978). The mitochondria were resuspended in 0.1 M-potassium phosphate, 5 mM-2-mercaptoethanol and 1 mM-ADP adjusted to pH 7.2 and extracted by freezing and thawing in liquid nitrogen three times. The mitochondria were then centrifuged for 30 min at 36,900 X g and 4°C. The supernatant was retained and the pellet was discarded.

2.2.4. Preparation of Polyclonal Antiserum to *M. methylotrophus* IDH

Antibodies were raised in a female Californian rabbit. 0.3 mg of antigen in 0.75 ml 20 mM-Tris, 2.4 mM-EDTA, 10 mM-$MgCl_2$ adjusted to pH 8.0 (MET-8.0) were mixed with an equal volume of Freund's complete adjuvant in two 5-ml glass syringes connected by a short piece of tubing until a creamy emulsion was formed. The rabbit was bled from the right ear to obtain a pre-immune serum and then injected at two sites intra-dermally with the adjuvant-antigen mixture. After 2 weeks, the animal was re-immunised with an emulsion of Freund's incomplete adjuvant and 0.22 mg of antigen (1:1, v/v) at two sites intramuscularly. At 3 and 4 weeks after the first immunisation, the two-week immunisation was repeated at four intramuscular sites. A test

bleed was carried out at the last immunisation and a week later the rabbit was bled under anaesthetic by cardiac puncture.

The blood was kept at room temperature for 1 h in a glass vessel. The clot was chilled overnight at 4°C and removed. Any remaining serum in the clot was extracted by centrifugation at 4°C and 2,500 X g for 10 min. The supernatant sera were pooled and stored frozen at -20°C.

2.2.5. Buffer Exchange

Buffers were exchanged by dialysis in dialysis tubing or by gel filtration using Sephadex G-25. Dry G-25 was swollen overnight in water. The fines were removed by decanting. The swollen gel was then poured to give a 45 X 2.5 cm column (bed volume 250 ml) which was equilibrated at 4°C in buffer. Up to 8 ml of sample were applied to the surface of the drained gel bed and the column was eluted with equilibration buffer at a flow rate of 46 $ml.h^{-1}$. Fractions (3 ml) were collected in an LKB 2112 RediRac fraction collector (Pharmacia-LKB Biotechnology). For sample volumes of 1 ml or less, commercially packed 10 ml-columns of Sephadex G-25 (PD 10s) were used. Columns were equilibrated with 50 ml of buffer and run with a sample as described above, except that columns were eluted under gravity.

2.2.6. Sample Concentration

Samples were concentrated by ultrafiltration at 4°C through a 10,000 M_r cut-off membrane. Samples (up to 50 ml) were placed in an Amicon model 850 ultrafiltration cell and a pressure of 4.36 $kg.cm^{-2}$ nitrogen was applied. Once the volume of the sample had been reduced by the desired amount, the pressure was released gradually and the sample was removed.

2.2.7 Protein Purification Procedures

2.2.7.1. Ammonium Sulphate Precipitation. Ammonium sulphate was crushed and added slowly to the extract on ice with stirring. After addition, the extract was allowed to equilibrate for 20 min. The

precipitate was removed by centrifugation at 15,000 x g for 30 min at 4°C. The quantity of ammonium sulphate added was calculated from the table of Green and Hughes (1955).

2.2.7.2. Protamine Sulphate Precipitation. Protamine sulphate in water (10 $mg.ml^{-1}$) was added drop-wise to a stirred extract on ice until a ratio of 1 mg protamine sulphate per 10 mg protein was reached. The extract was equilibrated for 10 min and then centrifuged at 20,000 X g for 30 min at 4°C. The precipitate was discarded.

2.2.7.3. Acid Precipitation. 0.1 M-HCl was added drop-wise to a stirred extract on ice. After equilibration for 15 min the precipitated protein was removed by centrifugation in an Eppendorf 5415 bench-top centrifuge (Eppendorf Geratebau Netheler and Hinz GmbH Hamburg, W. Germany) at room temperature for 2 min at 16,000 X g.

2.2.7.4. Thermal Precipitation. Extracts were heated in glass-ware suspended in a water-bath maintained at the required temperature for the required time. Precipitated protein was removed by centrifugation at 4°C for 30 min and 20,800 X g or for 2 min at room temperature and 16,000 g in an Eppendorf bench-top centrifuge.

2.2.7.5. DEAE-Anion Exchange Chromatography. DEAE-Sephacel anion exchanger was poured as a thick slurry into a 250-ml column (45 X 2.5 cm). The column was equilibrated with buffer at 4°C before use. Once the pH of the eluate was equal to that of the buffer entering the column, samples were applied. The column was washed with equilibration buffer and elution of unbound protein was monitored by following the change in A_{280}. When the absorbance had fallen below 0.05, a linear gradient of increasing KCl concentration over two column volumes at a flow rate of 10 $ml.h^{-1}$ was applied. The eluted protein was collected in 5-ml fractions using an LKB 2112 RediRac fraction collector.

2.2.7.6. Triazine Dye-Ligand Chromatography. Dye-ligand chromatography is a recent addition to the techniques used for purification of proteins. In many cases it has replaced affinity chromatography as it

can have the bio-specificity that affinity chromatography offers without many of the problems inherent in the latter.

Dextran conjugates of Cibacron Blue-F3GA have been used for many years as void volume markers for gel filtration. However, it was found that blue dextran co-chromatographed with glutathione reductase, lactate dehydrogenase, phosphofructokinase and pyruvate kinase (Kopperschläger et al., 1968; Staal et al., 1969, 1971; Ryan and Vestling, 1974). This was caused by binding of the enzyme to the blue dextran chromophore (Kopperschläger et al., 1968). It is now known that triazine dyes bind many proteins (Dean and Watson, 1979).

The triazine dyes were developed at ICI in 1954 for use in the textile industry. They are based on 1,3,5-trichloro-triazine (cyanuric chloride; Figure 12) of which they are substituted derivatives, where up to two chlorine atoms are substituted for polyaromatic chromophores linked to the ring by -NH- bridges. ICI make two types of dye: the Procion MX range that contain dichlorotriazinyl groups and the Procion H range which contain monochlorotriazinyl groups. The most common dyes in protein purification (Figure 12) are Procion Red HE-3B and Cibacron Blue F3GA (manufactured by Ciba-Geigy, and ICI as Procion Blue H-B).

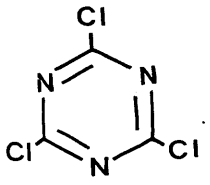
A particular advantage that triazine dyes have is their ease of coupling to solid supports. The chlorine atom(s) of the triazine are easily substituted by hydroxyl groups of polysaccharide-based supports under basic conditions (Dean and Watson, 1979). This contrasts with the hazards of cyanogen bromide immobilisation. Other advantages that triazine dyes have over use of coenzymes or other biochemicals as ligands are economy, stability, re-usability and a high capacity for protein binding (Turner, 1981; Hey and Dean, 1981).

The mechanism of dye-protein binding is complex and varied. In many cases, binding of enzyme to dye is probably biospecific, that is, the dye mimics some natural ligand of the enzyme. Fulton (1980) has noted the similarity in overall shape and charge between Cibacron Blue F3GA and NAD^+. The binding of this dye to horse liver alcohol dehydrogenase has been studied by X-ray crystallography and it appears that the dye interacts at the nucleotide binding site (Biellmann et al., 1979). Competitive inhibition has been observed between free dye and nucleotide (see Fulton, 1980) suggesting an interaction between

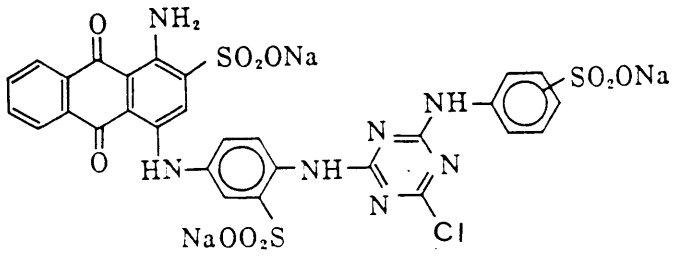
Figure 12

Triazine Based Dye Structures

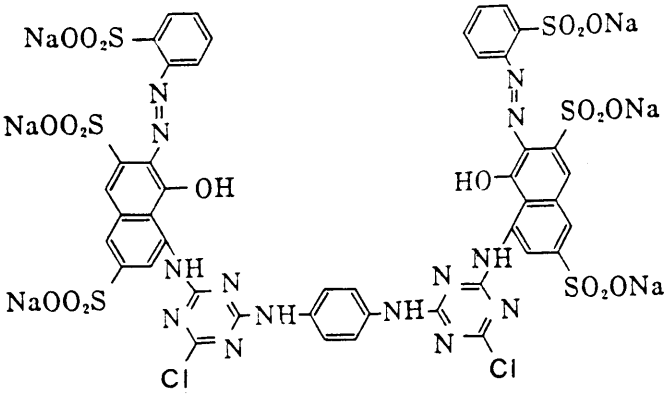
1,3,5 Trichlorotriazine, the basic element of triazine dyes



The structure of Cibacron Blue F3GA (Procion Blue H-B)



The structure of Procion Red HE-3B



the dye and the nucleotide binding site. It has often been concluded that a biospecific interaction between dye and enzyme has occurred if the enzyme is eluted by a concentration of ligand that is lower than the salt concentration required to elute the enzyme (Dean and Watson, 1979); however, this has been questioned by Yon (1977).

Binding of proteins to dyes may not always be bio-specific. Indeed, as well as nucleotide binding sites, Cibacron Blue F3GA binds to, for example, chymotrypsin and albumins (Hey and Dean, 1979). It should be noted that triazine dyes have multiple sulphonate and aromatic groups which may allow non-specific electrostatic or hydrophobic interactions to occur (Turner, 1981; Hey and Dean, 1981).

As triazine dyes often interact biospecifically, they can achieve spectacular purification factors, e.g. yeast orotidylate decarboxylase was purified 6590-fold by elution from Cibacron Blue F3GA with UMP (Reyes and Sandquist, 1978). In view of this potential, triazine dyes were employed in the present work for protein purification. In this instance a variety of dyes was screened for protein binding. As there are many reasons why proteins bind to triazine dyes (Fulton, 1980; Dean and Watson, 1979; Hey and Dean, 1981; Turner, 1981), it was considered that this approach could maximise the chances of obtaining a successful purification protocol (Hey and Dean, 1981, 1983).

2.2.7.6.1. Linkage of Triazine Dyes to Sepharose 4B and Their Use. Triazine dyes were linked to Sepharose 4B according to Dean and Watson (1979). 10 ml of a slurry of Sepharose 4B was resuspended in 35 ml of water. To this was added 100 mg of a triazine dye. After stirring slowly for 10 min, 15 ml of 20% (w/v) NaCl were added. The mixture was then stirred for 30 min. 7.5 ml of 5 M-NaOH were then added. The mixture was stirred slowly for 4 days at room temperature. The gel was then washed in a sintered glass funnel with water until no dye was eluted, and then washed sequentially with 2 l of 6 M-urea, 2 l of 2 M-urea and 2 l of water. The gel was stored at 4°C in 0.01% (w/v) sodium azide. Columns were packed in 5-ml syringe barrels plugged with glass wool and equilibrated with buffer at 4°C until the eluate pH was equal to that of the buffer entering the column. Protein was applied to the drained gel and overlaid with buffer. Columns were washed until no

protein could be detected in the eluate. Bound protein was then eluted by increasing KCl concentrations or biospecifically by substrate, product or combination thereof of the enzyme being purified.

2.2.7.6.2. Commercially Available Agarose-Immobilised Triazine Dyes. Matrex Red Gel A was poured as a thick slurry into 30 X 1.7 cm or 10 X 0.5 cm columns and washed with buffer until the eluate was free of unbound dye and was the same pH as the buffer entering the column. Columns were loaded, washed, and eluted with a salt gradient as for DEAE-anion exchange chromatography or eluted with substrates and/or products of the enzyme being purified. 1-ml fractions were collected.

2.2.7.7. Gel Filtration. A thick slurry of Sephacryl S-200 was poured into a column and allowed to settle. The surface was stirred and the process repeated until a 102 X 2.5 cm column was poured. The top of the gel bed was fitted with an adjustable plunger and the column was then equilibrated at 4°C with buffer at 10 $ml.h^{-1}$. Samples were applied in 2 ml equilibration buffer and 10% (w/v) sucrose. 1.2-ml fractions were collected by a LKB 2070 UltroRac II fraction collector.

2.3. Analytical Techniques

All spectrophotometric measurements described were made using a Pye-Unicam SP6 UV/Vis spectrophotometer.

2.3.1. Protein Estimations

Protein concentrations were determined with a commercially produced protein assay kit from Bio-Rad. The assay system was based on the Coomassie Brilliant Blue G-250 protein binding method of Bradford (1976). Samples of up to 25 µg protein made up to 0.8 ml in buffer or water were mixed with 0.2 ml of the Bio-Rad protein assay solution and left at room temperature for between 2 and 60 min. The absorbance of the solution at 595 nm was then determined relative to a protein-free blank. The protein standard used was bovine thyroglobulin.

2.3.2. Ammonia Estimation

2.3.2.1. Quantitative. Samples were made up to a final volume of 2 ml with 0.1 M-citrate/ phosphate buffer adjusted to pH 6.0 and treated with 0.5 ml of 15% (w/v) trichloroacetic acid (TCA). Samples were centrifuged at room temperature for 2 min at 1,600 X g to remove precipitated protein. The supernatant was treated with 1 ml of Nessler's reagent and read at 425 nm against a blank, omitting sample. The assay was calibrated between 0.06 and 0.6 μmol-NH_4^+ using NH_4Cl as standard. A standard curve is shown in Figure 13.

2.3.2.2. Qualitative. This test was mainly used to confirm the removal of $(NH_4)_2SO_4$ from resuspended precipitates of ammonium sulphate fractionations. 25 μl of sample was added to 0.5 ml water. 0.5 ml Nessler's reagent was then added. NH_4^+ caused the formation of a yellow colour or an orange precipitate depending on its concentration.

2.3.3. Enzyme Assays

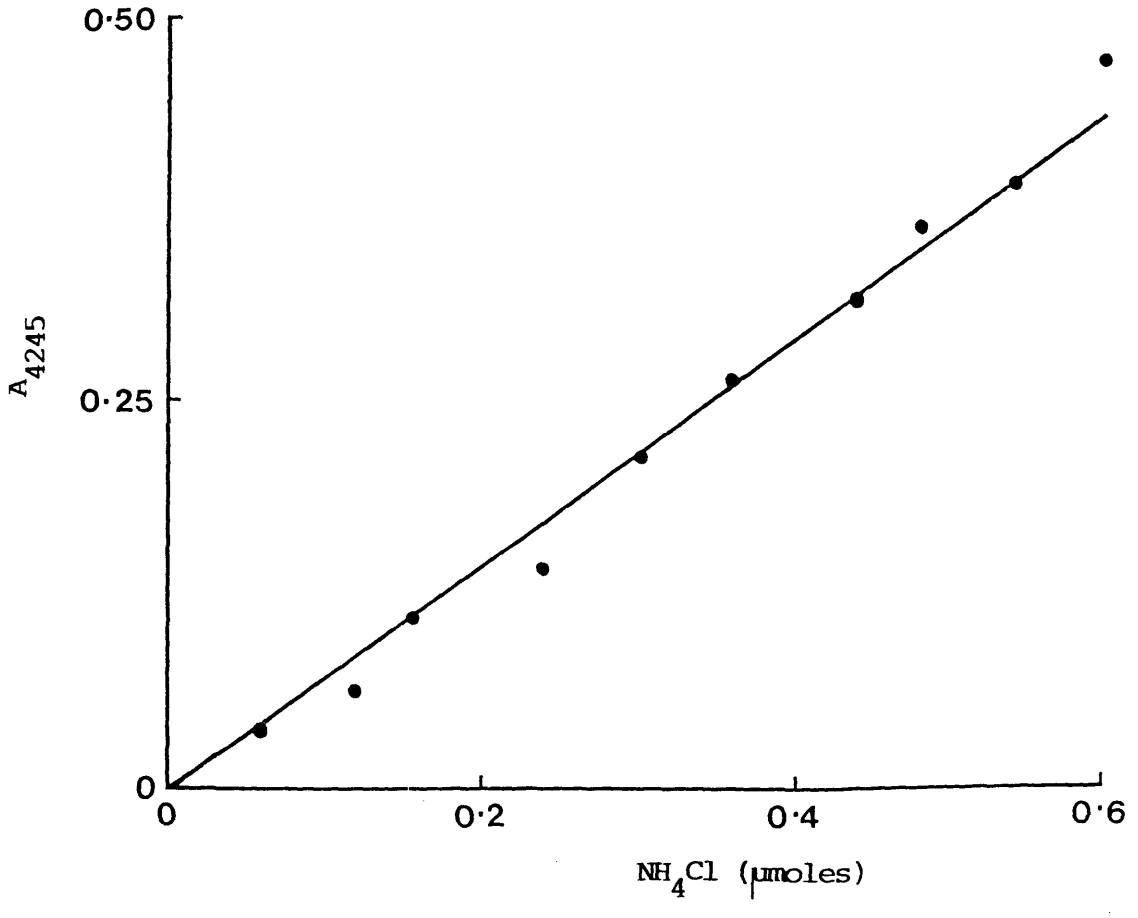
Unless specified otherwise, enzymes were continuously assayed at 25°C. Amounts of enzyme measured were such that the recorded rate was linear for several minutes and proportional to the enzyme added. 1 unit (U) is a quantity of enzyme activity catalysing a rate of 1 $\mu mol.min^{-1}$. Specific activity is defined as U per mg-protein ($U.mg^{-1}$).

2.3.3.1. Spectrophotometric Enzyme Assays. For assays carried out above 340 nm, plastic cuvettes were used, below this wavelength, quartz cuvettes were used. In both cases, cuvette volume and path lengths were 1 ml and 1 cm. All continuous enzyme rates were recorded on a Rikadenki chart recorder (Rikadenki Kogyo Co. Ltd., Tokyo, Japan). The molar absorption coefficients used are listed in Table 10.

2.3.3.1.1. Acetate Kinase (EC 2.7.2.1). This enzyme catalyses the ATP-dependent phosphorylation of acetate. It was assayed according to Wong and Wong (1981). The assay monitored ADP production by acetate kinase with pyruvate kinase and lactate dehydrogenase. The assay

Figure 13

Standard Curve for Determination of Ammonia Using Nessler's Reagent



Standards used for the construction of the above curve were treated in essentially the same way as samples of acetamide assays for estimation of ammonia (2.3.2.1.). Thus, the appropriate amount of ammonium chloride in 0.1 M-citrate/phosphate (pH 6.0) was added to 2 ml of the same buffer, treated with 0.5 ml of TCA, and then treated with 1 ml of Nessler's reagent. The absorbance was read at 425 nm against a blank omitting ammonium chloride.

Table 10

Published Values of Molar Absorption Coefficients Used in Enzyme Assays

Absorbing Species	Wavelength (nm)	Molar Absorption Coefficient ($10^{-3}.l.mol^{-1}.cm^{-1}$)	Reference
Acetoacetyl-CoA (Mg^{2+}: acyl Chelate)	303	20.5	Jaenicke and Lynen (1960)
Acetyl-CoA (thioester bond)	232	8.7	Dawson et al. (1986)
DCPIP (oxidised)	600	16.1	Dawson et al. (1986)
Gamma-glutamyl hydroxamate:$FeCl_3$	535	0.85	Rowe et al. (1970)
Glyoxylate phenyl hydrazone	324	17.0	Dixon and Kornberg (1959)
NAD(P)H	340	6.22	Dawson et al. (1986)
TNB anion	412	13.6	Ellman (1959)

mixture was composed of 67 mM-triethanolamine adjusted to pH 7.6, 10 mM-$MgCl_2$, 5.4 mM-ATP, 1.1 mM-PEP, 0.32 mM-NADH, 9.3 U pyruvate kinase, 13.8 U lactate dehydrogenase, and extract. Acetate kinase-independent NADH oxidation was measured at 340 nm and the acetate kinase was then assayed by addition of acetate to 0.3 M.

2.3.3.1.2. Acetyl-CoA Synthetase (Acetate: CoA Ligase [AMP forming/ADP forming] EC 6.2.1.1/6.2.1.?). This enzyme was assayed in two ways:

a) Direct Assay of thioester bond production at 232 nm, according to Webster (1969). The assay composition was 0.1 M-Tris adjusted to pH 8.0, 3 mM-$MgCl_2$, 24 µM-ATP, 1.6 mM-CoA and extract. Acetyl-CoA synthetase was measured on addition of sodium acetate to 1.8 mM.

b) Indirect Assay of acetyl-CoA synthetase by linking acetyl-CoA production to CS in the presence of an OAA generating system - malate dehydrogenase. Thus NADH generation was followed at 340 nm. The assay constituents and method were the same as above for the direct assay except 1 mM-malic acid, 0.5 mM-NAD^+, 60 U malate dehydrogenase and 11.6 U porcine CS were incorporated into the assay.

2.3.3.1.3. Amidase (Acylamide Amino-hydrolase EC 3.5.1.4). Amidase was assayed discontinuously by following acetamide-dependent production of ammonia with Nessler's reagent. The enzyme was incubated in 2 ml containing 5 mM-acetamide and 0.1-M citrate/phosphate (pH 6.0) for 10 min. Ammonia was then determined (**2.3.2.1.**) using a blank where 0.5 ml of 15% (w/v) TCA had been added to the reaction mixture prior to addition of the enzyme.

2.3.3.1.4. 3-Oxoacid CoA Transferase (Succinyl-CoA: 3-oxoacid CoA Transferase EC 2.8.3.5). The enzyme was assayed by monitoring the succinate-dependent fall in A_{303} due to the loss of the Mg^{2+}-chelate of acetoacetyl-CoA (Jaenicke and Lynen, 1960). The assay procedure was that of Ann-Page et al. (1971). Iodoacetamide was included in the assay to inhibit thiolase which would have otherwise interfered in the assay by breaking down acetoacetyl-CoA. The assay mixture contained: 50 mM-Tris and 10 mM-$MgCl_2$ adjusted to pH 8.5, 80.1 µM-acetoacetyl-CoA, 5 mM-iodoacetamide and extract. The reaction was followed for

succinate-independent decomposition of acetoacetyl-CoA. Transferase activity was then determined by addition of succinate to 5 mM.

2.3.3.1.5. Phosphotransacetylase (Acetyl-CoA: Orthophosphate Acetyl transferase EC 2.3.1.8). The enzyme was assayed in the direction of acetyl-CoA synthesis, monitoring either the increase in A_{232} due to acetyl-CoA production (Method a) or acetyl-CoA production with CS in the presence of OAA generated by malate dehydrogenase (Method b).

Method a. This method is based on that of Webster (1969). Extract was added to an assay mixture of 0.1 M-Tris adjusted to pH 7.4, 20 mM-$(NH_4)_2SO_4$, 2 mM-DTT and 0.4 mM-CoA. The phosphotransacetylase-independent change in A_{232} was followed and the reaction was initiated by the addition of acetyl-phosphate to 10 mM.

Method b. In addition to the above, the assay contained 1 mM-malate, 0.5 mM-NAD^+, 60 U porcine malate dehydrogenase and 11.6 U porcine CS. The assay was followed at 340 nm.

2.3.3.1.6. Pyruvate Carboxylase (Pyruvate: Carbon Dioxide Ligase [ADP-Forming] EC 6.4.1.1). Pyruvate carboxylase was assayed by linking OAA production to malate dehydrogenase, according to Seubert and Weicker (1969) with the modification by Aperghis (1981). The assay was composed of 70 mM-Tris pH 7.2, 10 mM-$MgCl_2$, 13 mM-$KHCO_3$, 0.2 mM-NADH, 1.3 mM-ATP, 1.3 mM-pyruvate, 30 U malate dehydrogenase and extract. The ATP-independent decrease in A_{340} was monitored and the pyruvate carboxylase reaction was then initiated by the addition of ATP.

2.3.3.1.7. Pyruvate Dehydrogenase (Pyruvate: Lipoate Oxidoreductase [Decarboxylating and Acceptor Acetylating] EC 1.2.4.1). The enzyme was assayed in 50 mM-potassium phosphate pH 8.0, 2 mM-$MgCl_2$, 0.2 mM-TPP.Cl, 2.6 mM-NAD^+, 0.15 mM-CoA, 3.4 mM-cysteine, 0.2 mM-pyruvate and extract. Pyruvate-independent NAD^+ reduction was monitored at 340 nm and the reaction was started by the addition of pyruvate.

2.3.3.1.8. Thiolase (Acetyl-CoA: Acetyl-CoA-Acetyl Transferase EC 2.3.1.9). Thiolase was assayed by the method used for 3-oxoacid CoA transferase (**2.3.3.1.4.**) omitting iodoacetamide and succinate.

2.3.3.1.9. Glutamate Dehydrogenase (L-Glutamate: $NAD(P)^+$ Oxidoreductase [deaminating] EC 1.4.1.3). The enzyme was assayed according to Windass et al. (1980) in 50 mM-Hepes pH 7.5, 1 mM-EDTA, 1 mM-2OG, 0.16 mM-NADH or NADPH and 10 mM-NH_4Cl. Extract was added in the absence of NH_4Cl and the rate of NH_4Cl-independent NAD(P)H oxidation was recorded. The reaction was then initiated with NH_4Cl.

2.3.3.1.10. Glutamine Synthase (L-Glutamine: 2-OG Aminotransferase [NAD(P)H Oxidising] EC 2.6.1.53/54). The enzyme was assayed as for glutamate dehydrogenase but with glutamine in place of NH_4Cl.

2.3.3.1.11. Glutamine Synthetase (L-Glutamine: Ammonia Ligase [ADP Forming] EC 6.3.1.1). The gamma-glutamyl transferase discontinuous assay (Shapiro and Stadtman, 1970) was used. The assay relies upon the ability of glutamine synthetase to catalyse the reaction

$$\text{Glutamine} + \text{Hydroxylamine} \longrightarrow \text{Glutamylhydroxamate} + NH_3$$

$FeCl_3$ can react with glutamylhydroxamate to form a complex that absorbs at 535 nm. The assay was carried out at 30°C in 0.5 ml and was composed of: 0.155 M-imidazole pH 7.4, 0.15 M-glutamine, 42 mM-hydroxamic acid (neutralised with KOH), 0.4 mM-$MgCl_2$, 0.4 mM-ADP and 20 mM-$KAsO_4$. The reaction was initiated with extract and the assay was incubated for 30 min. Control mixtures were set up containing all of the above components except ADP and arsenate to account for glutamine synthetase-independent activity. After incubation, 2 ml of 3.33% (w/v) $FeCl_3$ and 2% (w/v) TCA in 0.25 M-HCl was added. The solution was centrifuged in a bench centrifuge and the A_{535} was determined.

2.3.3.1.12. Aconitase (Citrate [Isocitrate] Hydro-Lyase EC 4.2.1.3). The enzyme was assayed in MET-8.0 buffer in the presence of 1 mM-citrate, 0.2 mM-NADP, 0.29 U porcine heart NADP-IDH and extract. Citrate-dependent reduction of $NADP^+$ was followed at 340 nm.

2.3.3.1.13. Citrate Synthase (Citrate Oxaloacetate-Lyase [CoA-Acetylating] EC 4.1.3.7). CS was usually assayed according to Srere et

al. (1963). The assay is based upon the non-enzymic, irreversible reaction of CoA with DTNB (Figure 14). The TNB anion is a resonance hydrid of structures I and II (Figure 14). Of these, the quinone-type structure (II) absorbs at 412 nm (Ellman, 1959). Thus the routine conditions for CS assays were: 20 mM-Tris and 1 mM-EDTA adjusted to the desired pH, - usually 8.0 (TE-8.0) or 8.6 (TE-8.6), 0.2 mM-acetyl-CoA, 0.1 mM-DTNB, 0.2 mM-OAA and extract. All components were added except OAA and the A_{412} was monitored to assay OAA-independent hydrolysis of acetyl-CoA and reactions with thiols in the extract. The CS reaction was then initiated by addition of OAA to the same cuvette.

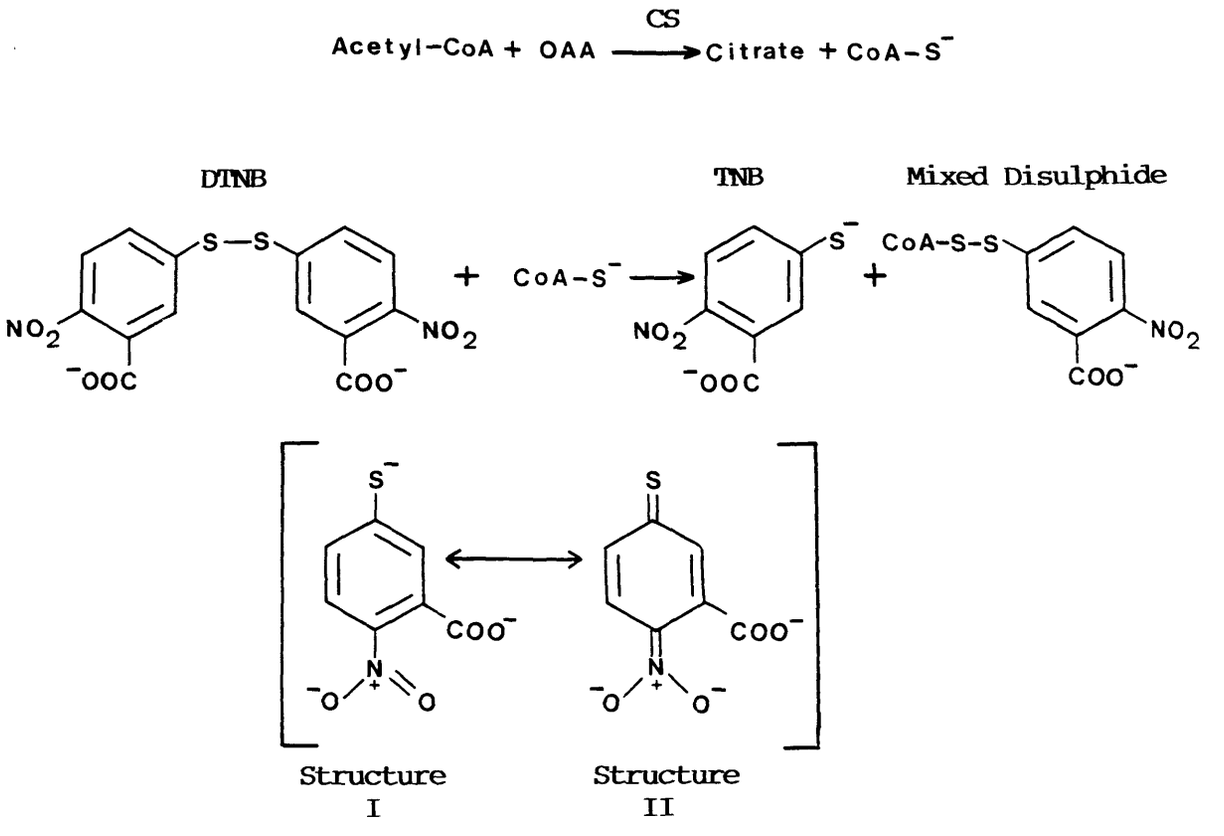
The deprotonation of the thiol is necessary for the absorbance of TNB chromophore at 412 nm. Furthermore, as the unprotonated CoA thiol (pKa = 9.6) reacts with DTNB (Dawson et al., 1986; Jocelyn, 1987), the assay is only useful at alkaline pH. The marked pH-dependence of the reaction of the thiolate ($-S^-$) anion with DTNB is emphasised by the behaviour of the second order rate constant for the reaction of DTNB with glutathione which drops 1000-fold between pH 8 and pH 5 (Palau and Dabán, 1974). This occurs because $-S^-$ is a far more potent nucleophile than -SH, and thus the former reacts far more rapidly with the disulphide of DTNB (Russo and Bump, 1988).

The restricted pH range over which this assay could be used was overcome by use of two other CS assays. One of these (a polarographic assay) is described later. The other uses an alternative disulphide chromogen: 2,2'-dithiobis (5-nitropyridine) (DTNP; Figure 14, III). This reaction of this reagent with thiol groups is not as pH dependent as with DTNB. This pH-independence is the product of two opposing effects. On the one hand, decreasing pH reduces the $-S^-$ concentration. However, this is countered by protonation of the pyridine ring nitrogen of DTNP (IV; Figure 14) which, due to its proximity to the disulphide, makes the adjacent sulphur atom far more electrophilic; indeed, monoprotonation of dithiobis pyridines increases their rate of reaction by 10^3 (Brocklehurst, 1982; Russo and Bump, 1988).

The assay protocol for the DTNP-linked CS assay was identical to that employed with the DTNB-linked assay except that 0.1 mM-DTNP (10 µl of a 10 mM solution in acetone) was added instead of DTNB. The acetone did not alter the measured rate in the DTNB-linked assay. The

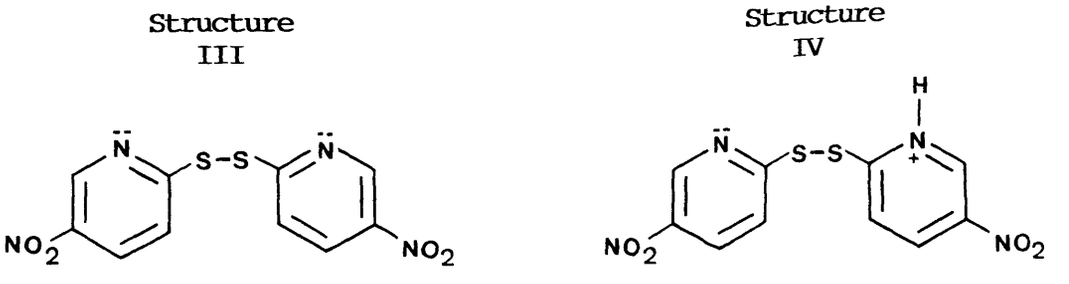
Figure 14

Disulphide Chromogens Used in the Assay of CS



Resonance Hybrid of the TNB Anion

DTNP



extinction coefficient for the leaving group (2-thio, 5-nitro-pyridone; TNP) produced on reaction of 0.29 M-2-mercaptoethanol with 0.102 mM-DTNP was estimated at pH 6.2 in 20 mM-Mops, 1 mM-EDTA and pH 8.0 in TE-8.0. After 15 min incubation at 25°C, the extinction coefficients at 412 nm were 14,300 ± 200 $l.mol^{-1}.cm^{-1}$ at pH 6.2 and 14,600 ± 300 $l.mol^{-1}.cm^{-1}$ at pH 8.0 (both ± S.E.M.; n = 14). This confirmed that TNP absorbance was independent of pH between 6.2 and 8.0 and the average of the two values (14,500) was used for calculation of activities.

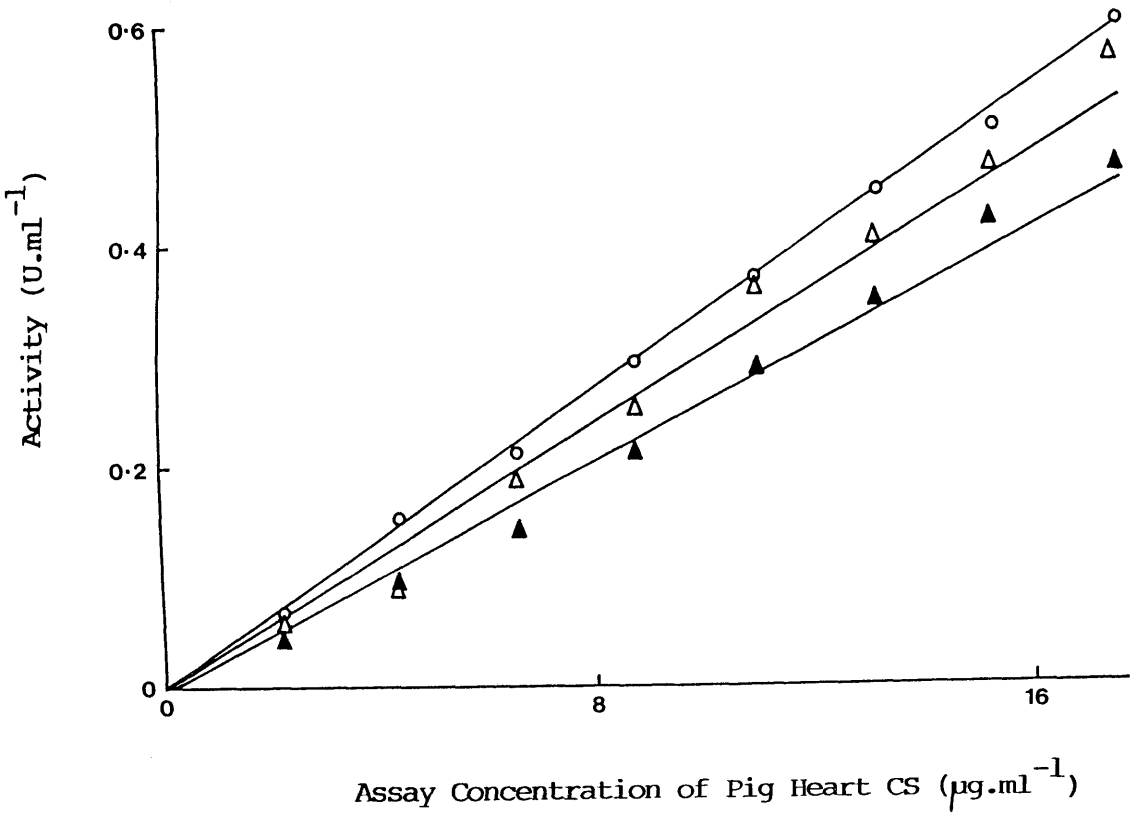
At pH 8.0, in TE-8.0, the DTNB and DTNP-linked assays gave comparable linear relationships between rate and pig heart CS added (Figure 15). The gradients of lines in Figure 15 were in reasonable agreement, giving specific activities for the enzyme assayed by DTNB and DTNP as 34 and 30 $U.mg^{-1}$ respectively. At pH 6.2, in 20 mM-Mops and 1 mM-EDTA, the DTNP-linked assay still displayed a linear relationship between rate and enzyme (Figure 15). Further, at pH 6.2, after addition of OAA, the assay entered a steady state for the initial increase in A_{412} due to porcine heart CS within 1 min. In comparison, the DTNB-linked assay detected hardly any CoA in the 2 min following OAA addition (Figure 16). Thus it seemed that DTNP was a suitable assay reagent for CS over a wide pH range.

2.3.3.1.14. Fumarase (L-Malate Hydrolase EC 4.2.1.2). Fumarase was assayed by coupling it to malate dehydrogenase and CS. CoA produced by CS could then be assayed with DTNB at 412 nm. The assay mixture was: 20 mM-Tris, 2.4 mM-EDTA, 10 mM-$MgCl_2$ adjusted to pH 8.6 (MET-8.6), 0.5 mM-NAD^+, 0.2 mM-acetyl-CoA, 0.1 mM-DTNB, 5.8 U porcine heart CS, 60 U malate dehydrogenase and extract. Fumarate-independent DTNB cleavage was recorded at 412 nm and 1 mM-fumarate was then added.

2.3.3.1.15. NAD-Linked Isocitrate Dehydrogenase (threo D_s-Isocitrate: NAD^+ Oxidoreductase [Decarboxylating] EC 1.1.1.41). The bovine heart mitochondrial enzyme was assayed using a method based on the results of Denton et al. (1978). The assay contained 50 mM-Mops pH 7.0, 10 mM-$MgCl_2$, 2 mM-NAD^+, 0.2 mM-ADP and 1 mM-D,L-isocitrate. The assay was initiated by isocitrate. Rotenone (2 $\mu g.ml^{-1}$) was initially added to

Figure 15

Relationship Between Porcine CS Concentration and the Initial Rate Measured by the DTNB and DTNP Linked Assays



o : DTNB Assay

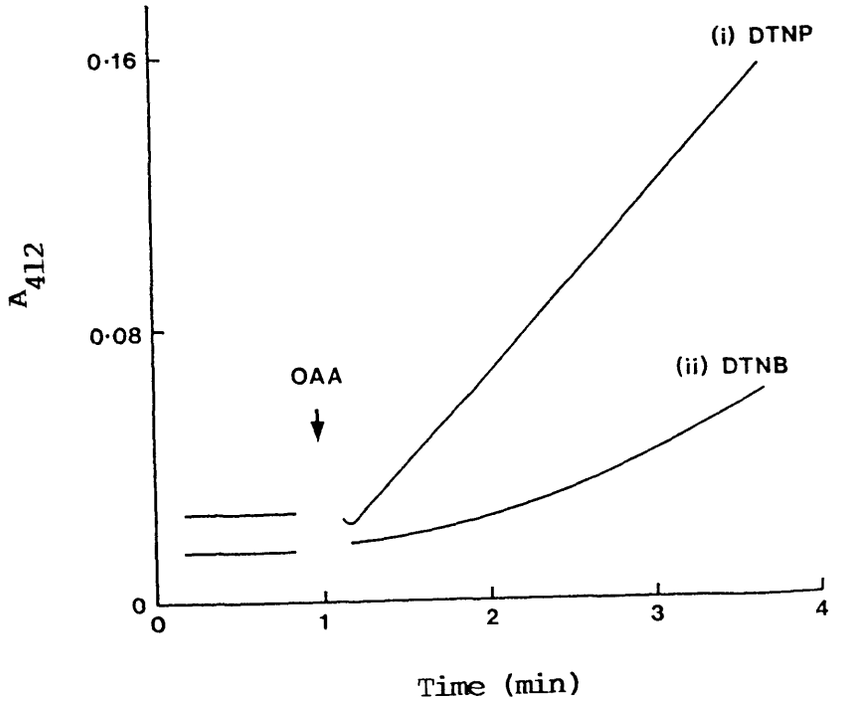
Δ ▲ : DTNP Assays

Open Symbols: Assays carried out in TE-8.0

Closed Symbols: Assays carried out in 20 mM-Mops and 1 mM-EDTA at pH 6.2

Figure 16

Effect of Substitution of DTNP for DTNB in the Spectrophotometric CS Assay at pH 6.2



(i) DTNP linked assay, (ii) DTNB linked assay. A Sigma preparation of porcine CS was used at a final assay concentration of 8.8 $\mu g.ml^{-1}$. The buffer was 20 mM-Mops and 1 mM-EDTA adjusted to pH 6.2. Acetyl-CoA and OAA were present at 0.2 mM, DTNP and DTNB were both present at 0.1 mM. OAA was added last at the point indicated.

The chart recorder was run at 3 $cm.min^{-1}$, with a full scale deflection of 0.4 absorbance units.

prevent NADH-oxidation by mitochondrial NADH-oxidase, but was found to have no effect on the rate and was omitted from subsequent assays.

The prokaryotic enzyme was assayed in MET-8.0 by following NAD^+ reduction. The assay mixture contained 0.2 mM-NAD^+ and extract. The assay was started by addition of 4 mM-D,L-isocitrate.

2.3.3.1.16. NADP-Linked Isocitrate Dehydrogenase (threo D_s-Isocitrate: $NADP^+$ Oxidoreductase [Decarboxylating] EC 1.1.1.42). This assay was done in MET-8.0, containing 0.2 mM-$NADP^+$, 4 mM-D,L-isocitrate and extract. The assay was started by isocitrate and followed at 412 nm.

2.3.3.1.17. Malate Dehydrogenase (L-Malate: NAD^+ Oxidoreductase EC 1.1.1.37). The enzyme was assayed by following OAA-dependent oxidation of NADH. The assay contained either MET-8.0 or MET-8.6, 0.2 mM-NADH, extract and 0.2 mM-OAA. Malate dehydrogenase was also assayed by linking its activity to CS and assaying CoA production with DTNB. The assay mixture contained either MET-8.6 or 20 mM-potassium phosphate buffer pH 7.5, 0.5 mM-NAD^+ (or 0.5 mM-$NADP^+$), 0.2 mM-acetyl-CoA, 0.1 mM-DTNB, 5.8 U porcine heart CS and extract. The assay was monitored at 412 nm for malate dehydrogenase-independent thiol production. The assay was started by addition of malate to a concentration of 1 mM.

2.3.3.1.18. Malate Oxidase (L-malate: Oxygen Oxidoreductase EC 1.1.3.3). This enzyme was assayed according to Francis et al. (1963). The assay mixture contained 66.7 mM-potassium phosphate pH 7.2, 6.7 mM-potassium cyanide, 0.0003% DCPIP, 6.7 mM-malate and extract. The assay was started by the addition of malate, the oxidation of which was followed as reduction of DCPIP at 600 nm.

2.3.3.1.19. 2-Oxoglutarate Dehydrogenase (2-Oxoglutarate Dehydrogenase: Lipoate Oxidoreductase [Decarboxylating and Acceptor Succinylating] EC 1.2.4.2). The assay for this enzyme was similar to that of PDH (**2.3.3.1.7.**) except for the substitution of 0.2 mM-2-OG in place of 0.2 mM-pyruvate.

2.3.3.1.20. Succinate Dehydrogenase (Succinate: [Acceptor]

Oxidoreductase EC 1.3.99.1). Succinate dehydrogenase was assayed according to Müller et al. (1968). Succinate oxidation was linked to the DCPIP reduction with N-methyl phenazine methosulphate. The assay conditions were 0.1 M-potassium phosphate pH 7.5, 0.1 mM-DCPIP, 1.5 mM-N-methyl phenazine methosulphate, 3 mM-sodium cyanide, extract and 30 mM-succinate. Succinate-independent DCPIP reduction by the extract was recorded and the assay was initiated by addition of succinate.

2.3.3.1.21. Succinate Thiokinase (Succinate-CoA Ligase [ADP-Linked] EC 6.2.1.5). The enzyme was assayed in two ways:

a) The Discontinuous Assay of Kaufman et al. (1953). This depends on the cleavage of enzyme-produced succinyl-CoA with hydroxamic acid to CoA and succinyl-hydroxamate. The latter forms a chromophoric complex with ferric chloride. The assay mixture contained 50 mM-Tris pH 7.4, 50 mM-succinate, 5 mM-ATP, 65 µM-CoA, 10 mM-DTT, 5 mM-$MgCl_2$ and 0.5 M-hydroxylamine hydrochloride. Extract was added, and the final volume was 2 ml. The mixture was incubated at 37°C for 30 min. The control contained all the above constituents except ATP. After incubation, 3 ml of a freshly prepared solution of 1.67% (w/v) ferric chloride and 4% (w/v) TCA in 1 M-HCl were added. The solution was then clarified by centrifugation at 2,500 X g for 10 min at 4°C. The absorbance of the supernatant was then read at 540 nm. The assay was used qualitatively to detect the presence or absence of ADP-STK.

b) The Assay of Cha (1969). ADP produced by STK was detected with pyruvate kinase and lactate dehydrogenase at 340 nm. The assay mixture contained MET-8.0, 0.2 mM-CoA, 1 mM-ATP, 10 mM-succinate, 0.32 mM-NADH, 1.1 mM-PEP, 9.3 U pyruvate kinase, 13.8 U lactate dehydrogenase and extract. The assay was initiated with succinate, the rate being the difference between assay with and without succinate.

2.3.3.1.22. Isocitrate Lyase (threo D_s-Isocitrate Glyoxylate Lyase EC 4.1.3.1). The enzyme was assayed by following glyoxylate production. Glyoxylate was detected by the reaction of its carbonyl group with phenylhydrazine to form the phenylhydrazone which absorbs at 333 nm (Dixon and Kornberg, 1959). The assay mixture was 50 mM-Mops pH 7.3, 1 mM-EDTA, 5 mM-$MgCl_2$, 4 mM-D,L-isocitrate and extract.

2.3.3.2. Polarographic Enzyme Assays. I am indebted to Dr. T.M. Jenkins for performing all the polarographic enzyme assays in this thesis. Due to the limited use of the polarograph in biochemistry, a brief account of this technique is given below:

Polarography exploits current production at a polarizable micro-electrode that has been induced by a potential difference (PD). The PD is applied between the polarizable micro-electrode (in this case, a mercury dropping electrode) and a non-polarizable calomel electrode connected by a salt bridge. Reduction of an electro-active species at the dropping mercury electrode causes a 'cathodic current'. Oxidation of an electro-active species causes current flow in the opposite direction which is called an 'anodic current'.

Raising the PD from zero causes little current flow between the dropping electrode and the electro-active species until the PD required for oxidation or reduction of the latter is approached. From this point over a small rise in PD, a very large current increase is observed. Further increases of PD allow the electro-active species to be oxidised or reduced as fast as it reaches the electrode. At this point, the current (now a 'limiting current') to and from the electro-active species is determined by the diffusion rate of the latter to the dropping electrode. Thus the limiting current measures the electro -active species concentration. Polarography has been used to assay a number of enzymes using electroactive species like carbonyl groups, CoASH, lipoamide, dihydrolipoamide and $NAD(P)^+$ (Weitzman, 1976).

2.3.3.2.1. Polarographic Assay of CS and Succinate Thiokinase. All assays were carried out at 25°C in 1 ml, with a Radiometer PO_4 polarograph (Radiometer, Copenhagen, Denmark.). The instrument was equipped with a glass cell (containing the assay solution) fitted to a polythene stopper through which the dropping mercury electrode, salt bridge (a tube containing 2% (w/v) agar in saturated potassium chloride), inlet and outlet holes for nitrogen and a sample port passed. Both STK and CS were assayed by following CoA production at a PD of -0.3 V. At this PD, CoA donated electrons to mercury;

$$Hg + 2\ CoASH \longrightarrow Hg(CoA)_2 + 2\ H^+ + 2\ e^-$$

with the production of a limiting current. The response of the electrode was calibrated with 1 mM-CoA such that a response of 1.63 μA = 1.00 μM-CoA. Assays were carried out under nitrogen to prevent interference from oxygen which, under these conditions would have been a reducible electro-active species (Morris, 1974).

For CS, the assay mixture composition was identical to that of the spectrophotometric assay (**2.3.3.1.13.**) with the omission of DTNB. OAA-dependent current production was measured continuously.

For STK, the assay composition was 20 mM-potassium phosphate, pH 7.0, 0.15 mM-succinyl-CoA, 10 mM-$MgCl_2$ and 1 mM-ADP, IDP or GDP. Nucleotide-dependent current production was continuously monitored.

2.3.3.3. Estimation of K_m and V_{max}. This was carried out using the direct linear plot of Eisenthal and Cornish-Bowden (1974). As recommended by these authors, the data are shown in half-reciprocal form (Hanes, 1932). It should be noted that K_m and V_{max} values were determined at saturating but finite concentrations of the co-substrate and are apparant values but are referred to as "K_m" and "V_{max}" here.

2.3.4. Polyacrylamide Gel Electrophoresis

2.3.4.1. Non-denaturing Conditions. This was carried out according to the method of Davies et al. (1964). Gels were cast in 9 X 0.5 cm glass tubes. The gel composition was as follows: 7% (w/v) polyacrylamide (6.82% (w/v) acrylamide, 0.18% (w/v) bis-acrylamide), 0.378 M-Tris, 38 mM-glycine pH 7.9, 0.058% (v/v) TEMED and 0.7% (w/v) ammonium persulphate. Gel solutions were poured into glass tubes, overlain with water and set overnight at room temperature. Samples were dried down under vacuum and resuspended in 50 μl 50 mM-Tris, 38 mM-glycine and 10% (w/v) sucrose, adjusted to pH 8.9 and coloured with bromophenol blue. After sample application, electrophoresis was conducted in a running buffer of 50 mM-Tris and 38 mM-glycine (pH 8.9) using a Bio-Rad rod gel electrophoresis cell (Bio-Rad Laboratories Ltd.). Samples were electrophoresed initially at a constant current of 0.5 $mA.gel^{-1}$ until the sample had entered the gel and then at 2 $mA.gel^{-1}$. Gels were fixed and stained as described in **2.3.4.4.**.

2.3.4.2. Continuous Denaturing Conditions. The phosphate-based method of Shapiro et al. (1967) was used. 7.5% (w/v) polyacrylamide gels (7.3% (w/v) acrylamide and 0.2% (w/v) bis-acrylamide) were cast as a slab gel of dimensions 14 X 14 X 0.15 cm. Gels were polymerised in 0.1 M-sodium phosphate adjusted to pH 6.7, 0.1% (w/v) SDS, 0.05% (v/v) TEMED and 0.1% ammonium persulphate. Once the gel had been poured, it was overlain with water and set at room temperature overnight. Protein samples were dried down under vacuum and suspended in 50 µl of 0.1 M-sodium phosphate, adjusted to pH 6.7, 10% (v/v) glycerol, 0.1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol, coloured with a small quantity of bromophenol blue (loading buffer). After heating in boiling water for 2 min, samples were cooled and applied to gels. Electrophoresis was carried out in an Atto standing slab gel apparatus (Atto Corporation, Tokyo, Japan), in a running buffer composed of 0.1 M-sodium phosphate and 0.1% SDS (w/v), adjusted to pH 6.7 and at a constant current of 2 mA per track until the sample had entered the gel. Electrophoresis was then carried out at 6 $mA.gel^{-1}$ at room temperature.

2.3.4.3. Discontinuous Denaturing Conditions. The method of Laemmli (1970) was used. The resolving gel was poured as a gradient of 9% to 20% (w/v) acrylamide as follows: 19.6% (w/v) acrylamide, 0.4% (w/v) bis-acrylamide, 12% (w/v) sucrose and 0.02% (w/v) ammonium persulphate in 0.241 M-Tris pH 8.8 was placed in one chamber of a gradient maker. The second chamber contained a similar solution except that acrylamide, bis-acrylamide and sucrose were 8.82, 0.08 and 6% (w/v) respectively. The chamber with the higher acrylamide concentration was placed on a magnetic stirrer and its outlet was fed to the bottom of a 14 X 14 X 0.15 cm standing slab gel mould. 0.0007% (v/v) TEMED was added. The connection between the two chambers was opened and the gel solution was pumped into the mould. Once the gel had been poured, the gel surface was overlain with water and the gel was allowed to polymerise overnight. Once the resolving gel had set, the water was removed and the resolving gel was overlain with a stacking gel of 0.13% (w/v) bis-acrylamide, 4.91% (w/v) acrylamide, 0.05% (w/v) ammonium persulphate and 0.2% (v/v) TEMED in 0.143 M-Tris pH 6.8. The stacking gel was allowed to polymerise and the gel was mounted in an

Atto standing slab gel apparatus. Samples were prepared as described in **2.3.4.2.** and were applied to the stacking gel. Electrophoresis was carried out in a running buffer of 50 mM-Tris, 38 mM-glycine and 1% (w/v) SDS, adjusted to pH 8.3, at a constant current of 50 mA to 60 mA per gel.

2.3.4.4. Gel Staining. The gel was immersed in 0.1% (w/v) Coomassie Blue in methanol, water and acetic acid (1:17.5:1.5) for 1 h. Excess stain was removed by methanol:water:acetic acid (same proportions).

2.3.4.5. Polypeptide M_r Determination. Polypeptide M_r values were deduced from the migration of standard proteins in denaturing conditions. Ovotransferrin, bovine serum albumin, ovalbumin, bovine erythrocyte carbonic anhydrase, equine cytochrome c and equine myoglobin of polypeptide M_r values 76,000, 66,250, 45,000, 30,000, 17,200 and 12,300 respectively were standards. Their relative mobilities were determined and plotted vs. $\log_{10}M_r$. This relationship was then used to determine the Mr of sample polypeptides.

2.3.5. Native M_r Determination by Gel Filtration

A 102 X 2.5 cm Sephacryl S-200 column was packed (**2.7.2.3.**) and was equilibrated at 4°C with elution buffer at 10 $ml.h^{-1}$ until the pH of the eluate was that of the buffer entering the column. The void volume of the column was then estimated from the elution volume of blue dextran (M_r is about 3 X 10^6). 1 ml of a 3 $mg.ml^{-1}$ solution of blue dextran in elution buffer was loaded onto the column and eluted in the same buffer at 10 $ml.h^{-1}$. Fractions were collected using an LKB 2070 UltroRac II fraction collector.

The column was calibrated with M_r standards. These were loaded in up to 1 ml in buffer and eluted at 10 $ml.h^{-1}$. Elution of standards was monitored thus allowing the relationship between $\log_{10}M_r$ and elution volume to be determined. The elution volume of the enzyme was then estimated from the position of elution of its activity. The M_r of the enzyme to be found from the log relationship between protein standard M_r and elution volume.

2.3.6. HPLC.

Nicotinamide nucleotide purity was assayed by HPLC with a LDC-Milton Roy HPLC system (LDC-Milton Roy, Staffs, U.K.) equipped with a 15 X 0.46 cm Hypersil C-18 reverse phase column (5 µm particle diameter; Phasesep, Chester, U.K.) The column was equilibrated in 10 mM-potassium phosphate pH 6.8 (equilibration buffer) and loaded with NAD^+ (Sigma) and $NADP^+$ (Boehringer Mannheim) in equilibration buffer (0.1 mg nucleotide per 20 µl injection). The column was eluted with equilibration buffer at 2 $ml.min^{-1}$ for 10 min and then a gradient of 0-40% (v/v) methanol in the same buffer applied over 15 min at the same flow rate. $NAD(P)^+$ elution was followed at 260 nm.

2.3.7. Amino Acid Analysis

I am indebted to Miss S. O'Neil for performing the protein sample hydrolysis and operation of the amino acid analyser.

2.3.7.1. Sample Reduction and S-Carboxymethylation. This was carried out according to Gibbons and Perham (1970). Samples were dialysed overnight in TE-8.0 and then dried down in acid-washed glass tubes (50 µg protein per tube). Samples were resuspended in 1 ml of 6 M-guanidine hydrochloride in 0.1 M-Tris, adjusted to pH 8.5. 20 µl of fresh 0.1 M-DTT was added. The mixture was then degassed with a vacuum pump and flushed with nitrogen. The tube was sealed and incubated at 37°C for 1 h. 20 µl of 0.5 M-iodoacetic acid was added and the sample was degassed and flushed with nitrogen as before. The sample was then incubated in the dark for 30 min at 37°C. The reaction was quenched by adding 10 µl 2-mercaptoethanol and the mixture was dialysed overnight in 50 mM-Tris and 2 M-urea, adjusted to pH 8.5. Finally the sample was dialysed for 48 h against water after which it was dried down.

2.3.7.2. Protein Hydrolysis in 6M-Hydrochloric Acid. Samples were resuspended in 0.2 ml of 6M-HCl and 0.05% (v/v) 2-mercaptoethanol, evacuated and sealed. Hydrolysis was then carried out at 105°C for 24, 48 and 72 h. Samples were then dried down under vacuum and resuspended

in 0.2 ml of 25 mM-HCl for amino acid analysis.

2.3.7.3. Protein Hydrolysis in 3 M-Mercaptoethane Sulphonic Acid. Due to the instability of tryptophan in 6 M-HCl (Perham, 1978), samples were hydrolysed for 24 h in 0.2 ml of 3 M-mercaptoethane sulphonic acid as for hydrolysis in 6 M-HCl. After incubation, the sample was neutralised by the addition of 0.2 ml of 1 M-NaOH.

2.3.7.4. Amino Acid Separation and Analysis. Amino acid analysis was carried out using a Rank-Hilger Chromospek J180 amino acid analyser (Rank-Hilger Ltd, Kent, U.K.). Hydrolysates (0.2 ml) were separated at 60°C on the exchange column of the analyser with a non-linear gradient between an acidic buffer (0.1 M-citric acid, 0.2 M-sodium chloride, 0.35% (w/v) BRIJ-35, 0.0625% (v/v) thiodiglycol, 10% (v/v) propan-2-ol; pH 2.2) and a basic buffer (45 mM-disodium tetraborate, 0.1 M-sodium hydroxide, 0.116% (w/v) BRIJ-35, 4% (w/v) EDTA; pH 11.5). Amino acids were detected by reaction with 1% (w/v) ninhydrin in 3.33 M-sodium acetate, 40% (v/v) 2-methoxyethanol, 1% (w/v) BRIJ-35 and 0.001% (w/v) KCN. The amino acids were identified by comparison with elution of standard amino acids (10 nmole each).

2.3.8. Electron Microscopy

Samples (0.05-0.22 $mg.ml^{-1}$ in TE-8.0) were applied dropwise onto glow-discharge treated carbon-coated electron microscope grids (Reissing and Orrell, 1970). After 1 min the grids were washed and negatively stained by dropwise addition of 50 µl of 1% (w/v) uranyl acetate. Grids were then blotted and dried. Electron microscopy was carried out with a Jeol 2000fx electron microscope (Jeol, Japan) operating at an accelerating voltage of 200 KeV. All images were obtained at constant magnification, the value and variability of which were calibrated using images of negatively stained catalase crystals (Wrigley, 1968) taken at frequent intervals during the experiment. The magnification used was 53,300 $\pm$ 1%. I am indebted to Dr. J. Berriman (School of Material Sciences, University of Bath) for performing all the electron microscopy described in this thesis.

CHAPTER 3

The State of the Citric Acid Cycle in *Methylophilus methylotrophus*

3.1. Introduction

The CAC of M. methylotrophus has been partially characterised (Section C, Chapter 1). However, there are certain points concerning the CAC of this organism that need attention. Thus, the supply of acetyl-CoA to the CAC of M. methylotrophus has not been established; however, OAA may be supplied by pyruvate carboxylase (Aperghis, 1981). Further, examination of the CAC's enzyme complement has been limited to CS, IDH, OGDH, and malate dehydrogenase. The presence of any other CAC enzyme has not been ascertained. Thus the CAC enzymes were assayed to assess the integrity of the CAC in methanol-grown M. methylotrophus.

Additionally, involvement of the CAC in nitrogen assimilation was examined by assay of those enzymes that effect amination of 2-OG.

The response of the M. methylotrophus CAC or the CAC of any other obligate methylotroph to heterotrophic substrates has not been studied. This area has become of interest with the recent discovery of an acetamide-induced amidase in M. methylotrophus (Silman et al., 1989). This enzyme hydrolyses acetamide to acetate and ammonia:

$$CH_3CONH_2 + H_2O \longrightarrow CH_3COOH + NH_3$$

As amidase may cause acetate to accumulate in M. methylotrophus, the CAC may have some role in disposing of this accumulation. Thus, the effects of acetamide and acetate on acetate activation to acetyl-CoA, the CAC enzymes and the normal routes of supply of acetyl-CoA and OAA to the CAC were investigated.

3.2. Results

Unless otherwise stated, assays were performed on methanol-grown cultures of the organism harvested in late exponential phase (Section 2.3.)

3.2.1. Enzymes Synthesising Acetyl-CoA and OAA in M. methylotrophus

These results are summarised in Table 11.

Table 11

Specific Activities of CAC Enzymes that Convert OAA to Succinyl-CoA and Synthesise Acetyl-CoA and OAA in Methanol-Grown M. methylotrophus

Enzyme	Specific Activity ($mU.mg^{-1}$)	Number of Extracts Assayed
Acetyl-CoA Synthetase	6.1	3
Acetate Kinase	0	3
Thiolase	0	1
3-Oxo-Acid CoA-Transferase	0	1
Phosphotransacetylase	0	3
PDH	36.1	3
Pyruvate Carboxylase	4.6	3
CS	28.0	3
Aconitase	2.0	1
NAD-IDH	19.4	3
NADP-IDH	2.0	3
OGDH	0	3

On extraction of late exponential M. methylotrophus cells into MET-8.0, pyruvate carboxylase had a specific activity of 4.6 $mU.mg^{-1}$. The presence of this enzyme would allow supply of the CAC with OAA. PDH was also detectable in these extracts at a specific activity of 36.1 $mU.mg^{-1}$ and would therefore facilitate the synthesis of acetyl-CoA from pyruvate.

Acetyl-CoA can also be synthesised directly from acetate: **a)** via acetyl-phosphate using acetate kinase (i) and phosphotransacetylase (ii), or b) via activation of acetate directly to acetyl-CoA:

a) Acetate + ATP ⟶ Acetyl-phosphate + ADP (Acetate Kinase; i)

Acetyl-phosphate + CoA ⟶ Acetyl-CoA + Pi (Phosphotransacetylase; ii)

Sum: Acetate + ATP + CoA ⟶ Acetyl-CoA + ADP + Pi

b) Acetate + ATP + CoA ⟶ Acetyl-CoA + AMP + PPi (Acetyl-CoA Synthetase)

Extracts of M. methylotrophus prepared in MET-8.0 contained no acetate kinase. The direct assay of phosphotransacetylase by following acetyl-CoA thioester bond formation at 232 nm (**2.3.3.1.5.** a) was not possible because of the high absorbance of the assay mixture. However, this was not so with the indirect coupled assay (**2.3.3.1.5.** b) but even with this, phosphotransacetylase was not detected, thus ruling out route (a). ATP and CoA dependent synthesis of acetyl-CoA (acetyl-CoA synthetase) was, however, detected (specific activity of 6.1 $mU.mg^{-1}$) by a similar indirect coupled assay of acetyl-CoA production. The direct assay for acetyl-CoA synthetase suffered similar problems to those encountered with the direct phosphotransacetylase assay. The above results suggest that route (b) could operate.

Acetyl-CoA may also be produced by thiolysis of acetoacetyl-CoA:

Acetoacetyl-CoA + CoA ⟶ 2 Acetyl-CoA

The reaction is catalysed by thiolase. There was no thiolase activity in extracts of M. methylotrophus prepared in MET-8.0, nor was there any activity of the enzyme that synthesises acetoacetyl-CoA, 3-oxo-

acid CoA transferase in similarly prepared extracts.

3.2.2. Enzymes of the CAC and Glyoxylate Cycle

3.2.2.1. Enzymes Converting OAA to Succinyl-CoA. CS, aconitase and IDH were present in extracts prepared in MET-8.0 (Table 11). No OGDH was detected. IDH seemed to be mostly NAD-linked; however, $NADP^+$ could replace NAD^+ with 10.3% of the NAD-linked activity. Examination of the $NADP^+$ used here by HPLC (**5.1.3.3.1.**) confirmed that it was not contaminated by NAD^+ and so did not cause an erroneous observation. This is pursued further (**5.1.3.3.**).

3.2.2.2. Enzymes Interconverting Succinyl-CoA and OAA. The results recorded below are summarised in Table 12.

3.2.2.2.1. Succinate Dehydrogenase. In M. methylotrophus grown overnight no succinate dehydrogenase could be detected in either crude extract prepared in 0.1 M-potassium phosphate, pH 7.5, or the pellet obtained in the preparation of the crude extract (insoluble fraction).

3.2.2.2.2. Succinate thiokinase. ATP, GTP and ITP-linked STK activity was assayed in various ways in extracts prepared in various buffers or toluenised cells from overnight cultures:

In extract prepared with 20 mM-potassium phosphate (adjusted to pH 7.0), the polarographic assay detected an ADP-linked STK activity of 1.0 $mU.mg^{-1}$. No ADP-linked activity was found in the insoluble fraction. Further, no activity was observed when ADP was replaced with the same concentration of GDP or IDP in the crude extract. Repetition of the experiment where both the assay buffer and the extraction medium were supplemented with 20% (v/v) glycerol gave identical results to those obtained without the incorporation of glycerol.

The low STK specific activity may have resulted from extraction. Thus, instead of extracting the enzyme, the cells were permeabilised to its substrates by treatment with toluene, and the enzyme was assayed polarographically in situ. However, no ADP-STK activity could be detected in cells treated in this manner.

Table 12

Specific Activities of ICL and Enzymes of the CAC of M. methylotrophus that interconvert Succinyl-CoA and OAA

Enzyme	Fraction	Specific Activity ($mU.mg^{-1}$)	Number of Extracts Assayed
Succinate Dehydrogenase	C*	0	1
	I*	0	1
STK(P)			
ADP-linked	T*	0	1
ADP-linked	C	1.0	2(i)
ADP-linked	I	0	2(i)
GDP-linked	C	0	2(i)
IDP-linked	C	0	2(i)
STK(S)			
ATP-linked	C	1.6	2
Fumarase	C	47.3	3
Malate Dehydrogenase			
NAD-linked	C	0	1
NADP-linked	C	0	1
NADH-linked	C	0	5(ii)
NADPH-linked	C	0	2(iii)
Malate Oxidase	C	0	1
	I	0	1
ICL	C	0.4	3

*: C=Crude extract; T=Toluene treated cells; I=Insoluble Fraction
(P)=Polarographic Assay; (S)=Spectrophotometric Assay.
(i) signifies one determination was carried out in the absence of glycerol; the other determination was carried out in the presence of glycerol.
(ii) Signifies that 3 extracts were assayed at pH 8.0, and 2 extracts were assayed at pH 8.6
(iii) Signifies that 1 extract was assayed at pH 8.0 and the other was assayed at pH 8.6.

To attempt to verify the results obtained by polarographic assay, extracts of M. methylotrophus were assayed spectrophotometrically using either the discontinuous method of Kaufman et al. (1953) or the continuous method of Cha (1969). These assays detect the reaction reverse to that detected by the polarographic assay. ATP-linked STK activity was found only in extracts of late-exponential cultures (prepared in MET-8.0) by the assay of Cha (1969). The STK specific activity observed by this method was 1.6 $mU.mg^{-1}$.

3.2.2.2.3. Fumarase. The enzyme was detectable in extracts prepared in 0.1 M-Tris, 20 mM-$MgCl_2$, 5 mM-EDTA and 20% (v/v) glycerol, adjusted to pH 7.5. Under these conditions, fumarase was present at 43 $mU.mg^{-1}$.

3.2.2.2.4. Malate Dehydrogenase. Malate dehydrogenase was assayed in extracts of M. methylotrophus (cultured overnight) prepared in MET-8.6. Assays that followed OAA-dependent oxidation of NADH or NADPH were performed at pH 8.6 and 8.0 in MET-8.6 and MET-8.0. In both assay media, neither NADH nor NADPH-linked activity could be detected. The second assay used for the detection of this enzyme involved coupling NAD(P)-linked malate-dependent OAA production to CS. Thus any enzyme activity could be followed as DTNB reduction. However, as with the previous assay method, no activity was detectable.

3.2.2.2.5. Malate Oxidase. No activity was observed in either the insoluble fraction or crude extract of an overnight culture of M. methylotrophus extracted into 20 mM-potassium phosphate pH 7.0.

3.2.2.2.6. Isocitrate lyase. An apparent specific activity of 0.3 $mU.mg^{-1}$ was detected in extracts prepared in MET-8.0.

3.2.3. Synthesis of Glutamate from 2-OG

Extracts of overnight cultures of M. methylotrophus prepared in 20 mM-potassium phosphate (pH 7.0) were assayed for GS, NADH-GOGAT, NADPH-GOGAT, NADH-GDH and NADPH-GDH activity. As can be seen from Table 13, only GS and NADH-linked GOGAT were present.

Table 13

Specific Activities of Enzymes Responsible for Ammonia Assimilation in M. methylotrophus

Enzyme	Specific Activity ($mU.mg^{-1}$)	Number of Extracts Assayed
GS	1086	3
GOGAT		
NADH-linked	47.2	2
NADPH-linked	0	2
GDH		
NADH-linked	0	1
NADPH-linked	0	1

3.2.4. Effect of Acetate and Acetamide on the CAC of M. methylotrophus

To compare the CAC of M. methylotrophus grown under different conditions, it had to be established that comparisons were made between cells that had reached the same stage of growth. For these experiments, this was the late exponential phase of growth. Therefore, the growth characteristics of M. methylotrophus were determined.

Cultures of M. methylotrophus were pre-adapted on solid media to growth on methanol in the presence of 10 mM-sodium acetate or 10 mM-acetamide (**2.1.1.2.**). The organism was also adapted to growth on solid medium that was deficient in the normal nitrogen source in the presence of 10 mM-acetamide and methanol.

Flasks of 100 ml of AS1 medium, methanol and additions of 10mM-acetate or 10 mM-acetamide (+/- $(NH_4)_2SO_4$) were inoculated from solid media with a loopful of M. methylotrophus culture. After growth for 39 h, 5 ml of these cultures were used to inoculate 250 ml fresh AS1 media (supplemented as previously). The growth was then followed spectrophotometrically at 680 nm (**2.1.1.3.**).

The growth of M. methylotrophus on methanol with and without acetate or acetamide (+/- NH_4^+) is shown in Figure 17. These curves suggested that in batch culture, M. methylotrophus grown in the presence of methanol reached late exponential phase at approximately 14 h, while with acetate or acetamide (+/- NH_4^+) supplements, the same stage of growth was reached after 16 h incubation. Thus to provide cell material for enzyme assay, 39 h starter cultures (100 ml) were used to provide 2% (v/v) inocula for 250 ml cultures that were grown until late exponential phase and then harvested.

It was determined that no growth occurred in batch culture in the presence of 10 mM-acetate or 10 mM-acetamide unless methanol was present. Thus, it was unlikely that the results recorded below were affected by the presence of a contaminating heterotrophic organism. All cell-free extracts were prepared in MET-8.0.

3.2.4.1. Effect of Acetate on the CAC and Related Enzymes. There were increases in CS, NAD-IDH, acetyl-CoA synthetase and pyruvate carboxylase relative to their activities without acetate (Table 14).

Figure 17

Growth of M. methylotrophus on Methanol in the Presence and Absence of Acetamide, NH_4^+ and Acetate

(*): Regime A in text

(*'): Regime B in text

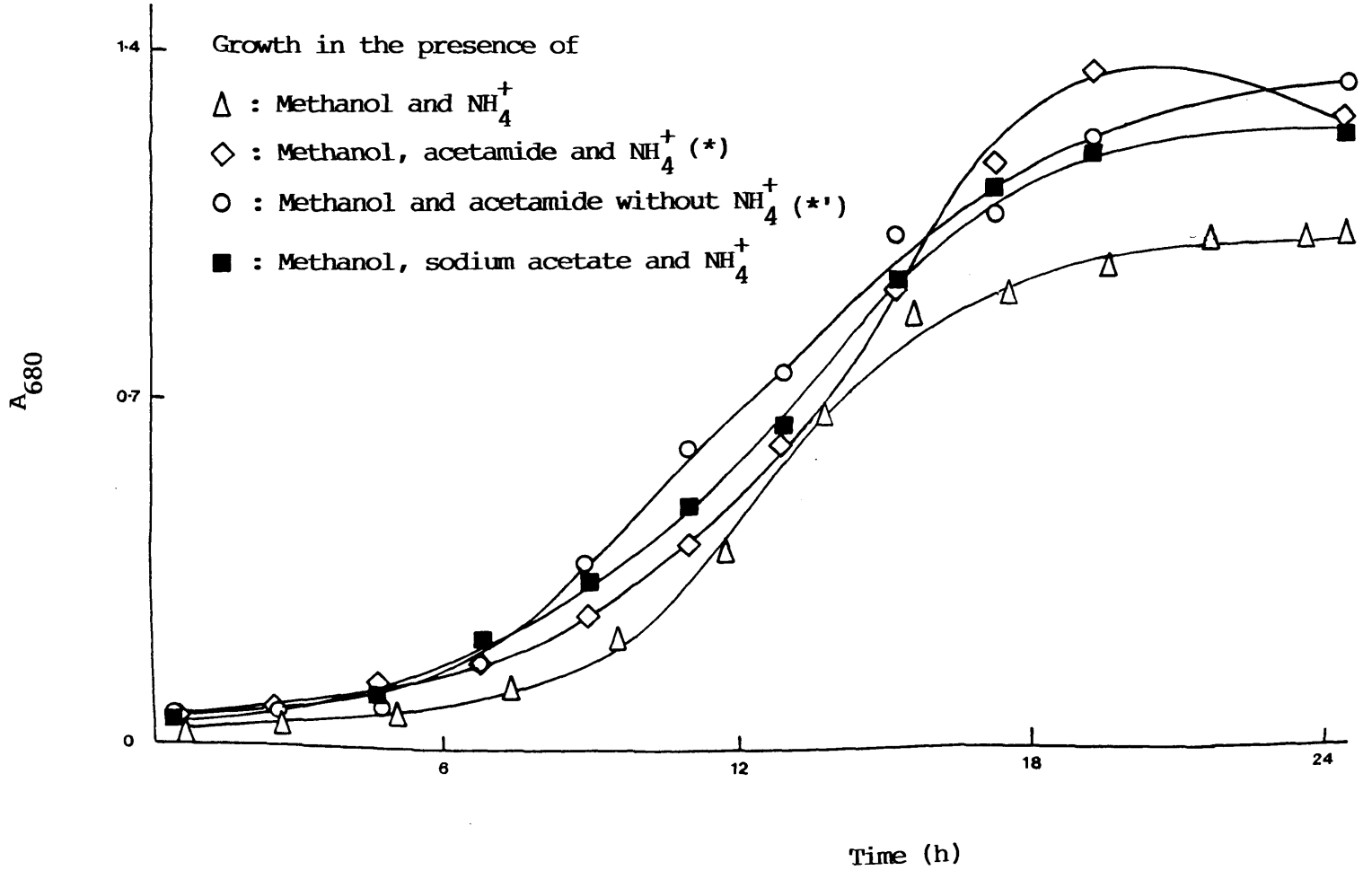


Table 14

The Effect of 10mM-Acetate on the Specific Activities of the CAC and Related Enzymes from Methanol-Grown M. methylotrophus

Enzyme	Specific Activity ($mU.mg^{-1}$)	Number of Extracts Assayed	Activity Relative to Activity on Methanol Alone*
Acetyl-CoA Synthetase	14.2	2	2.3
Phosphotrans-acetylase	0	2	-
Acetate Kinase	0	2	-
Pyruvate Carboxylase	8.1	2	1.8
PDH	17.5	2	0.48
CS	51.0	2	1.8
Aconitase	0.6	2	0.3
NAD-IDH	44.5	2	2.3
NADP-IDH	1.6	2	0.8
ICL	0.1	2	0.33
OGDH	0	2	-

*: Value quoted is the specific activity of the enzyme recorded in in this table divided by the value of the Specific activity given in Table 11.

The activity of aconitase was unaffected by acetate. No OGDH was detected. PDH fell by 48% (Table 14). As with growth on methanol, no acetate kinase or phosphotransacetylase was detected in M. methylotrophus during growth in the presence of acetate.

Relative to their activities on methanol, the NADP-linked IDH showed no change in specific activity and the apparent ICL activity dropped in the presence of acetate (Table 14).

3.2.4.2. Effect of Acetamide on the CAC and Related Enzymes. The experiments with acetamide were carried out with two growth regimes: **A**, where M. methylotrophus was grown in the presence of acetamide in normal AS1 medium; **B**, where M. methylotrophus was grown in the presence of acetamide in AS1 medium deficient in the normal nitrogen source ($(NH_4)_2SO_4$). Organisms grown under regime **B** were presumed to be obliged to use acetamide as a nitrogen source whereas organisms grown under regime **A** were not.

3.2.4.2.1. Regime A. A large increase in amidase activity (5.9-fold over that found during growth without acetamide) was observed (Table 15). Of those enzymes that might have dealt with the increased amount of acetate generated by the increased amidase activity, no acetate kinase or phosphotransacetylase was detected. However, as was the case with growth in the presence of acetate, acetyl-CoA synthetase activity increased 1.8-fold over the value found without acetamide. In a similar manner, but to a lesser extent than that observed in the presence of acetate, CS, NAD-IDH and pyruvate carboxylase all increased in specific activity (Table 16 c.f. Table 14). Also, as with acetate, PDH activity dropped by 23%. The specific activities of aconitase and ICL also fell slightly.

3.2.4.2.2. Regime B. Although amidase activity rose under these growth conditions, its increase over that found without acetamide (Table 15) (2.0-fold) was less than that found in regime **A**. However, generally, changes in CAC and related enzymes relative to activities in methanol-growth were similar but slightly larger than those found in regime **A** (Table 16). In accord with this, PDH activity fell by 51%. Both

Table 15

The Effect of 10 mM-Acetamide in the Presence and Absence of NH_4^+ on the Specific Activity of Amidase in Methanol-Grown M. methylotrophus

Growth Conditions	Amidase Specific[@] Activity ($mU.mg^{-1}$)	Amidase Activity Relative to Growth on Methanol + NH_4^+
Methanol + NH_4^+	11.6	1
*Methanol + NH_4^+ + Acetamide	68.1	5.9
$Methanol - NH_4^+ + Acetamide	22.8	2.0

@: Each value is a value determined from two extracts.

*: This growth condition corresponds to "Regime A" in the text.

$: This growth condition corresponds to "Regime B" in the text.

Table 16

The Effects of 10mM-Acetamide on the CAC and Related Enzymes of Methanol-Grown M. methylotrophus

Enzyme	Specific Activity ($mU.mg^{-1}$)		Activity Relative to Growth on Methanol Alone		Number of Extracts	
	*Regime A	*Regime B	Regime A	Regime B	Regime A	Regime B
Acetate Kinase	0	0	-	-	3	3
Phosphotransacetylase	0	0	-	-	3	3
Acetyl-CoA Synthetase	11.0	13.5	1.8	2.2	3	3
Pyruvate Carboxylase	6.4	6.9	1.4	1.55	3	3
PDH	27.9	17.5	0.77	0.49	3	3
CS	34.4	32.9	1.2	1.2	3	3
Aconitase	0.9	1.7	0.45	0.85	1	1
NAD-IDH	27.1	32.9	1.4	1.7	3	3
NADP-IDH	2.1	3.4	1.1	1.7	3	3
OGDH	0	0	-	-	3	3
ICL	0.2	0.9	0.67	3.0	3	3

*: Regime A=Growth with $(NH_4)_2SO_4$ and acetamide as N-sources; Regime B=Growth with acetamide as sole N-source.

aconitase and ICL dropped slightly in activity.

3.3. Discussion

3.3.1. Acetyl-CoA and OAA Synthesis

In agreement with Aperghis (1981), M. methylotrophus can synthesise OAA from pyruvate with pyruvate carboxylase. This supply is crucial as the OGDH lesion in this organism (see below) means the CAC cannot make its own OAA. The results also suggest that during growth on methanol, pyruvate decarboxylation by PDH is a source of acetyl-CoA. PDH has been observed in other methylotrophs, although there is doubt as to whether it supplies the CAC of serine pathway-utilisers (**1.9.1.**). The activity of PDH in M. methylotrophus was far greater than that of methylotrophically-grown Methylobacterium extorquens AM1 (Bolbot and Anthony, 1980). This may reflect the greater importance of PDH as a supplier of acetyl-CoA in obligate RuMP cycle utilisers such as M. methylotrophus relative to its importance in methylotrophically growing serine pathway organisms such as Methylobacterium extorquens AM1 (**1.9.1.**). As well as PDH, acetyl-CoA synthetase was found in M. methylotrophus during growth on methanol alone.

Other facultative methylotrophs like Methylobacterium and Hyphomicrobium X also have acetyl-CoA synthetase in C_1-growth. They resemble M. methylotrophus in that they do not use the other route of acetyl-CoA synthesis via acetate kinase/ phosphotransacetylase (Attwood and Harder, 1974; Taylor and Anthony, 1976b). It should be noted that although the acetyl-CoA synthetase assay used here and by Attwood and Harder (1974) detected ATP and CoA dependent acetyl-CoA synthesis, it did not distinguish the pyrophosphate cleavage:

$$\text{Acetate} + \text{CoA} + \text{ATP} \longrightarrow \text{Acetyl-CoA} + \text{AMP} + \text{PPi}$$

from the more reversible reaction:

$$\text{Acetate} + \text{CoA} + \text{ATP} \longrightarrow \text{Acetyl-CoA} + \text{ADP} + \text{Pi}$$

However, it has been shown that Methylobacterium extorquens AM1 uses the pyrophosphate cleavage reaction (Taylor and Anthony, 1976b).

In facultative methylotrophs acetyl-CoA synthetase is induced by and necessary for growth on carbon sources that are metabolised via acetate (Attwood and Harder, 1974; Taylor and Anthony, 1976b). However, the role in C_1-growth for this enzyme is obscure because Taylor and Anthony (1976b) found acetyl-CoA synthetase-deficient mutants of Methylobacterium can grow on reduced C_1 compounds. Thus, it is even more difficult to suggest a role for the enzyme in obligate C_1-utilisers like methanol-grown M. methylotrophus.

Thus, without a known means of acetate-generation to supply acetyl-CoA synthetase, it seems likely that OAA and acetyl-CoA can be supplied to the CAC of M. methylotrophus by the disproportionation of 2 molecules of pyruvate derived from methanol by the RuMP cycle. This is catalysed by pyruvate carboxylase (i) and PDH (ii):

(i) **Pyruvate** + ATP + CO_2 ⟶ **OAA** + ADP + Pi

(ii) **Pyruvate** + CoA + NAD^+ ⟶ **Acetyl-CoA** + CO_2 + NADH + H^+

SUM: 2 **Pyruvate** + ATP + CoA + NAD^+ ⟶ **Acetyl-CoA** + **OAA** + ADP + Pi + NADH + H^+

The lack of thiolase or 3-oxo-CoA transferase suggested that (a) during growth on methanol, acetoacetyl-CoA was not a source of acetyl-CoA, and (b) M. methylotrophus may not use poly-3-hydroxybutyrate as a carbon or energy reserve. This contrasts with facultative organisms like Methylobacterium which may use the polymer for such purposes during C_1-growth and have both enzymes (Taylor and Anthony, 1976b).

3.3.2. The CAC and Apparent ICL Activity of M. methylotrophus

3.3.2.1. Conversion of Acetyl-CoA and OAA to 2-OG. As in other organisms (**1.8.2.1.**), possession of CS, aconitase and IDH enables the CAC of M. methylotrophus to synthesise 2-OG from OAA and acetyl-CoA. The levels of CS and NAD-IDH are similar to those found in this organism by Taylor (1977) and Large and Haywood (1981). Likewise the levels of CS and NAD-IDH found here in M. methylotrophus (Table 11)

are similar to those of other obligate methylotrophs like organisms W6, 4B6 and C2A1, the type I methanotrophs Methylococcus capsulatus (Bath and Texas strains), Methylomonas methanica, Methylomonas albus, Methylobacter bovis, Methylococcus minimus, Methylobacter vinelandii, Methylobacter chroococcum, and the type M RFMs W3A1 and W6A (Colby and Zatman, 1972; Davey et al., 1972; Colby and Zatman, 1975a; Patel et al., 1975; Babel and Hofmann, 1975; Trotsenko, 1976).

In agreement with Taylor (1977), the IDH in M. methylotrophus uses $NADP^+$ and NAD^+, where NAD^+ is the preferred substrate. However, this is inconsistent with the results of Large and Haywood (1981), who found no NADP-linked IDH in this organism.

The level of aconitase during growth of M. methylotrophus on methanol is much lower than the levels of CS and IDH (Table 11), being present at 7% and 10% the levels of these enzymes respectively. Although there was not enough time available to find the cause of this low activity, it has been noted that in other C_1-utilisers, aconitase has sometimes been difficult to measure: Methylococcus capsulatus (Texas) has no assayable aconitase; however, its presence is suggested by the incorporation patterns of [1-^{14}C]acetate (Patel et al., 1975). Likewise the enzyme from the type I methanotroph Methylomonas albus resembles M. methylotrophus in that its levels of aconitase are well below those of its CS and IDH activities (Davey et al., 1972).

3.3.2.2. Interconversion of 2-OG and OAA. M. methylotrophus is no exception to the well established pattern of OGDH occurrence (**1.8.2.1.**). As would be expected of an obligate C_1-utiliser that does not use the serine pathway of C_1-assimilation, M. methylotrophus has no OGDH (Table 11). This result is in agreement with those of Large and Haywood (1981) and Taylor (1977).

Thus it seems likely that, as has been suggested for the incomplete CACs of other obligate RuMP cycle utilisers, the function of the M. methylotrophus CAC is biosynthetic, as the lack of OGDH disables its oxidative capacity (Eccleston and Kelly, 1973; Colby and Zatman, 1975a; Smith and Hoare, 1977; Zatman, 1981; Anthony, 1982). In this context, the data here are consistent with Taylor (1977) who was unable to detect acetate oxidation by M. methylotrophus. Thus, the

role of CS, aconitase, and IDH in M. methylotrophus is to provide C_5 skeletons (2-OG) for biosynthesis. This suggestion is supported by the alternative 'specialist' dissimilatory pathways that M. methylotrophus employs for catabolism of methanol (**1.7.2.**).

Of the enzymes assayed that interconvert succinyl-CoA and OAA, only STK and fumarase were present (Table 12). This result is at variance with the distribution of STK. Exclusive use of adenine nucleotides by STK is thought to be a Gram-positive characteristic (Weitzman, 1981, 1987); however, M. methylotrophus is Gram-negative.

Thus it seems that the CAC of M. methylotrophus cannot interconvert malate and OAA, or oxidise succinate to fumarate. This suggests there is no means of supplying either fumarase substrate (fumarate or malate). These results are similar to those obtained in other obligate non-methanotrophic methylotrophs (e.g. C2A1, 4B6) and type M RFMs (Colby and Zatman, 1972, 1975a), which also have enzyme lesions in this section of the CAC.

As OGDH is absent and M. methylotrophus may need succinyl-CoA for biosynthesis, it is possible that OAA would be reduced to succinyl-CoA by reversal of the oxidative CAC. However, the lack of malate oxidase or dehydrogenase reduces the likelihood of this. Otherwise, it has been noted that anaerobically-grown E. coli can reduce OAA to fumarate without malate reduction by using aspartate aminotransferase and aspartase. Fumarate can then be reduced to succinate via fumarate reductase (Miles and Guest, 1987). The enzymes required for this have not been assayed in any other methylotroph or here. However, attempts to show aspartate-dependent ammonia generation in M. methylotrophus (aspartase; results not shown) with the qualitative ammonia test (**2.3.2.2.**) were not successful. If this method of reduction of OAA to fumarate were ever demonstrated in M. methylotrophus, the function of the fumarase would still be cryptic.

The operation of fumarate reductase in M. methylotrophus was not pursued. The lack of succinate dehydrogenase activity would be expected if the enzyme was operating in a reverse direction relative to succinate oxidation, i.e. as a fumarate reductase (Gest, 1987); however, this is not proof for the operation of this enzyme. There are, however, other ways to produce succinate and hence succinyl-CoA.

3.3.2.3. Isocitrate Lyase. A low level of ICL was detected in M. methylotrophus (Table 12). The ICL also occurs in small quantities in other RuMP cycle utilisers such as organisms C2A1, 4B6, type M RFMs W3A1 and W6A (Colby and Zatman, 1972, 1975a). However those findings and the current data are undermined by the work of Attwood (1977) who found that the assay used here may cause artefactual observations of ICL in the presence of IDH. If, however, the presence of ICL in M. methylotrophus was confirmed, it would, in conjunction with STK, allow the CAC to produce succinyl-CoA for use in biosynthesis, independent of the interconversion of OAA and succinate (c.f. **1.10.1.**).

3.3.3. Glutamate Synthesis From 2-OG

In Gram-negative heterotrophs, GS is controlled by covalent modification. Adenylylation inhibits GS, while deadenylylation reactivates it. Regulation of GS is thus achieved through the regulation of the enzyme activities that control its adenylylation state. Two effectors that can do this that are relevant here are 2-OG and glutamine. The former stimulates deadenylylation and stimulation of GS, while glutamine stimulates adenylylation and thus inactivation of GS (Tyler, 1978). This regulatory mechanism has also been found amongst the methanotrophic GSs. Murrell and Dalton (1983a) purified Methylococcus capsulatus (Bath) GS and found it to be adenylylated. However, not all methylotrophic GSs are adenylylated, e.g. the Methylobacterium extorquens AM1 GS (Bellion and Bolbot, 1983).

The gamma-glutamyl transferase assay measures an activity that is independent of the adenylylation state of GS (Tyler, 1978). Thus, the results reported here with M. methylotrophus (Table 13) may reflect the total activity of GS and exceed that of the enzyme in vivo if it is adenylylated. If this was so, it could go some way to explaining why GS activity was far higher (23-fold) than the next enzyme involved in assimilation of ammonia, GOGAT (Table 13). Murrell and Dalton (1983b) have obtained similar results with the glutamyl-transferase assay. Indeed when a GS assay reflecting the adenylylation state of the enzyme was used, the values obtained in other methylotrophs more closely resembled the activities of GOGAT (Shishkina and Trotsenko,

1979; Murrell and Dalton, 1983b).

M. methylotrophus GOGAT was linked to NADH. As IDH in this organism is mainly NAD-linked, it may be that as well as 2-OG, IDH provides the NADH required by the GS/GOGAT ammonia assimilatory pathway. Thus NADH would act catalytically in ammonia assimilation. Such close coupling of reducing equivalents between the CAC and ammonia assimilation via GOGAT/GS does not seem to be essential as the type II methanotrophs like Methylocystis parvus and Methylosinus trichosporium have an NADP-IDH and an NADH-linked GOGAT (Davey, et al., 1972; Shishkina and Trotsenko, 1979; Murrell and Dalton, 1983b).

No GDH activity was detected in M. methylotrophus, in agreement with the results of Windass et al. (1980). When Windass et al. (1980) inserted NADP-linked GDH into M. methylotrophus and eliminated NADH-GOGAT, any cycling of NAD/NADH between the CAC and ammonia assimilation may have been disrupted. Again, that the organism could grow with NADP-linked GDH shows that coupling between the CAC and ammonia assimilation via NAD/NADH may not be essential for growth.

The distribution of the GS/GOGAT and GDH mechanisms for ammonia assimilation show no pattern with regard to nutritional status. One or both mechanisms are found in obligate and facultative methylotrophs (Colby and Zatman, 1972, 1975a; Shishkina and Trotsenko, 1979; Bellion and Bolbot, 1983; Murrell and Dalton, 1983b; Bellion and Tan, 1984).

3.3.4. The Effect of Acetate and Acetamide on M. methylotrophus

3.3.4.1. Growth Characteristics. Acetate and acetamide increased the absorbance of M. methylotrophus cultures to a small extent (Figure 17). It is impossible to conclude from this that any acetamide or acetate was incorporated into cell material because the relationship between cell dry weight and absorbance was not determined. However, if the increase in absorbance was the result of acetate incorporation, the absence of growth in the absence of methanol would underline the requirement of C_2-assimilation for the C_1-substrate (**1.9.2.**).

3.3.4.2. Effect of Acetate on the CAC and Associated Enzymes. The increase in acetyl-CoA synthetase (Table 14) is generally less than

that seen in facultative methylotrophs such as Methylobacterium extorquens AM1 and Hyphomicrobium X when they were transferred to growth on compounds metabolised via acetate to acetyl-CoA (Attwood and Harder, 1974; Taylor and Anthony, 1976b). Nevertheless, acetate may stimulate synthesis of M. methylotrophus acetyl-CoA synthetase, which would be consistant with use of acetate as a source of acetyl-CoA.

If M. methylotrophus made acetyl-CoA directly from acetate, the decarboxylation of pyruvate assimilated from methanol would be a waste of carbon. Possibly for this reason, the activity of PDH was halved (Table 14). Further, acetyl-CoA synthetase had risen enough to ensure that the amount of acetyl-CoA synthesis by PDH and the synthetase were almost equal to PDH activity in M. methylotrophus on methanol alone (Table 14). Analogous modulation of PDH activity has been observed before in E. coli and the photosynthetic bacterium Rhodobacter capsulatus (Walsh and Koshland, 1985; Miles and Guest, 1987; Willison, 1988).

Acetate caused the PDH:pyruvate carboxylase activity ratio to fall. This ratio represents the distribution of pyruvate between the synthesis of acetyl-CoA and OAA in M. methylotrophus. The lower the ratio is, the more pyruvate is directed towards carboxylation. During growth on methanol, the results (Table 17) showed the PDH:pyruvate carboxylase ratio was 7.9. When M. methylotrophus was grown under the same conditions, the ratio dropped 3.4-fold to 2.3. Thus it seems possible that in the presence of acetate, M. methylotrophus re-directs pyruvate derived from methanol into OAA production to allow entry of acetate carbon into the CAC.

The observed rise in pyruvate carboxylase (Table 14) is probably caused by synthesis of more enzyme in response to acetate. Although the enzyme from some sources is activated by acetyl-CoA, Aperghis (1981) has shown that M. methylotrophus pyruvate carboxylase is relatively insensitive to acetyl-CoA. From these results it seems unlikely that the activation of pyruvate carboxylase seen here could be caused by residual acetyl-CoA in the crude extract.

CS and IDH were induced about two-fold by acetate (Table 14). This would be consistent with the response of the CAC of the facultative methylotrophs Methylobacterium extorquens AM1, Pseudomonas

Table 17

Effect of Acetate and Acetamide on the PDH: Pyruvate Carboxylase Ratio of Methanol-Grown M. methylotrophus

Growth Conditions	PDH: Pyruvate Carboxylase Ratio.
Methanol	7.9
Methanol + 10 mM-Acetate	2.3
Methanol + 10 mM-Acetamide	
Regime A	4.6
Regime B	2.5

Values quoted for cells grown in the presence of methanol (with or without acetamide) are pooled from 3 cultures grown under each condition.

Values quoted for cells grown in the presence of methanol and acetate are based on results from 2 cultures.

3A2, Pseudomonas MA, Methylobacterium R6, Organism 5B1 and Hyphomicrobium X to growth on C_2 compounds (Colby and Zatman, 1972; Bellion and Hersch, 1972; Dunstan and Anthony, 1973; Harder and Attwood, 1974; Patel et al., 1978). However, there are two differences between the response of the CAC of these facultative organisms and M. methylotrophus: (a) the degree of activation of CS and IDH in these facultative methylotrophs is generally much greater than that of M. methylotrophus; and (b) facultative methylotrophs express far higher activities of CAC enzymes in the presence of compounds metabolised via acetate than does M. methylotrophus.

The presumed ICL activity was not stimulated by acetate (Table 14). In other icl^+ facultative methylotrophs, ICL is induced during growth on acetate to levels that are two orders of magnitude greater than those observed in M. methylotrophus in the presence of acetate, e.g. organism 5B1 (Colby and Zatman, 1972) and Pseudomonas MA (Bellion and Hersch, 1972). These features suggest that the CAC of M. methylotrophus cannot be modified so that it can adopt an energy generating role or assimilate acetate via the CAC.

Thus, the response of the CAC of M. methylotrophus to the presence of a heterotrophic carbon source (acetate) strongly resembled the response of the type M RFM's to heterotrophic carbon sources (Colby and Zatman, 1975a) in its inability to induce CAC activities or the activity of ICL to levels required for heterotrophic growth.

3.3.4.3. Effect of Acetamide on the CAC and Related Enzymes. The induction of acetamide amidase in M. methylotrophus grown under regime A conditions (5.9-fold; Table 15) was the greatest change in activity seen in cells grown under regime A. These results were similar to those obtained with M. methylotrophus by Silman et al. (1989) except that these authors found no amidase activity when ammonia was the sole nitrogen source. As amidase activation may cause the intracellular accumulation of acetate, the response that the CAC and related enzymes made to the presence of acetate in the growth medium was anticipated. Generally, this anticipation was realised (Table 16), and the same conclusions could be drawn about modification of the levels of the CAC and associated enzymes in the presence of

acetamide as were drawn regarding their response to acetate (Tables 14-17). However, it should be noted that the increase of the CAC and related enzymes was far less than the amidase. In particular, the activity of the amidase was 6-fold greater than the activity of acetyl-CoA synthetase. Therefore, even in cells adapted to the presence of acetamide, acetate may have accumulated.

M. methylotrophus grown under regime B (ammonia-deficient) was assumed to be fully dependent upon acetamide for nitrogen. Thus now, M. methylotrophus would have to deal with a molecule of acetate for every molecule of nitrogen incorporated. If the CAC was involved in acetate-dissipation, further increases in CAC enzyme activities might have been expected. Under regime B, the increases in the activity of some enzymes were slightly greater than under regime A (Table 16; e.g. acetyl-CoA synthetase, NAD-IDH). Also, the ratio of PDH to pyruvate carboxylase showed a greater drop relative to its value on methanol than it did under regime A (Table 17). This effect might be interpreted as an increasing use of pyruvate for synthesis of OAA, as the organism became more dependent upon acetamide for acetyl-CoA synthesis. However, levels of other CAC and related enzymes in regime B were similar to those obtained in regime A.

Further, the presumed increased dependence on acetamide was not expressed in an increase in amidase activity on removal of $(NH_4)_2SO_4$ from the medium (regime B.). Indeed, the amidase activity fell from its level in regime A (Table 15). This is the converse of the results of Silman et al. (1989) and is difficult to explain. It does suggest that the interpretation of these data in terms of an increased opportunity or requirement for M. methylotrophus to metabolise acetate derived from acetamide as a result of dependence upon acetamide nitrogen may be too simplistic.

3.4. Further Work

The results leave some points that ought to be addressed:

1. That the CAC of M. methylotrophus is incomplete and OGDH-deficient needs to be confirmed by study of the metabolism of [1-^{14}C]acetate.
2. The reactions that interconvert OAA and succinyl-CoA during growth

of M. methylotrophus on methanol need closer scrutiny. Particularly: (a) the possession by M. methylotrophus of a fumarate reductase needs to be evaluated; (b) the conversion of OAA to fumarate by the aspartase and aspartate transaminase route may merit investigation; and (c) the function of fumarase needs investigation, possibly by following, in extracts, the fate of ^{14}C-labelled malate or fumarate in radiolabelling studies, or by ^{13}C-NMR with the ^{13}C-malate or fumarate.

3. The detection of ICL in M. methylotrophus requires verification by identification of the assay products. It would also be of interest to determine if M. methylotrophus has malate synthase.

4. The results above were obtained during growth on methanol. Large and Haywood (1981) showed that during growth on methylated amines, M. methylotrophus produces malate dehydrogenase and NAD-IDH rises three-fold over the activity it has on methanol. Thus study of the CAC during growth on these substrates may be of interest.

5. Wild-type M. methylotrophus used GS and GOGAT and not GDH for NH_3 assimilation. The relative activities of GS and GOGAT in vitro suggested that, in vivo, GS activity may be lower. Thus, it seems sensible to check M. methylotrophus GS is adenylylated. This may be done by snake venom phosphodiesterase treatment of GS, and assay of the deadenylylated GS by a method which is affected by GS adenylylation. M. methylotrophus had no GDH, but this is not the only methylotrophic amino acid dehydrogenase. Thus, assaying M. methylotrophus for alanine dehydrogenase which has been found in facultative organisms may be informative (Bellion and Tan, 1987; Colby and Zatman, 1975a).

6. In view of the disparity of results obtained here concerning induction of amidase by acetamide and those of Silman et al. (1989), it seems that a more careful study of amidase induction is warranted.

7. M. methylotrophus is generally viewed as an obligate methylotroph (e.g. Taylor, 1977; Byrom, 1981; Large and Haywood, 1981; Dawson and Jones, 1981; Anthony, 1982; Greenwood et al., 1986; Page and Anthony, 1986 and elsewhere). There is, however, one published report of growth of M. methylotrophus on glucose as a sole carbon and energy source (Jenkins et al., 1987). If this situation can be reproduced, it would be of interest to examine the CAC under these conditions.

CHAPTER 4

Methylophilus methylotrophus Citrate Synthase

4.1. Introduction

The CS from M. methylotrophus is of interest for two reasons. First, it is the initiating step in the formation of 2-OG and perhaps even succinyl-CoA. It thus initiates the supply of C_4 and C_5 skeletons to a number of processes such as nitrogen assimilation and possibly also porphyrin synthesis. Thus, CS is a logical point at which to exert regulatory control. Secondly, no methylotrophic CS (or any other CAC enzyme) has ever been purified to homogeneity. This is of interest because, unusually, the M. methylotrophus CS and other CSs from obligate methylotrophs are apparantly NADH-insensitive, but may be similar in size to their heterotrophic counterparts (**1.12.12.1-2.**). If this is so, then ultimately, they would be a useful control protein in the study of the NADH-sensitive CSs. Therefore, the purification of the M. methylotrophus CS was pursued. The enzyme was partially characterised and then scrutinised for regulatory properties. Its lack of response to NADH was studied in a number of ways. The NADH-sensitive CS from Acinetobacter calcoaceticus was used as means for comparison with the M. methylotrophus CS.

4.2. Results

Unless otherwise specified, all M. methylotrophus and porcine heart CS assays were performed as described in **2.3.3.1.13.** in TE-8.6. The A. calcoaceticus CS was assayed in TE-8.0.

4.2.1. Purification of CS

CS in crude extracts of methanol-grown M. methylotrophus is present at 0.028 $U.mg^{-1}$. This is less than 5% of the value for CS from other Gram-negative aerobes during heterotrophic growth (e.g. Eidels and Preiss, 1976; Morse and Duckworth, 1980). Assuming the specific activity of pure M. methylotrophus CS was similar to that from other sources (see e.g. Danson and Weitzman, 1973; Higa et al., 1978; Morse and Duckworth, 1980), it seemed that purification of M. methylotrophus CS would require large amounts of crude extract. Thus, frozen pastes

of methanol-grown cells supplied by ICI were used as a source of CS.

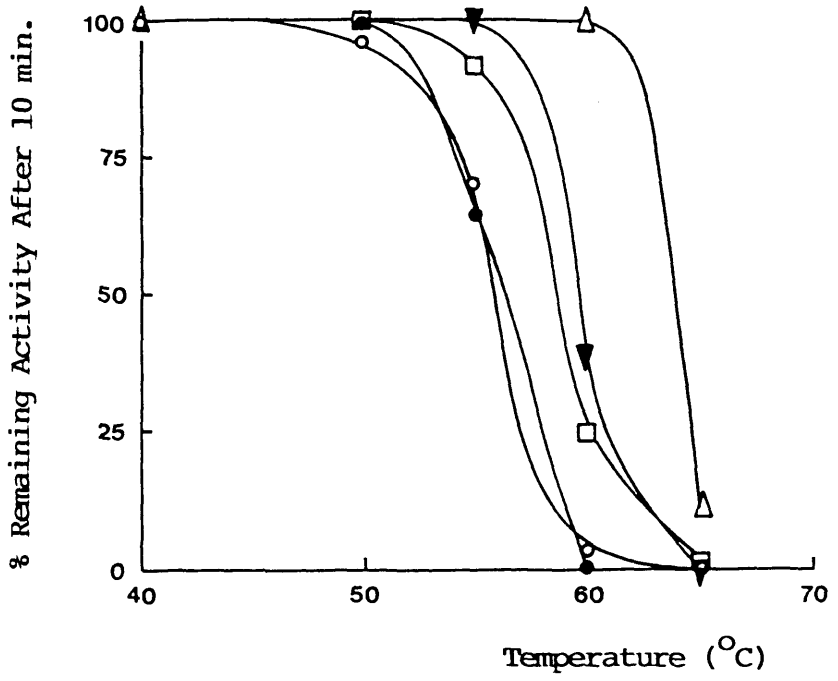
4.2.1.1. Fractionation by Thermal and Acid Precipitation. Raised ionic strength stabilises CS from E. coli and this observation has been used in purification protocols for the enzyme (Weitzman, 1966b; Faloona and Srere, 1969). Thus, the effect of raised ionic strength on the thermal stability of CS in crude extracts of M. methylotrophus was examined. Crude extracts were prepared in TE-8.6. Extracts (0.3 ml) were then added to 0.7 ml pre-warmed TE-8.6 and incubated for 10 min at a final protein concentration of 7.6 $mg.ml^{-1}$ at between 40°C and 65°C. Samples were then chilled on ice and centrifuged with an Eppendorf bench-top centrifuge. The supernatants were assayed for remaining activity. The temperature causing 50% inactivation (T_{50}) of M. methylotrophus CS over 10 min was about 57°C. 0.1 M-KCl enhanced the stability of the enzyme (Figure 18). The experiment was repeated where KCl was replaced by 1 mM-OAA, 1 mM-CoA or a mixture of 1 mM-OAA and 1 mM-CoA. These additions marginally stabilised CS activity, the most effective being OAA + CoA, although none was as effective as KCl (Figure 18).

The effect of KCl (0.05 M to 0.25 M) on the inactivation kinetics at 57°C was examined. Incubations were carried out as above except that the incubation time was 16 min and samples (0.2 ml) were taken every 4 min. The results were plotted as ln[remaining activity] vs. time (Figure 19). Assuming first-order kinetics, the rate constants of thermal inactivation at each KCl concentration were calculated from the semi-log plots in Figure 19 and are plotted vs. KCl concentration in Figure 20. From these data it is apparent that KCl protects CS activity by decreasing the rate constant of thermal inactivation. This effect is most marked at KCl concentrations up to 0.2 M above which there is no further significant gain in stability of CS activity.

Having established the protective effect of KCl, its use in the purification of CS from M. methylotrophus was evaluated. Incubations of crude extract were carried out as above over 10 and 20 min at 57°C with 0.2 M-KCl at a final protein concentration of 7.6 $mg.ml^{-1}$. After incubation and centrifugation, the specific activities of CS in the supernatants were determined. Results are shown in Table 18. From these data it is apparent that incubation over 10 min was preferable

Figure 18

The Effect of KCl, OAA and CoA on the Thermal Stability of CS from M. methylotrophus

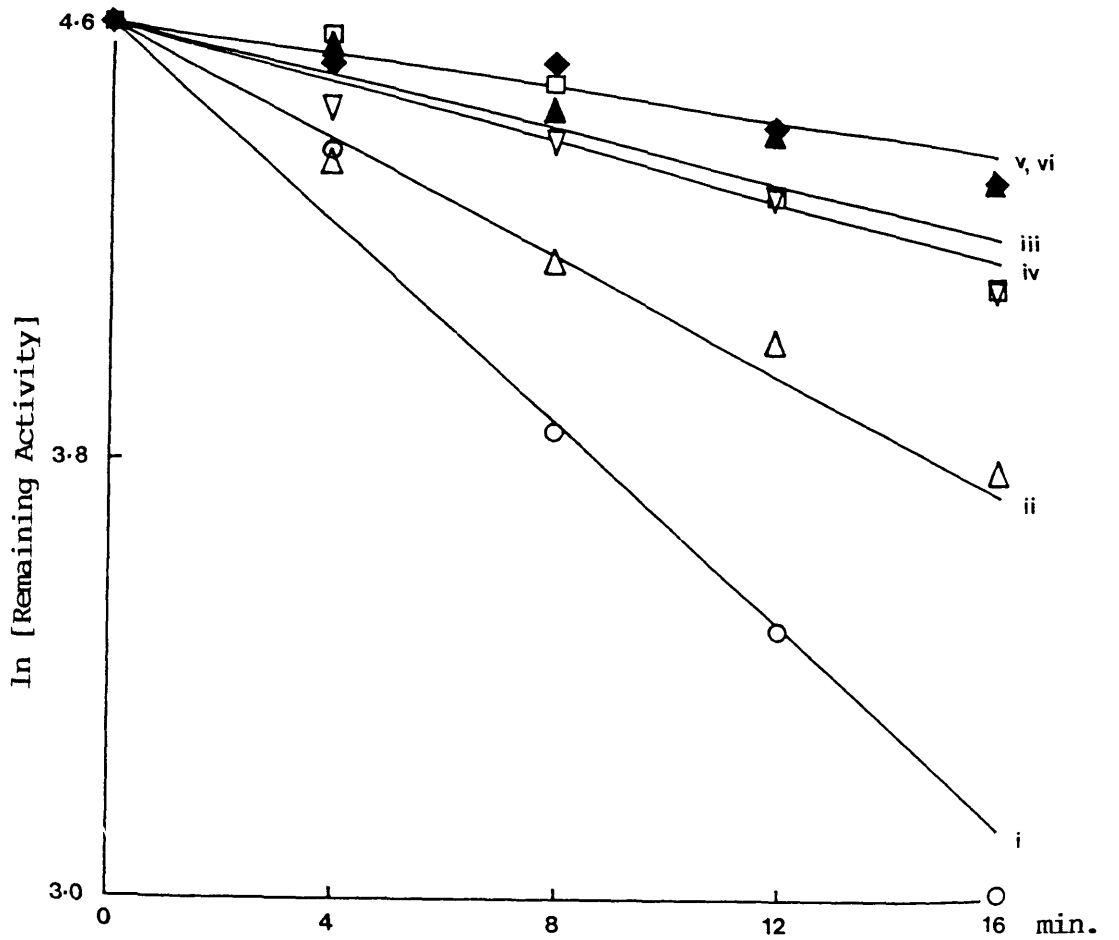


Points represent % remaining activity during incubation with:

- TE-8.6 alone
- TE-8.6 + 0.1 M-KCl
- TE-8.6 + 1 mM-OAA
- TE-8.6 + 1 mM-OAA + 1 mM-CoA
- TE-8.6 + 1 mM-CoA

Figure 19

The Effect of KCl on the Progress of Thermal Inactivation of M. methylotrophus CS at 57°C

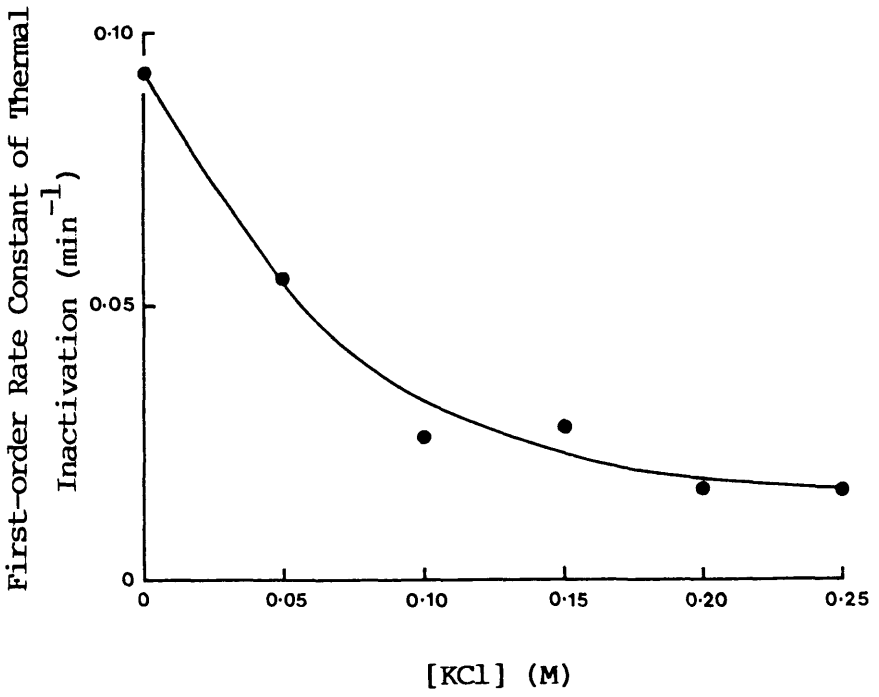


Enzyme incubated in the presence of:

- TE-8.6 alone (line i)
- TE-8.6 + 0.05 M-KCl (line ii)
- TE-8.6 + 0.1 M-KCl (line iii)
- TE-8.6 + 0.15 M-KCl (line iv)
- TE-8.6 + 0.2 M-KCl (line v)
- TE-8.6 + 0.25 M-KCl (line vi)

Figure 20

The Effect of KCl on the First-order Rate Constant of Thermal Inactivation of M. methylotrophus CS at 57°C



Each point was obtained from the gradient of the appropriate semi-log plot of the data shown in Figure 19.

Table 18

The Effect of Incubation at 57°C on the Activity and Specific Activity of CS in Crude Extracts of M. methylotrophus in the Presence of 0.2 M-KCl

Time of Incubation (min)	Total CS (U)	Total Protein (mg)	CS Specific Activity ($U.mg^{-1}$)	Purification (-fold)	Yield (%)
0	0.663	19.1	0.035	-	100
10	0.608	9.5	0.064	1.8	91
20	0.503	9.11	0.055	1.6	76

Incubation volume 2.5ml; 0.75 ml crude extract added to 1.75 ml pre-warmed TE-8.6 + 0.29 M-KCl. CS and protein were determined in crude extract without further fractionation, CS and protein in samples from 10 and 20 min incubations were determined after sedimentation of precipitated protein.

to incubation over 20 min. Under the conditions employed, 90% of the activity was retained with a 1.8-fold purification. As a result, treatment of the enzyme with 0.2 M-KCl and incubation for 10 min at 57°C was used as a step in the final purification.

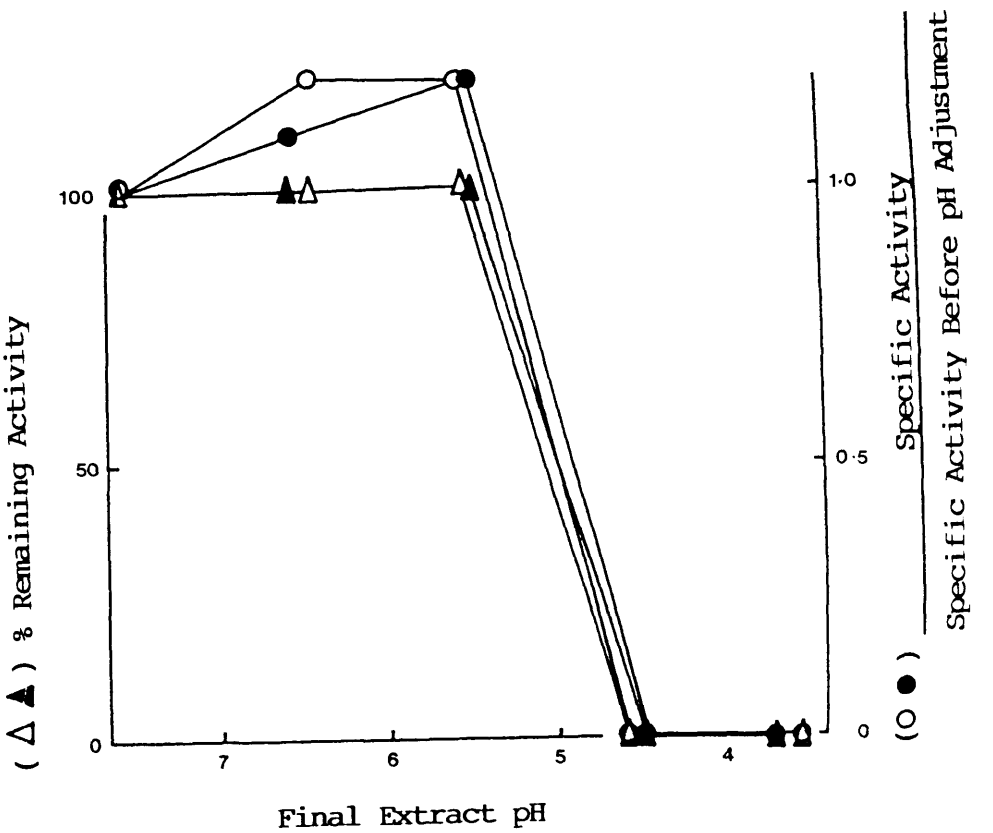
The stability of CS in crude extracts towards decreasing pH was examined to assess the use of acid precipitation in the purification of CS. Acid precipitation was carried out (**2.2.7.3.**) on an extract prepared in TE-8.6 and CS and protein were measured in the resultant supernatants. Most of the activity was lost between pH 5.5 and 4.5. The experiment was repeated with 0.3 mM-OAA; however, this did not protect activity against inactivation (Figure 21). As the purification was small (less than 1.2-fold in the presence of 0.3 mM-OAA), this technique was abandoned as a method of purification.

4.2.1.2. The Purification Protocol for M. methylotrophus CS. In addition to heat treatment, triazine dye-ligand chromatography with immobilised Red HE3B (Mātrex Red Gel A produced by Amicon) has been found useful in the purification of bacterial CSs (Mitchell and Weitzman, 1983b; Weitzman and Ridley, 1983; Robinson et al., 1983a, b). This latter step was also incorporated into the final protocol for purification of M. methylotrophus CS. One problem that had to be overcome was the instability of CS activity during dialysis. Overnight dialysis in TE-8.6 + 0.1 M-KCl of a 35-60% ammonium sulphate cut of crude extract led to a 40% loss of CS activity. This restricted the available means of buffer exchange to gel filtration on G-25 columns. This, in turn, reduced the volume of material that could be easily processed. As large amounts of starting material were required to purify the enzyme, steps such as ammonium sulphate precipitation were performed later in the purification than is usually the case, in order to avoid having to desalt large volumes of resuspended pellets.

The purification protocol was as follows. Unless otherwise specified, all steps were carried out at 4°C. Crude extract was prepared from a suspension of cell paste in TE-8.6 + 0.4 M-KCl (1 g cell paste per ml of buffer). The extract was then added to an equal volume of TE-8.6 pre-warmed to 57°C. The temperature of the extract was then readjusted as quickly as possible to 57°C and maintained at

Figure 21

The Effect of Acidification of Crude Extracts of M. methylotrophus on CS Activity



Extracts were initially prepared in TE-8.6 prior to pH adjustment.

Closed symbols refer to the response of CS activity to decreasing pH where no other additions were made to the extract before pH adjustment.

Open symbols refer to the response of CS activity to decreasing pH where OAA was added to a final concentration of 0.3 mM before pH adjustment.

that level for 10 min. The extract was chilled and centrifuged (**2.2.7.4.**). The supernatant was fractionated by protamine sulphate (**2.2.7.2.**). The supernatant from this step was then diluted with TE-8.6 to a KCl concentration of about 0.1 M.

Depending on the amount of protein remaining, extract was loaded onto one or two DEAE-Sephacel columns that had been pre-equilibrated in TE-8.6 + 0.1 M-KCl. The CS activity bound and, after washing in TE-8.6 + 0.1 M-KCl, the column(s) was (were) eluted over two column volumes with a 0.1-0.5 M-KCl gradient in TE-8.6. The peak of CS activity was eluted at between 0.3 M- and 0.35 M-KCl. Fractions with a specific activity greater than 0.2 $U.mg^{-1}$ were pooled.

The CS preparation was then diluted to a KCl concentration of 0.1 M and then fractionated on ice with ammonium sulphate. The material precipitating between 35% and 60% saturation was resuspended in TE-8.6 + 0.1 M-KCl and desalted into the same buffer using 250 ml-columns of G-25. Removal of ammonium sulphate was confirmed by the inability to detect NH_4^+ with Nessler's reagent in the active fractions.

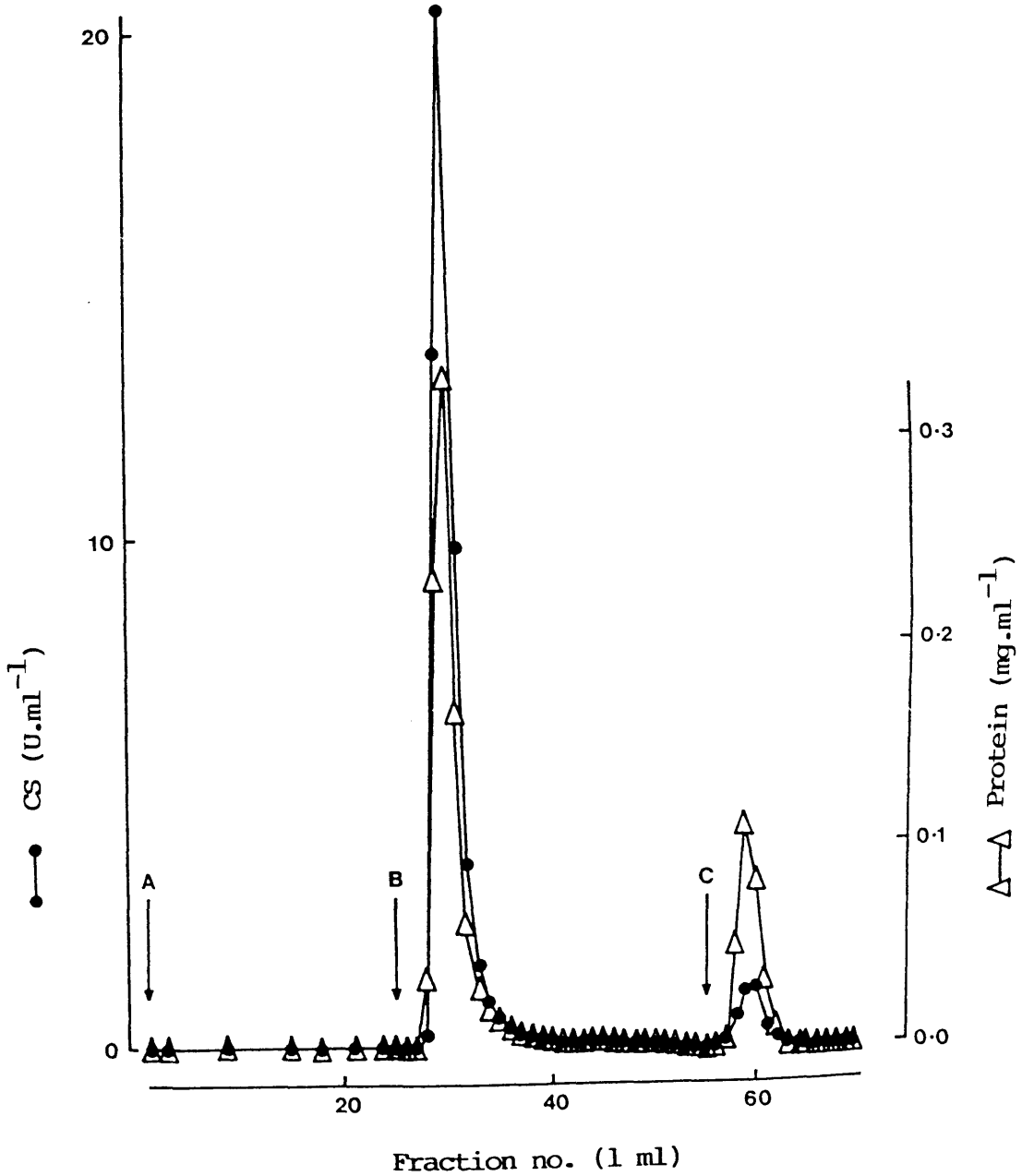
The CS preparation was then diluted two-fold, and loaded onto a column of Red Gel A (30 X 1.7 cm) pre-equilibrated in TE-8.6 (without KCl). All the CS activity bound. The column was washed with TE-8.6 + 0.1 M-KCl and a 400-ml (6 column volumes) gradient of 0.1-0.7 M-KCl in TE-8.6 was then applied. CS activity was eluted at approximately 0.4 M-KCl. Fractions with specific activities greater than 5.2 $U.mg^{-1}$ were pooled and diluted to approximately 0.05 M-KCl with TE-8.6.

The diluted fractions reserved from the previous step were loaded onto a 10 X 0.5 cm column of Red Gel A pre-equilibrated in TE-8.6 (without KCl). The column was washed in the same buffer + 0.1 M-KCl until no protein eluted. The column was then washed with 20 ml of TE-8.6 + 0.1 M-KCl containing 0.2 mM-CoA. CS was eluted as a sharp peak with TE-8.6 + 0.1 M-KCl containing 0.2 mM-CoA and 0.2 mM-OAA (Figure 22). The column was finally washed with TE-8.6 + 2 M-KCl.

A typical purification is summarised in Table 19. Its final product had a specific activity of 46.6 $\pm$ 4.9 $U.mg^{-1}$ ($\pm$ S.E.M.; n=3). Yields were from 9% to 20% of the starting activity. SDS-PAGE of the enzyme after the final Red Gel step according to Shapiro *et al.* (1967) and non-denaturing PAGE (**2.3.4.1.**) showed that in both systems, there

Figure 22

Biospecific Elution of M. methylotrophus CS from Immobilised Red HE-3B (Matrex Red Gel A)



Eluant was TE-8.6 + 0.1 M-KCl which was supplemented at A by 0.2 mM-CoA; at B by 0.2 mM-CoA + 0.2 mM-OAA and at C by 2 M-KCl.

Table 19

Purification of CS from M. methylotrophus

Step	Volume (ml)	Total CS (U)	Total Protein (mg)	Specific Activity ($U.mg^{-1}$)	Yield (%)	Purification (-fold)
Crude Extract	1660	813.9	47,630	0.017	100	-
Heat Treatment	1480	555.4	25,380	0.022	68.2	1.3
Protamine sulphate	1684	518.9	17,320	0.030	64.8	1.8
DEAE-Sephacel	327.9	273.4	761.7	0.359	33.6	20.9
35-60% $(NH_4)_2SO_4$ and desalting	219	336.4	574.8	0.585	41.3	34.2
Red Gel A 0.1 M-0.7 M-KCl gradient	213.6	164.3	13.5	12.2	20.2	711
Red Gel A 0.2 mM-OAA and 0.2 mM-CoA	13.9	72.9	1.75	41.6	9.0	2435

Starting material was 1500g (wet weight) cell paste.

was only one major species (Figures 23 and 24). Thus the protein was basically homogeneous and composed of subunits of identical M_r. A 2435-fold purification was needed to obtain a homogeneous preparation of CS (Table 19). Thus the enzyme represented only 0.04% (w/w) of the protein in the soluble fraction of the cell-free extract.

The protocol was not as rapid as the IDH purification (**5.1.1.3.**). In particular, the crude extract was difficult to handle, as even after the protamine sulphate step, there was material that markedly reduced the flow rate through DEAE-Sephacel.

4.2.1.3. Purification of CS from A. calcoaceticus. To compare the properties of M. methylotrophus CS with those of a heterotrophic CS, the A. calcoaceticus CS was purified. This enzyme is typical of those from Gram-negative aerobic heterotrophs (**1.12.3.**; **1.12.6.**). The purification was similar to that for the M. methylotrophus CS, except for the following differences. TE-8.0 replaced TE-8.6, and TE-8.0 + 50 mM-KCl replaced TE-8.6 + 0.1 M-KCl. Crude extract was prepared in TE-8.0 + 50 mM-KCl and treated with protamine sulphate. The heat step, $(NH_4)_2SO_4$ fractionation and G-25 buffer exchange were omitted. The first Red Gel step was modified so the salt gradient was now 0.05-0.5 M-KCl and was followed by preparative gel filtration (**2.2.7.7.**) in TE-8.0 + 0.05 M-KCl. This was followed by the last step which was the second Red Gel step using biospecific elution with OAA + CoA. The purification is summarised in Table 20. Analysis of 25 µg of protein from the final step by SDS-PAGE under the same conditions used to analyse the M. methylotrophus CS showed that the purification protocol produced a homogeneous preparation of Acinetobacter CS (Figure 24).

4.2.2. Quaternary Structure of M. methylotrophus CS

4.2.2.1. Subunit M_r. This was determined by SDS-PAGE. The two commonly employed methods use either phosphate (Shapiro et al., 1967) or Tris-glycine (Laemmli, 1970) as the running buffer. Both methods have been evaluated for reliability in estimating polypeptide M_r values of CS by Robinson (1984) who showed that phosphate-based methods were less prone to error. Thus, this method (**2.3.4.2.**) was used here. 25 µg of

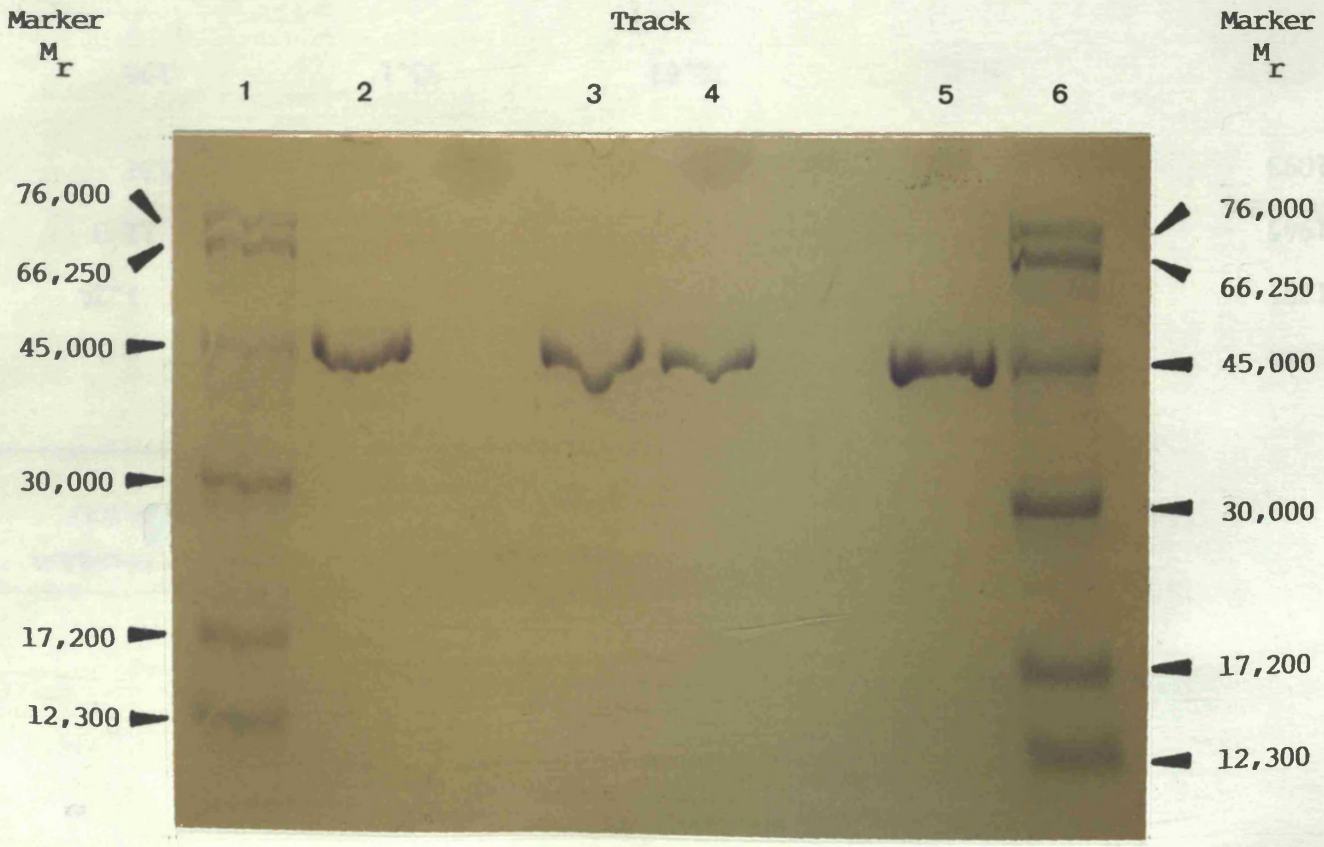
Figure 23

Analysis of M. methylotrophus CS by PAGE
Under Non-Denaturing Conditions

20 µg of purified M. methylotrophus CS was electrophoresed as described in 2.3.4.1. and stained with Coomassie Blue.

Figure 24

Analysis of CSs from M. methylotrophus and A. calcoaceticus by SDS-PAGE



10 µg of each M_r marker was loaded. The following was loaded onto each track:

1 and 6: M_r markers

2: 25 µg of M. methylotrophus CS.

3: 25 µg of M. methylotrophus CS.

4: 25 µg of A. calcoaceticus CS.

5: 25 ug of A. calcoaceticus CS mixed with M. methylotrophus CS (25 µg) prior to loading.

Table 20

Purification of CS from A. calcoaceticus

Step	Volume (ml)	Total CS (U)	Total Protein (mg)	Specific Activity ($U.mg^{-1}$)	Yield (%)	Purification (-fold)
Crude Extract	168	1274	3444	0.370	100	-
Protamine sulphate	190	1291	2812	0.459	101	1.24
DEAE-Sephacel	34.3	1945	425.5	4.572	153	12.3
Red Gel A 0.05 M-0.5 M-KCl gradient	16.65	1093	215.8	50.68	85.8	137
Sephacryl S-200 gel filtration	13.50	676.5	9.32	72.63	53.1	196
Red Gel A 0.2 mM-OAA and 0.2 mM-CoA	5.8	527.6	5.8	90.97	41.4	246

Starting material was 75g (wet weight) cell paste.

CS was electrophoresed (Figure 24) with the M_r markers [M_r values in square brackets]: ovotransferrin [76,000], albumin [66,250], ovalbumin [45,000], carbonic anhydrase [30,000], myoglobin [17,200], cytochrome c [12,300]. The mobilities of the markers relative to bromophenol blue (R_f) are plotted against the $\log_{10}$ of their M_r values (Figure 25). CS migrated at about the same rate as ovalbumin. From these data, the subunit M_r of M. methylotrophus CS is 45,200 ± 1,900 (± S.E.M., n = 3).

Figure 24 also shows the migration of A. calcoaceticus CS under denaturing conditions. From this, it seems that the A. calcoaceticus and M. methylotrophus CSs have virtually identical subunit M_r values.

4.2.2.2. Native M_r. The native M_r of purified CS was found by analytical gel filtration through Sephacryl S-200 (**2.3.5.**). The eluting buffer was TE-8.6 + 0.1 M-KCl. The void volume of the column was 170 ml. The column was calibrated by following the elution of the M_r markers [native M_r values in square brackets]: equine spleen ferritin [450,000], bovine liver catalase [240,000], rabbit muscle lactate dehydrogenase [140,000], porcine heart CS [98,000] and porcine heart malate dehydrogenase [67,000]. Apart from ferritin, elution of the other proteins was followed by assay of their respective enzymic activities (Figure 26).

The relationship between $\log_{10}$ native M_r and elution of the markers beyond the void volume (V_e) for the M_r markers is shown in Figure 27. V_e for M. methylotrophus CS was found to be 26.5 ± 2.2 ml (± S.E.M., n = 4). Using this value, the native M_r interpolated from the data in Figure 27 was 266,000 ± 19,000 (± S.E.M., n = 4).

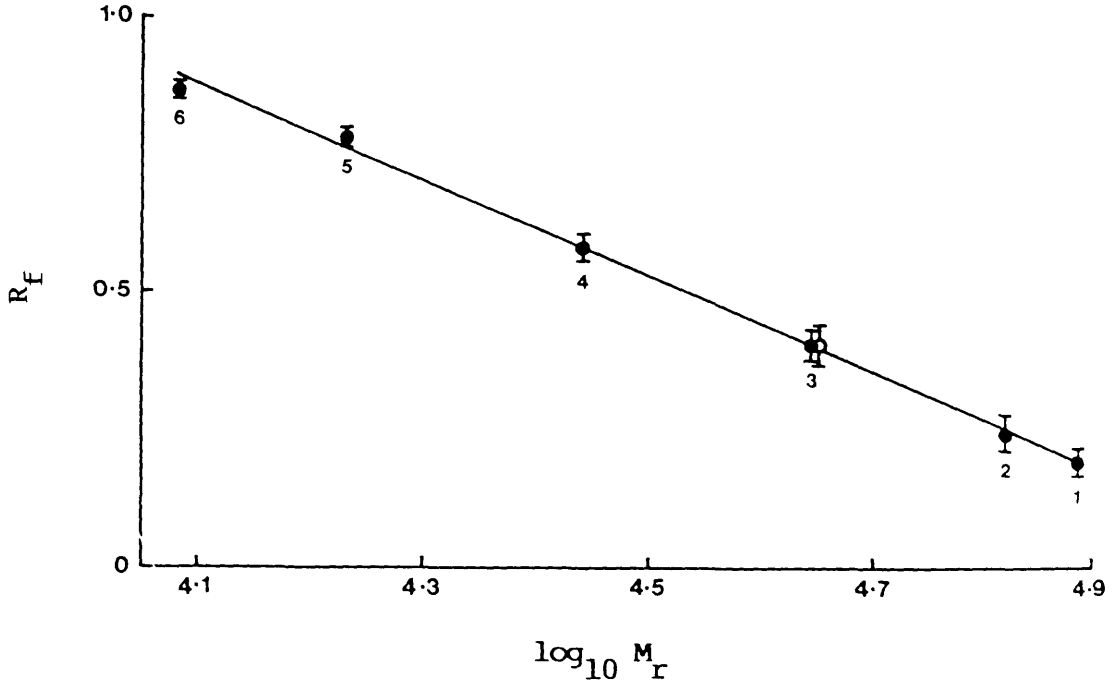
4.2.2.3. Subunit Stoichiometry. The data suggest a native M_r:subunit M_r ratio of 5.88. This suggests M. methylotrophus CS is a hexamer of subunits of M_r 45,200.

4.2.3. Amino Acid Analysis

Values for serine and threonine were obtained by extrapolation to zero time of values obtained after 24, 48 and 72 h hydrolysis. All other values are the means of triplicate samples taken at these times,

Figure 25

Determination of the Subunit M_r Value of M. methylotrophus CS by SDS-PAGE



Closed symbols labelled 1-6 represent the positions of migration of the following M_r markers relative to bromophenol blue [M_r values in square brackets]:

1. Ovotransferrin [76,000]
2. Albumin [66,250]
3. Ovalbumin [45,000]
4. Carbonic Anhydrase [30,000]
5. Myoglobin [17,200]
6. Cytochrome c [12,300]

The open symbol next to 3 is the position of migration of M. methylotrophus CS. All points are the mean values of three experiments and the bar is ± S.E.M.

Legend to Figure 26

The peaks labelled 1 to 5 correspond to the elution of the following protein native M_r markers [native M_r in square brackets]:

1. Equine spleen ferritin [450,000]
2. Bovine liver catalase [240,000]
3. Rabbit muscle lactate dehydrogenase [140,000]
4. Porcine heart CS [98,000]
5. Porcine heart malate dehydrogenase [67,000]

Elution of ferritin was followed by its absorbance at 280 nm, the elution of the other proteins was detected by the elution of enzyme activity.

Elution of catalase was assessed by assay of its activity using the following spectrophotometric method: Aebi, H. (1974) Catalase. In "Methods of Enzymatic Analysis" 2nd Edn. Academic Press Inc., London, U.K. pp. 673-684.

Elution of lactate dehydrogenase was assessed using the following spectrophotometric assay: The enzyme was assayed in MET-8.0 with 0.2 mM-NADH and 0.2 mM-pyruvate. Lactate dehydrogenase-independent NADH oxidation was monitored at 340 nm and the reaction was initiated by addition of pyruvate. The final assay volume was 1 ml.

Figure 26

Elution Profile of Native M_r Markers from Sephacryl S-200

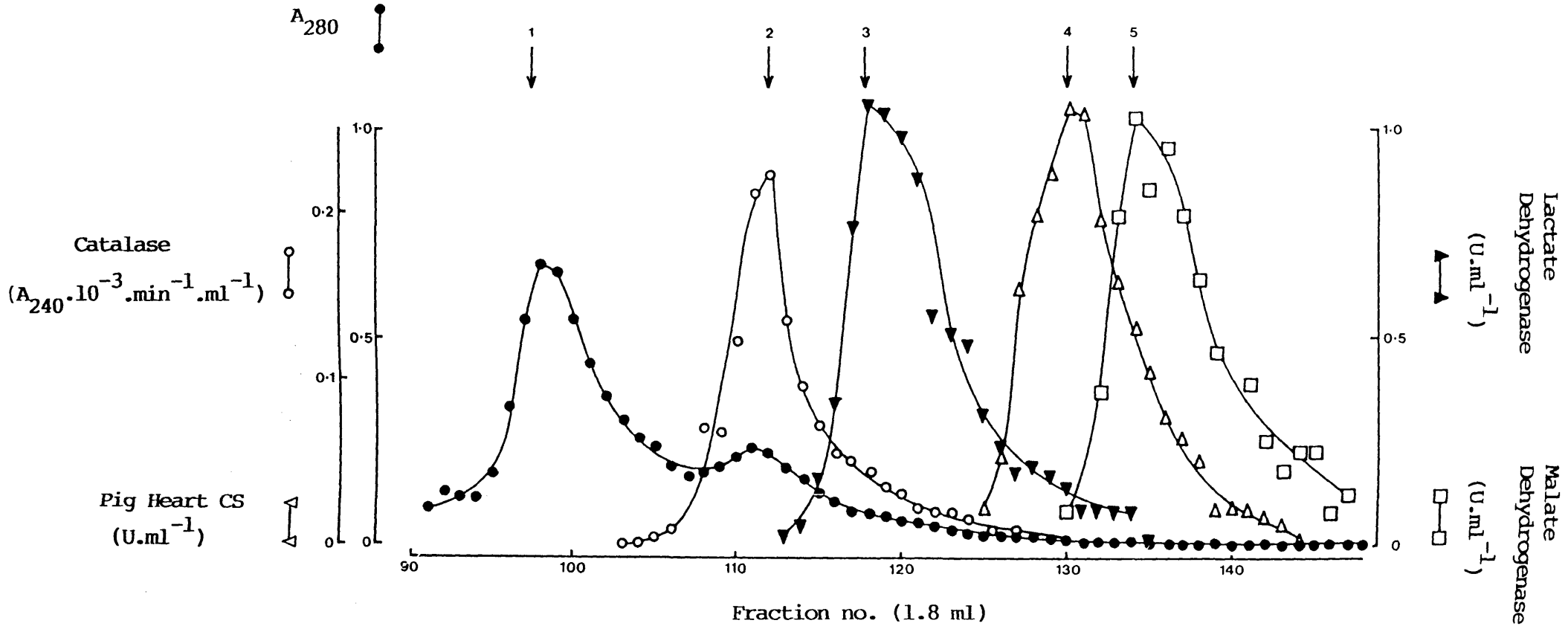
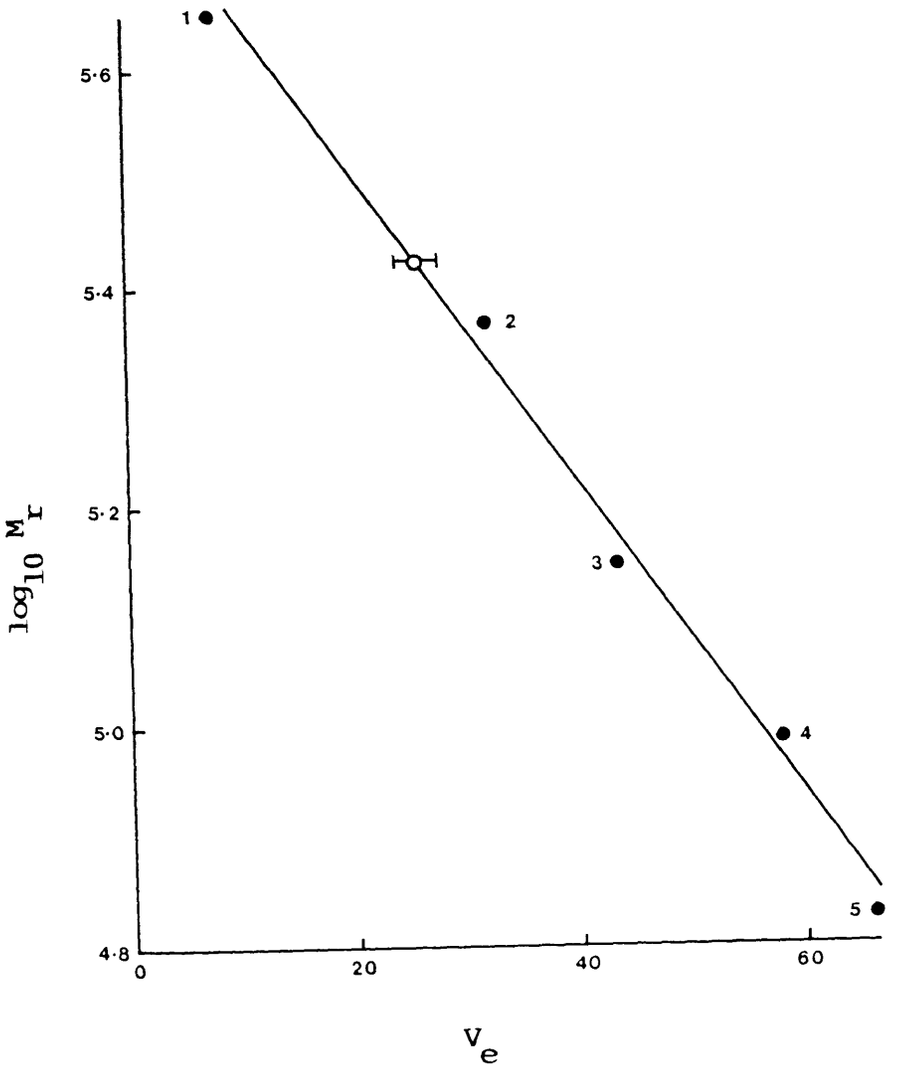


Figure 27

Determination of the Native M_r of M. methylotrophus CS by Gel Filtration through Sephacryl S-200



Position of elution of M_r markers are given by the closed symbols (numbered 1-5) which represent the mean from two experiments. Markers are as follows: 1 = ferritin, 2 = catalase, 3 = lactate dehydrogenase, 4 = porcine CS, 5 = malate dehydrogenase. The M_r values of these markers are given in the legend to Figure 26.

Position of elution of M. methylotrophus CS is given by an open symbol (between 1 and 2) and is the mean of four determinations ± S.E.M.

apart from tryptophan which is the result of a single hydrolysis at 24 h. Only the 72 h values were used for valine and isoleucine.

Most of the data show a strong similarity between amino acid compositions of A. calcoaceticus (392 residues) and M. methylotrophus (388 residues) CSs (Table 21). The exception to this are the values obtained for glycine, which show relatively large differences between the two enzymes. Further, the glycine values for M. methylotrophus CS have an unusually high S.E.M. relative to the rest of the data. A possible explanation for this is contamination. The obvious remedy, namely, to repeat the amino acid analysis, was unfortunately not possible because the time remaining for bench work after these results were obtained was insufficient. Thus, the best use of the available data must be made.

Cornish-Bowden (1977, 1983) has introduced an index for assessing composition divergence of two proteins of similar length. It is defined as half the sum of squares of the differences between the numbers of each residue of each type in the two proteins:

$$S\Delta n = \tfrac{1}{2}\Sigma(n_{iA} - n_{iB})^2$$

where n_{iA} and n_{iB} are the numbers of residues of the i^{th} type in proteins A and B. This index may be used as an estimator of the loci at which the two sequences are different and thus allows assessment of similarity in primary structure from amino acid compositions.

Application of this index as described by Cornish-Bowden (1977, 1983) to amino acid compositions in Table 21 gives a value for $S\Delta n$ of 168.8. This indicates that the Acinetobacter and M. methylotrophus CSs share a sequence homology of 100 - (168.8/392 X 100) = 57% with each other. Omission of glycine from the calculations gives an $S\Delta n = 39.2$ indicating a putative homology of 90%. It should be noted that, statistically, omission of glycine reduces the confidence with which this figure can be treated (Cornish-Bowden, 1977).

Homology between unrelated proteins by chance identity of loci amounts to 7% (Cornish-Bowden, 1983). Thus, the available data (with or without glycine) suggest that the CSs from M. methylotrophus and A. calcoaceticus are related in that they may share significant

Table 21

Amino Acid Compositions of CSs from M. methylotrophus and A. calcoaceticus

M. methylotrophus		*A. calcoaceticus*	
Amino Acid	**Residues/subunit*** (M_r = 45,200)	**Amino Acid**	**Residues/Subunit*** (M_r = 45,200)
Alanine	51.6 ± 0.8	Alanine	53.1 ± 0.8
Arginine	12.3 ± 0.6	Arginine	12.5 ± 0.1
Aspartate + Asparagine	33.2 ± 1.6	Aspartate + Asparagine	37.3 ± 0.7
Cysteine	2.2 ± 0.9	Cysteine	2.6 ± 0.9
Glutamate + Glutamine	28.8 ± 1.7	Glutamate + Glutamine	33.6 ± 1.2
Glycine	85.2 ± 10.1	Glycine	69.1 ± 1.3
Histidine	10.5 ± 0.6	Histidine	10.6 ± 0.8
Isoleucine	19.2 ± 0.8	Isoleucine	21.3 ± 0.5
Leucine	25.5 ± 1.7	Leucine	28.3 ± 0.6
Lysine	15.1 ± 1.3	Lysine	17.6 ± 0.2
Methionine	9.0 ± 0.8	Methionine	8.3 ± 0.5
Phenylalanine	10.2 ± 0.7	Phenylalanine	10.1 ± 0.4
Proline	19.8 ± 1.6	Proline	19.2 ± 1.0
Serine	22.2 ± 1.3	Serine	20.0 ± 1.4
Threonine	16.7 ± 0.8	Threonine	19.3 ± 0.6
Tryptophan*	1.0	Tryptophan*	1.0
Tyrosine	6.0 ± 0.9	Tyrosine	7.4 ± 0.4
Valine	18.7 ± 0.9	Valine	20.5 ± 0.3

*: Tryptophan determination is the result of a single incubation with 3-mercaptoethane sulphonic acid. All other results are quoted ± S.E.M.; for triplicate samples.

sequence homology. However, as will be considered in **4.3.**, wider application of the amino acid analysis in Table 21 shows that this data set (glycine values notwithstanding) may be unreliable.

4.2.4. Dependence of M. methylotrophus CS Activity on pH

The DTNB-linked assay was used to examine CS activity between pH 8.0 and 9.0. The polarographic assay was used (**2.3.3.2.1.**) to confirm the results of the DTNB assay and extend the range of pHs over which CS was examined to pH 6.2-9.7.

All DTNB-linked assays were conducted in 20 mM-Tris and 1 mM-EDTA. The polarographic assays were carried out in 1 mM-EDTA using 20 mM-Mops (pKa at 25°C = 7.2) between pH 6.2 and 7.6 and Tris (pKa at 25°C = 8.3) between pH 7.2 and 9.7. The results are shown in Figure 28. The relationship between activity and pH could approximately be described by a bell-shaped curve. Both assays indicate that the pH optimum for CS from M. methylotrophus lies between pH 8.2 and 8.6.

4.2.5. Substrate and Coenzyme Dependences

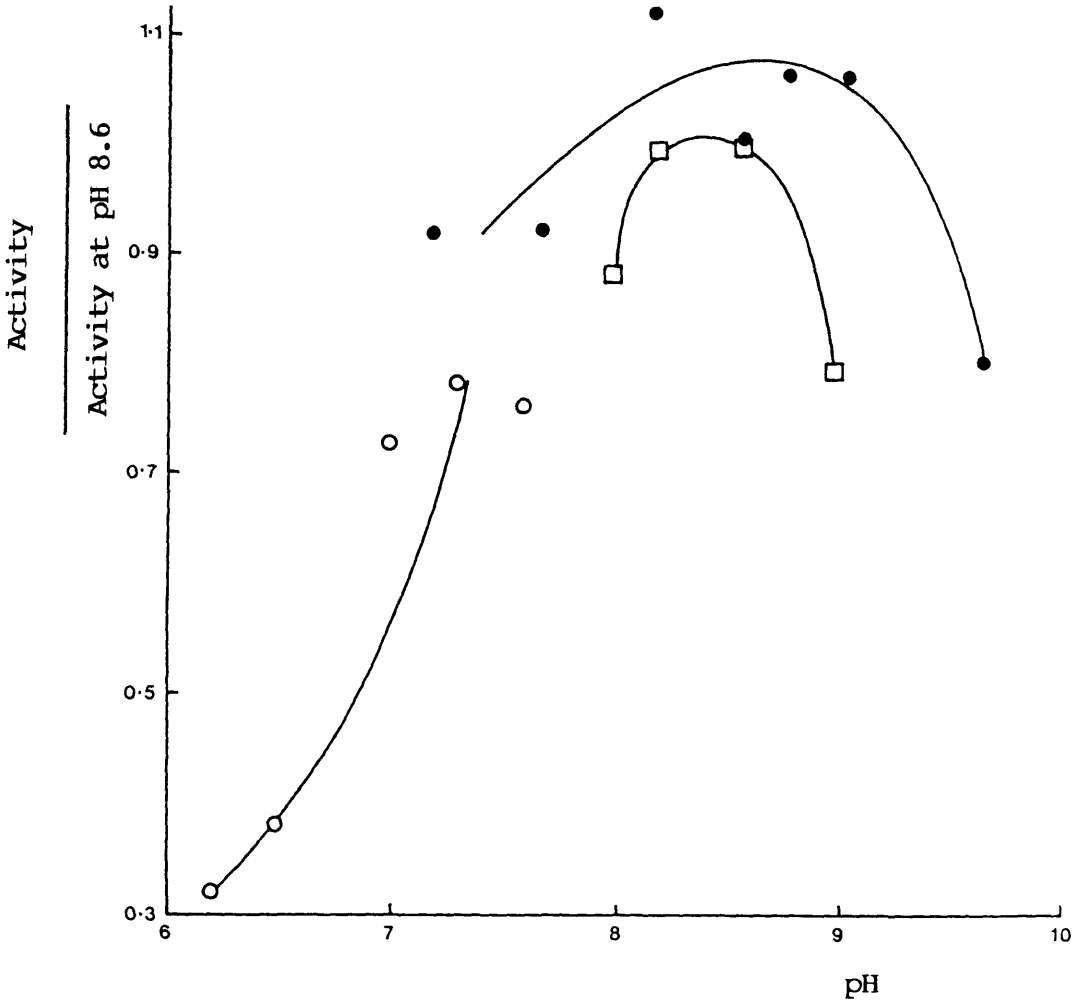
Substrate dependences were examined with CS that had been freed from CoA and OAA by buffer exchange into TE-8.6 with a PD-10 G-25 column (**2.2.5.**). Initial rates were measured over a concentration range of one substrate at a fixed concentration of the other. The dependence of CS on acetyl-CoA was determined at 0.2 mM-OAA, while the dependence of activity on OAA was determined at 0.83 mM-acetyl-CoA.

The dependence on acetyl-CoA is plotted in Figure 29a and shown in half-reciprocal form in Figure 29b. The dependence of initial rate on acetyl-CoA was apparently hyperbolic and this was confirmed by plotting the data as a Hill plot (Figure 30a). The Hill coefficient for acetyl-CoA was 1.14 $\pm$ 0.12 ($\pm$ S.E.), which showed that the dependence of CS activity on acetyl-CoA is probably not co-operative to any appreciable extent. The K_m and V_{max} values determined from the dependence of CS activity on acetyl-CoA were 119 μM and 52.2 $U.mg^{-1}$.

The dependence of activity on OAA was also determined and is plotted in Figure 31a and replotted as a half-reciprocal plot (Figure

Figure 28

The Effect of pH on the Activity of M. methylotrophus CS



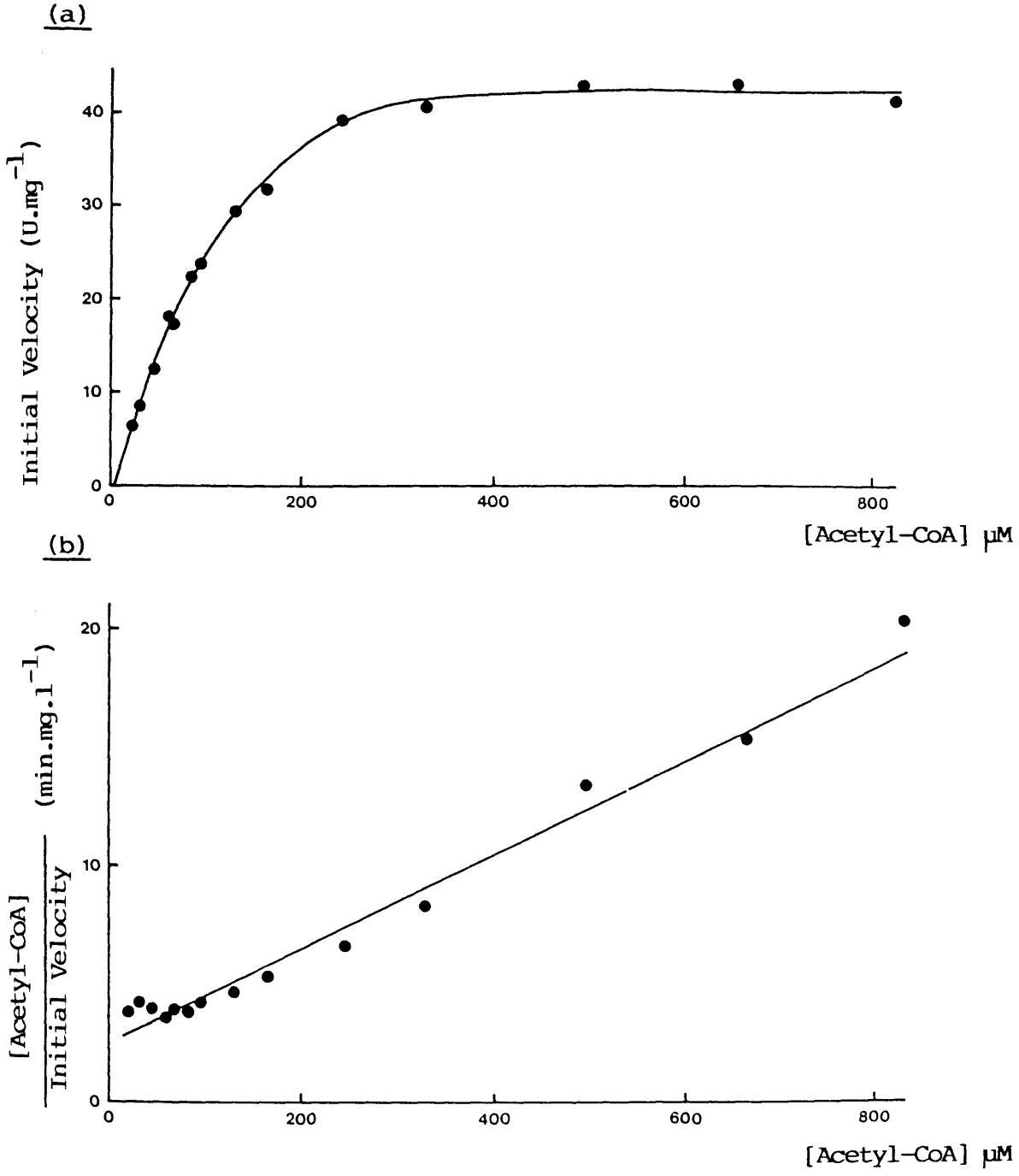
(●, ○) Polarographic and (□) DTNB-linked spectrophotometric assays

(○—○) Assays carried out in 20 mM-Mops and 1 mM-EDTA

(●—●, □—□) Assays carried out in 20 mM-Tris and 1 mM-EDTA

Figure 29

Relationship Between Acetyl-CoA Concentration and M. methylotrophus CS Activity

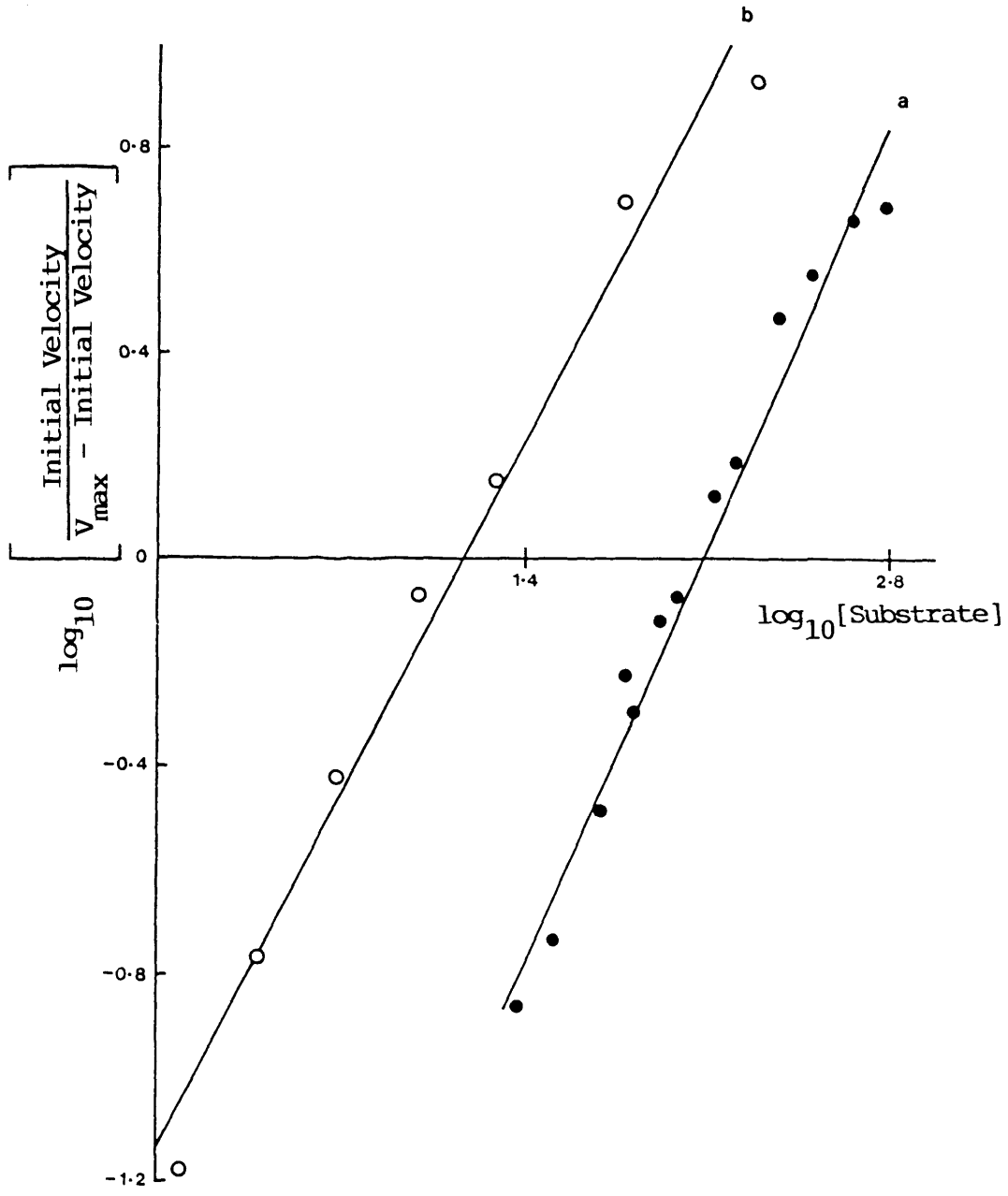


(a) Dependence of initial velocity on Acetyl-CoA concentration.

(b) Half Reciprocal plot of the data in (a).

Figure 30

Hill Plot of the Relationships Between Initial Velocity and Substrate Concentration shown by M. methylotrophus CS



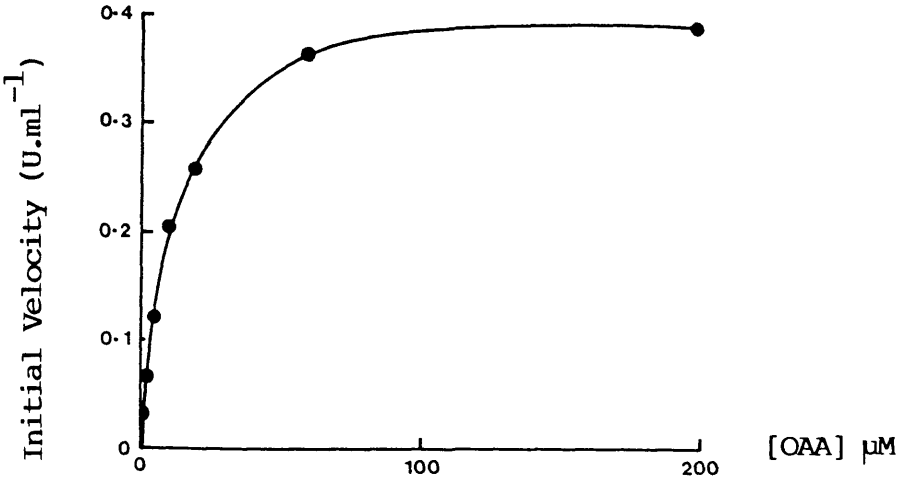
a: log10[Substrate] = $\log_{10}$[Acetyl-CoA]

b: $\log_{10}$[Substrate] = $\log_{10}$[OAA]

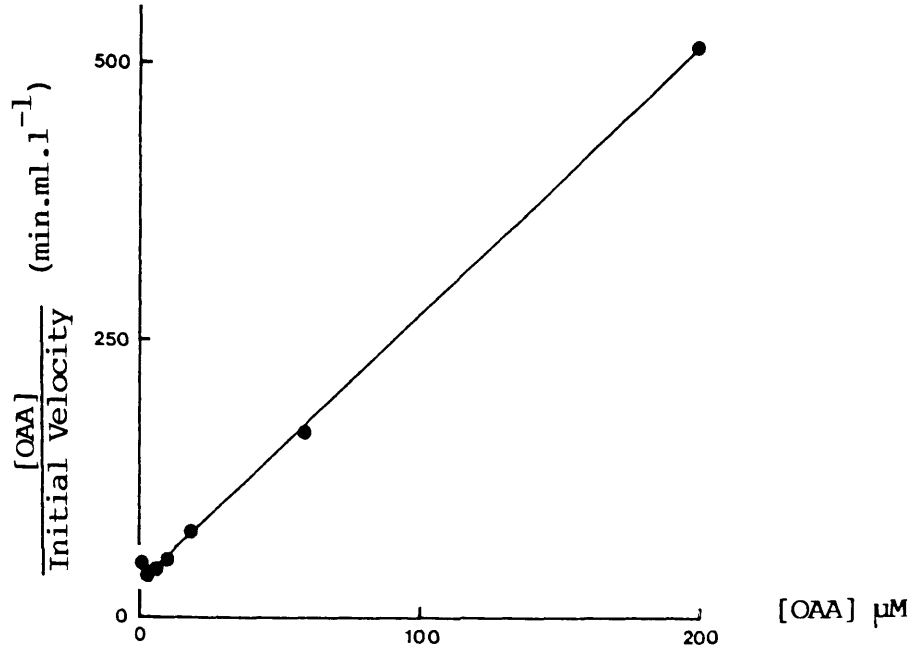
Figure 31

Relationship Between OAA Concentration and M. methylotrophus CS Activity

(a) : Dependence of Initial Velocity on OAA Concentration



(b): Half Reciprocal Plot of data in (a)



31b). Like acetyl-CoA, the dependence on OAA (Figure 31a) was examined by replotting the data as a Hill plot (Figure 30b). From these data, the Hill coefficient was 0.97 ± 0.05 (± S.E.). This value indicates that there is no co-operativity towards OAA and it is likely that CS shows Michaelis-Menten type kinetics in its dependence on OAA. Assuming that this is so, the K_m value for OAA was 12.2 μM.

4.2.6. Ionic Effects on M. methylotrophus CS Activity

4.2.6.1. The Effects of Monovalent Cations on CS Activity. The results in this section were obtained with purified CS that had been desalted into TE-8.6 (as in **4.2.5.** above).

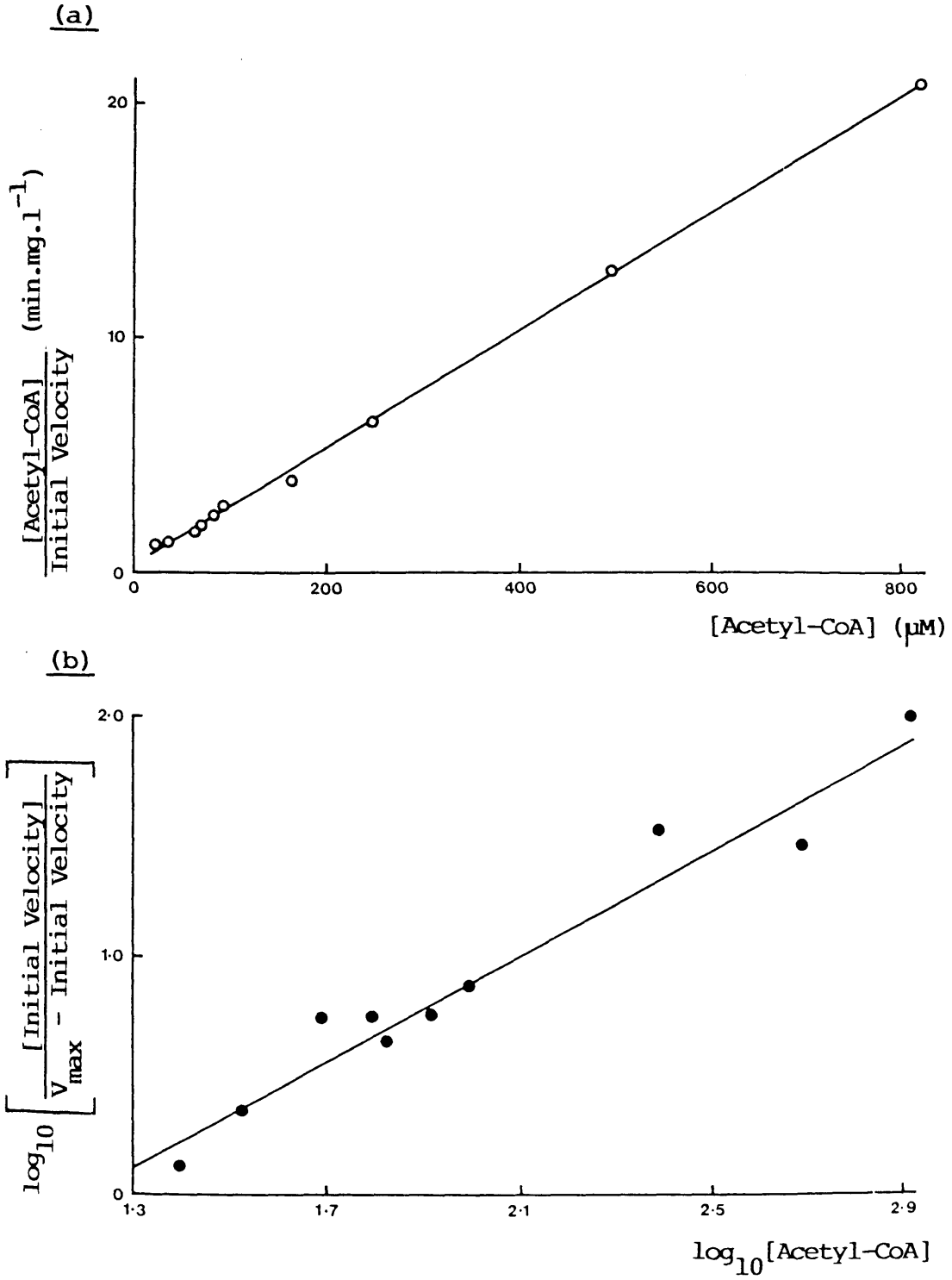
A variety of CSs are activated by monovalent salts, an effect primarily caused by a drop in the K_m for acetyl-CoA (Weitzman and Danson, 1976). The dependence of M. methylotrophus CS on acetyl-CoA at 0.1 M-KCl was studied in TE-8.6 at 0.2 mM-OAA. Figure 32a shows the data as a half-reciprocal plot. The K_m for acetyl-CoA was 19.5 μM. This is 6.1-fold smaller than the value in the absence of KCl. The V_{max} was 40.7 $U.mg^{-1}$ which is similar to the value in the absence of the salt (**4.2.5.**). Thus, KCl had a far smaller effect on V_{max} than on K_m. KCl had no effect on the Hill coefficient of the enzyme which, under these conditions, was 1.12 ± 0.10 (± S.E.) (Figure 32b).

The effect of KCl on K_m for the substrates was examined in more detail. Working between 0-0.1 M-KCl, the K_m values for acetyl-CoA (at 0.2 mM-OAA) and OAA (at 0.5 mM-acetyl-CoA) were determined. The K_m for acetyl-CoA dropped sharply with increasing KCl. A half-maximal effect was achieved by about 3 mM-KCl (Figure 33). In contrast, there was no marked change in the K_m value for OAA (Figure 33).

As the effect of KCl was directed towards a fall in the acetyl-CoA K_m, the ability of KCl to stimulate M. methylotrophus CS would be evident at concentrations of acetyl-CoA that, in the absence of KCl, were sub-saturating. This was indeed so, and at 49.9 μM-acetyl-CoA (0.42.K_m), KCl activated CS. This activation was hyperbolically dependent on KCl, and was half-maximal at 7 mM-KCl. Activation was essentially complete by 0.1 M-KCl, at which point the activity was increased 2.4-fold (Figure 34a).

Figure 32

Relationship Between M. methylotrophus CS Activity and Acetyl-CoA Concentration in the Presence of 0.1 M-KCl

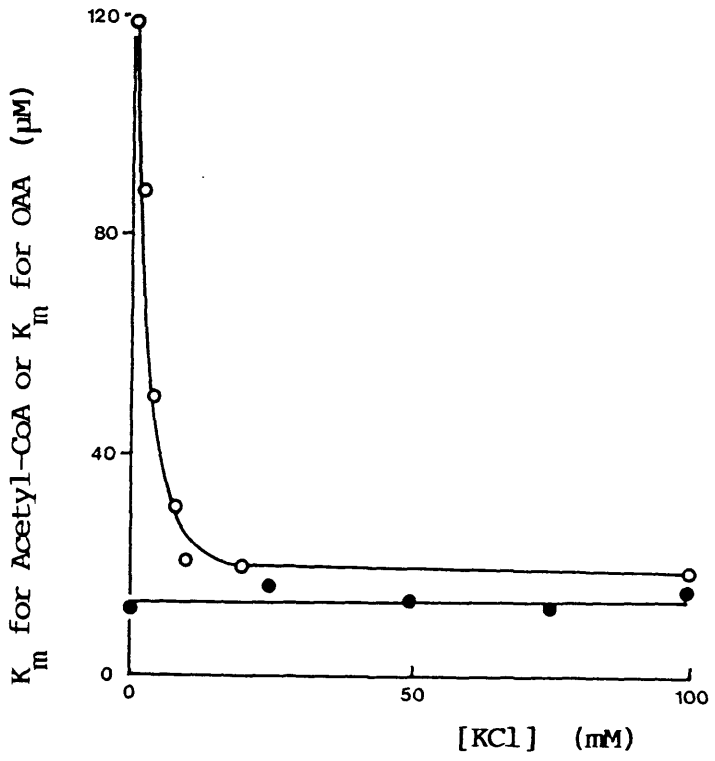


(a) : Half reciprocal plot of variation of initial velocity with acetyl-CoA concentration

(b) : Hill plot of the data represented in (a)

Figure 33

The Effect of KCl on the Substrate K_m Values of M. methylotrophus CS



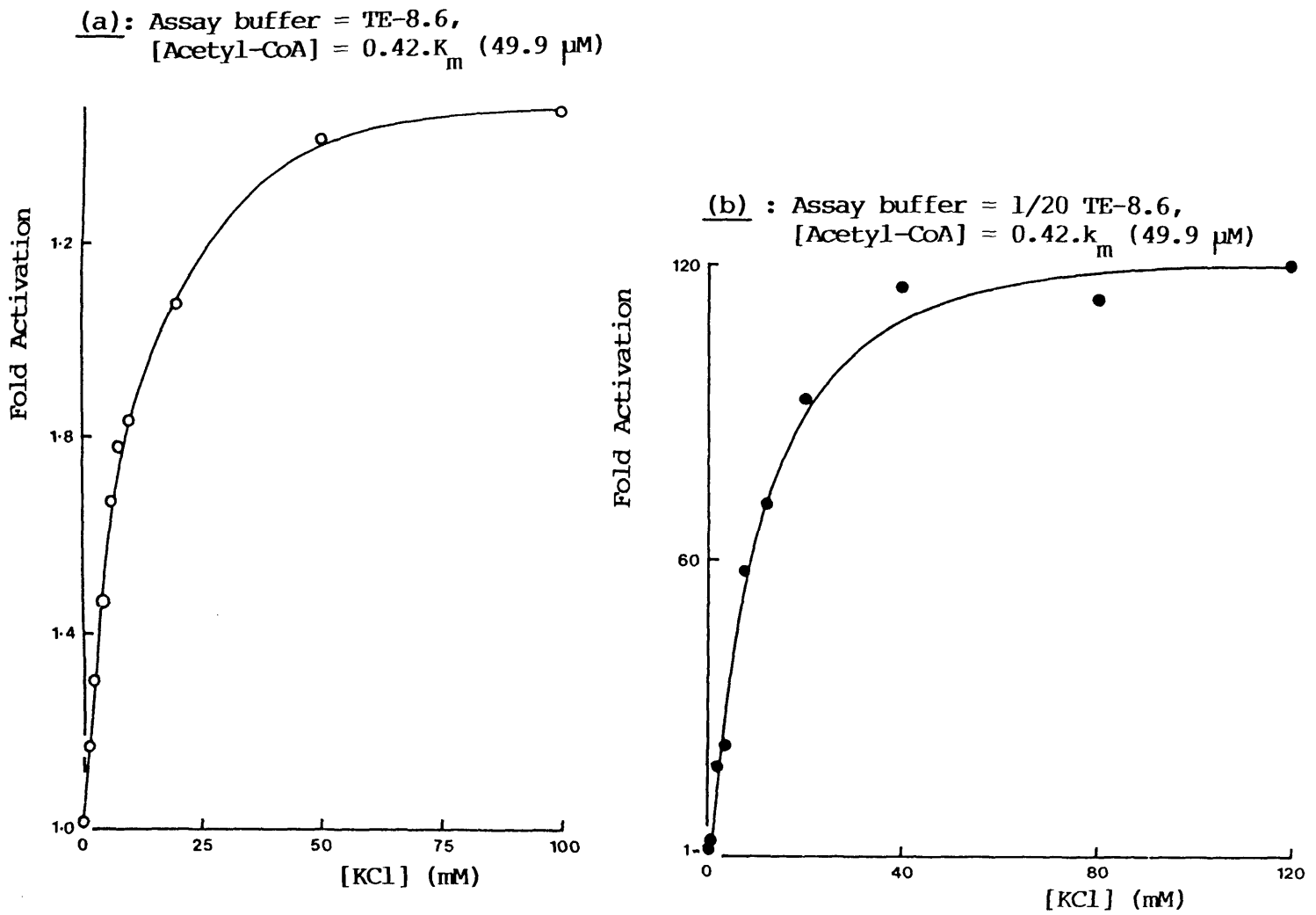
○—○ : Acetyl-CoA K_m values

●—● : OAA K_m values

Each K_m value was detemined from data obtained at 6 to 8 substrate concentrations. In the case of acetyl-CoA, the concentration range examined was 49.9 μM to 832 μM. In the case of OAA the concentration range was 1.25 μM to 200 μM.

Figure 34

The Effect of KCl on M. methylotrophus CS Activity at Subsaturating Acetyl-CoA Concentrations



The KCl effect could be greatly exaggerated if the concentration of the assay buffer was reduced. In 1 mM-Tris and 1 mM-EDTA adjusted to pH 8.6 (1/20.TE-8.6), at 0.42.K_m acetyl-CoA, a 120-fold increase in CS activity was achieved by addition of KCl (Figure 34b). That these effects were now more pronounced may have been because KCl was now also replacing the ionic strength that had been contributed by 20 mM-Tris. That the KCl effect was due to raised ionic strength was also suggested because activation was not specific to KCl. In TE-8.6 and at sub-saturating (0.42.K_m) acetyl-CoA, both 0.1 M-NaCl and 0.1 M-NH_4Cl shared the stimulatory properties shown by KCl (Figure 35).

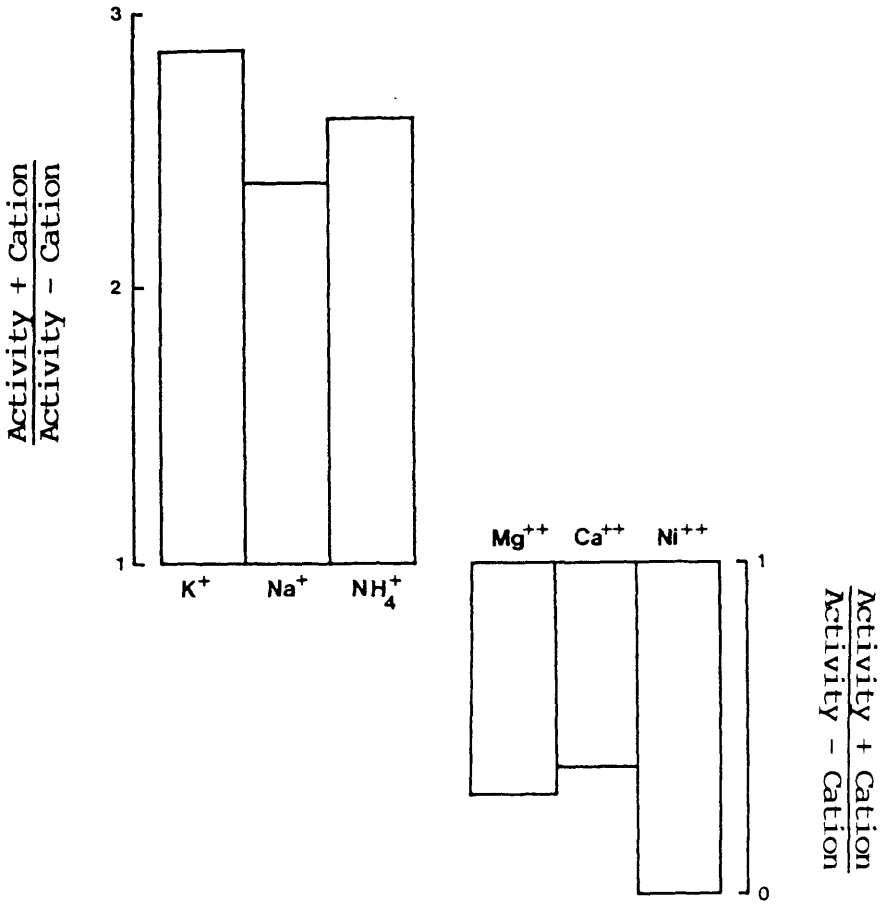
4.2.6.2. The Effect of Divalent Cations on CS Activity. At sub-saturating acetyl-CoA (49.9 μM) the effect of 0.1 M-$MgCl_2$ on CS was examined (for experiments here, the assay buffer was made up with $MgCl_2$ prior to adjustment to pH 8.6). $MgCl_2$ inhibited CS activity by 84%. This effect was not specific to Mg salts and could be mimicked by 0.1 M-$CaCl_2$ or 0.1 M-$NiCl_2$ (Figure 35). Thus it seemed that monovalent cations activated CS, while divalent cations were inhibitory.

This inhibitory effect has been noted before in eukaryotic and prokaryotic CSs (Kosicki and Lee, 1966; Lee and Kosicki, 1967; Faloona and Srere, 1967; Flechtner and Hanson, 1970). Examination of this effect with pig heart CS shows Mg^{2+} is competitive with acetyl-CoA (Kosicki and Lee, 1966). Examination of the effect of $MgCl_2$ on the CS from M. methylotrophus revealed a more complex interaction. In TE-8.6 without EDTA (T-8.6) and at sub-saturating acetyl-CoA (0.5.K_m; 59 μM), $MgCl_2$ actually activated CS (Figure 36a). This effect was maximal (1.83-fold activation) at 4 mM-$MgCl_2$. However, higher $MgCl_2$ concentrations caused the activity to fall and by 45 mM-$MgCl_2$ CS activity had returned to the level it possessed without $MgCl_2$. Further increases in $MgCl_2$ perpetuated the fall in activity so that at 0.1 M-$MgCl_2$, CS was 33% as active as in the absence of $MgCl_2$ (Figure 36a).

Thus, it seems there were two (at least) competing effects on CS activity. The fall in activity may been caused by competition of $MgCl_2$ with acetyl-CoA which increased the acetyl-CoA K_m. Conversely, the increase in activity caused by lower $MgCl_2$ concentrations may have been due to a drop in the acetyl-CoA K_m resulting from the inevitable

Figure 35

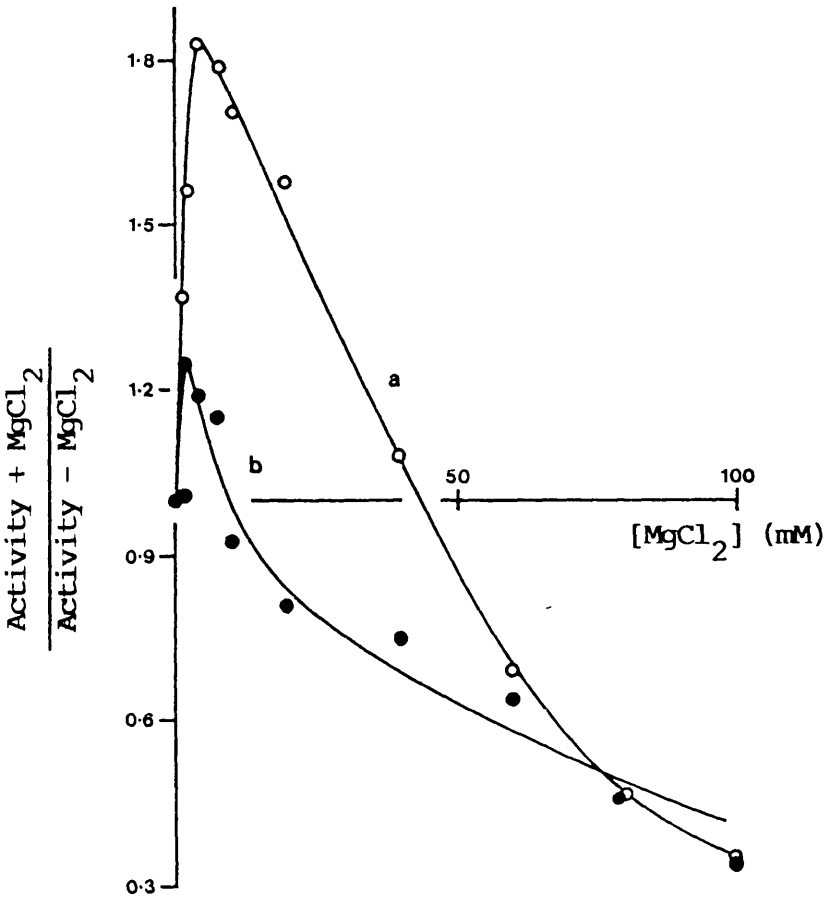
The Effect of Various Cations on M. methylotrophus CS Activity at Subsaturating Concentrations of Acetyl-CoA



[Acetyl-CoA] = 0.42.K_m (49.9 μM), counter ion = Cl^-.
[Metal cation] = 0.1 M.

Figure 36

The Effect of Increasing MgCl2 Concentration on M. methylotrophus CS at Subsaturating and Saturating Acetyl-CoA Concentrations



o——o : Response in the presence of 59 μM-acetyl-CoA ($0.5.K_m$)

●——● : Response in the presence of 500 μM-acetyl-CoA ($4.2.K_m$)

All assays were carried out in T-8.6.

rise in ionic strength on addition of $MgCl_2$. Another explanation for CS activation is prompted by Weitzman and Danson (1976) who have noted that $MgCl_2$ can stimulate the activity of a number of CSs. This results from formation of a Mg^{2+}:OAA chelate in which Mg^{2+} polarises the OAA carbonyl group and so encourages the nucleophilic attack on that group by acetyl-CoA. Thus, two approaches were adopted to investigate the activation of M. methylotrophus CS activity by $MgCl_2$.

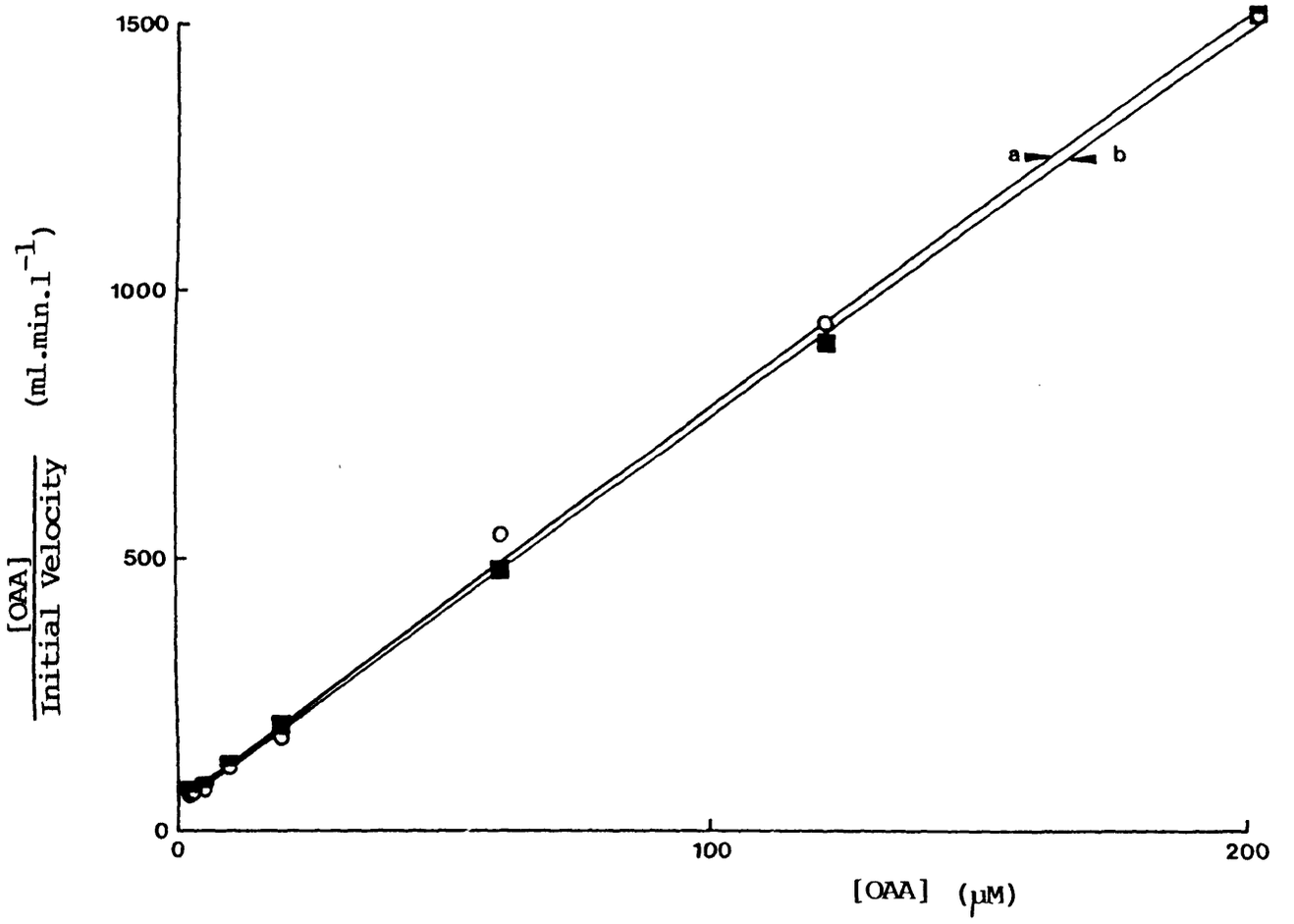
4.2.6.2.1. Effects of $MgCl_2$ on the OAA Dependence of CS. Dependence of activity on OAA was analysed in T-8.6 at 0.5 mM-acetyl-CoA and 0 or 4 mM-$MgCl_2$. Results for CS desalted into T-8.6 are plotted in Figure 37. The K_m and V_{max} values without $MgCl_2$ were 6.3 μM and 0.133 $U.ml^{-1}$. The same values with $MgCl_2$ were 8.1 μM and 0.139 $U.ml^{-1}$. Thus, the data show that the $MgCl_2$ concentration that maximally activates M. methylotrophus CS at sub-saturating acetyl-CoA has little influence on the dependence of CS activity on OAA concentration.

4.2.6.2.2. Effects of $MgCl_2$ on CS at Saturating Acetyl-CoA. If $MgCl_2$ activation of CS resembled activation by KCl in that it reduced the K_m for acetyl-CoA, then activation of CS by $MgCl_2$ should be reduced by increasing acetyl-CoA. At 0.5 mM-acetyl-CoA ($4.2.K_m$; $v = 0.81V_{max}$), low concentrations (< 15 mM) of $MgCl_2$ still activated CS (Figure 36b). However, activation was far less than at the lower concentration of acetyl-CoA (Figure 36a, c.f. 36b). This seemed consistent with the idea that low concentrations of $MgCl_2$ did activate CS as a result of raised ionic strength. The low level of activation seen with the higher acetyl-CoA concentration may have been because, even then, CS was not entirely saturated with acetyl-CoA.

4.2.6.3. The Effect of Anions on CS Activity. The effect of mono and divalent anions at 0.1 M on CS (cation = K^+) was studied at 49.9 μM-acetyl-CoA ($0.42.K_m$). Cl^-, Br^- and NO_3^- were added directly to the assay. Phosphate (about 98% HPO_4^{2-}) and carbonate (about 97% HCO_3^-) were buffered to pH 8.6 before addition. All anions activated CS [fold-activation]: Cl^- [2.87]; Br^- [1.81]; NO_3^- [1.91]; HCO_3^- [1.96] and HPO_4^{2-} [2.48]. These results indicate that, in contrast to the

Figure 37

The Effect of MgCl2 on the Dependence of M. methylotrophus CS on OAA



Assays were carried out without $MgCl_2$ (a; O) or with 4 mM-$MgCl_2$ (b; ■). The assay buffer was T-8.6.

effects of cations, the charge of the anion may be irrelevant.

4.2.7. Regulatory Properties of M. methylotrophus CS

4.2.7.1. The Effects of 2-OG. As M. methylotrophus probably has a biosynthetic CAC, analogy with organisms that have OGDH-deficient CACs would suggest that CS may be inhibited by 2-OG (**1.8.1.**; **1.12.6.2.1.**; **1.12.10.**; **3.2.2.1.**; Table 3). Thus the effect of 2-OG on CS was tested on M. methylotrophus CS. Pig heart CS was used as a 'control' as this CS operates in a CAC that functions catabolically and so would not be expected to be sensitive to 2-OG. M. methylotrophus CS was inhibited by 2 mM-2-OG. This effect was reversed by OAA showing its competitive nature (Figure 38a). Repetition of the experiment with pig heart CS showed that this CS was far less sensitive to 2-OG. Thus, at 16 μM OAA, M. methylotrophus and pig heart CSs were inhibited 50% and 6% respectively by 2 mM-2-OG (Figure 38b).

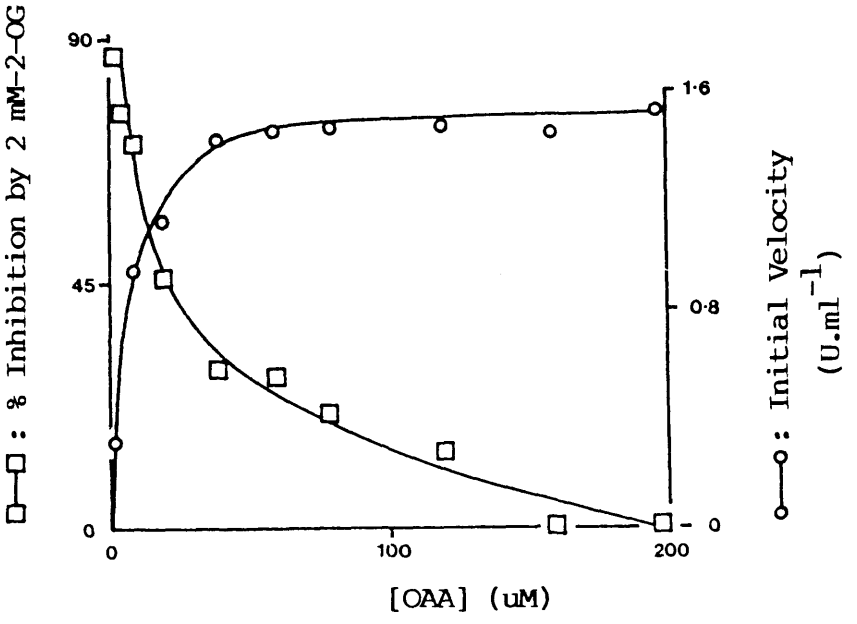
M. methylotrophus CS was assayed at 0.83 mM-acetyl-CoA at varying OAA concentrations at 0, 1, 2 and 6 mM-2-OG. When plotted as a double-reciprocal plot (Figure 39), all lines had a common ordinate intercept thus showing that 2-OG was a competitive inhibitor. Plotting the slopes of these lines against 2-OG gave a straight line (Figure 40). The K_i for 2-OG deduced from its negative intercept on the abscissa was 0.31 ± 0.02 mM (± S.E.). When OAA was held at 0.2 mM and acetyl-CoA was the varied substrate, assays were carried out in the presence of 0, 1, 2, 4 and 6 mM-2-OG. When the data were analysed as a double-reciprocal plot, all lines had a common intercept on the abscissa (Figure 41). This suggests that 2-OG was a non-competitive inhibitor of M. methylotrophus CS with respect to acetyl-CoA.

4.2.7.2. The Effects of Succinyl-CoA. Pending confirmation of the presence of ICL in M. methylotrophus, succinyl-CoA may be an end product of a pathway initiated by CS. Thus, the sensitivity of CS towards succinyl-CoA was examined. Using freshly prepared succinyl-CoA, assays were carried out with and without KCl. Inhibition was dependent on the presence of 0.1 M-KCl (Table 22). Under these conditions, inhibition was inversely proportional to acetyl-CoA

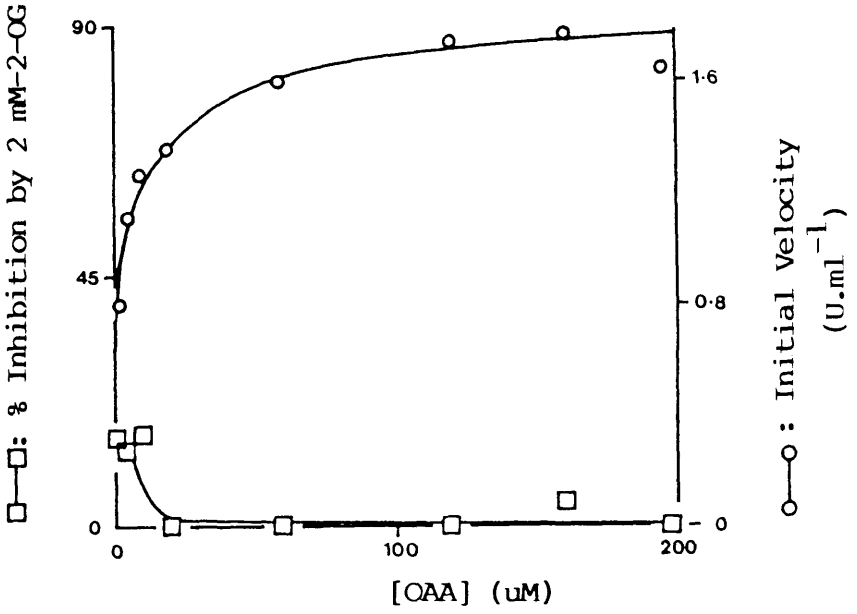
Figure 38

2-OG Inhibition of CS from M. methylotrophus Compared with 2-OG Inhibition of Porcine Heart CS

(a)



(b)



(a) : Effect of increasing [OAA] on inhibition of M. methylotrophus CS by 2 mM-2-OG.

(b) : Effect of increasing [OAA] on inhibition of porcine heart CS by 2 mM-2-OG.

Figure 39

Double Reciprocal Plot of the Dependence of M. methylotrophus CS on OAA at Various Concentrations of 2-OG

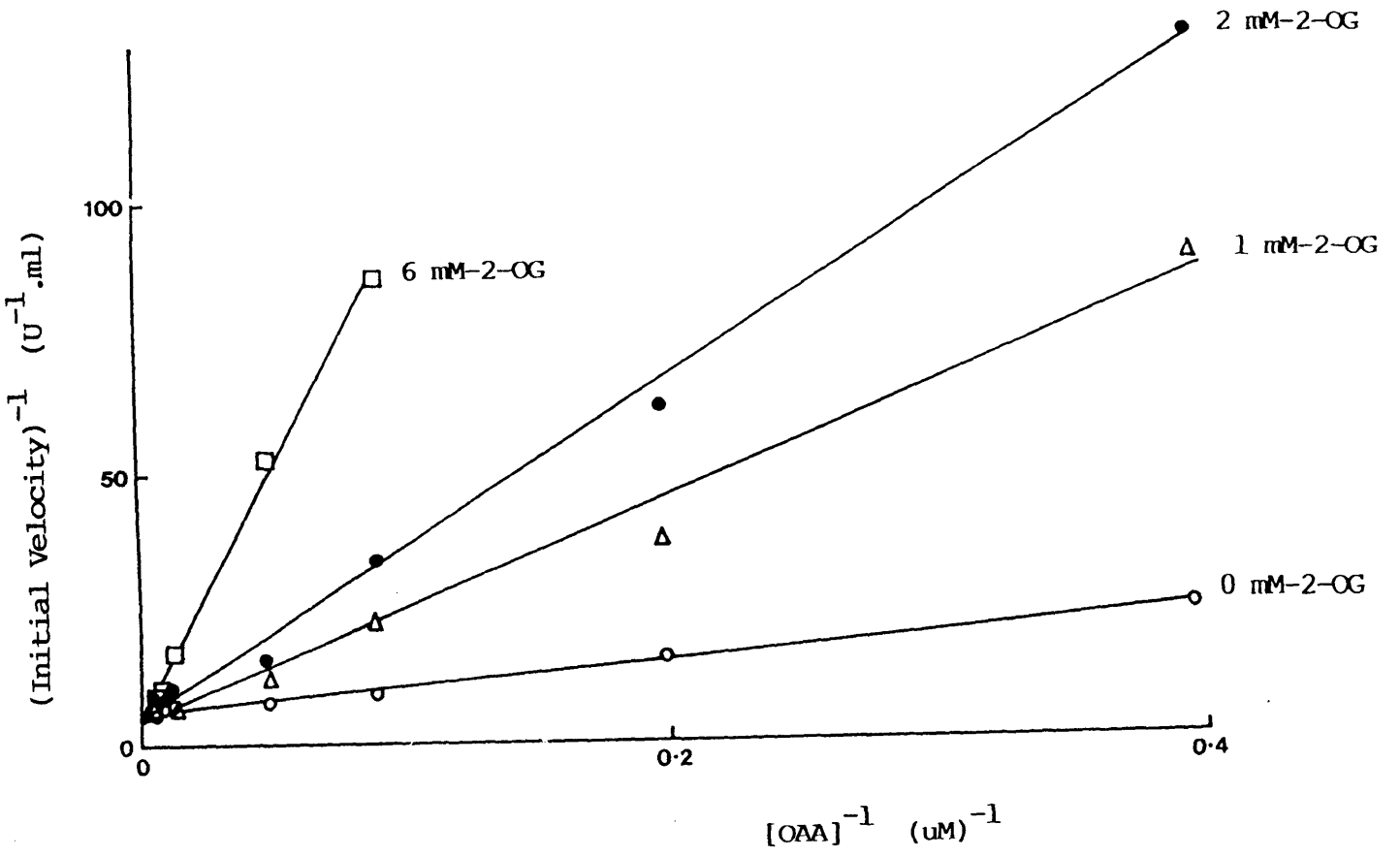
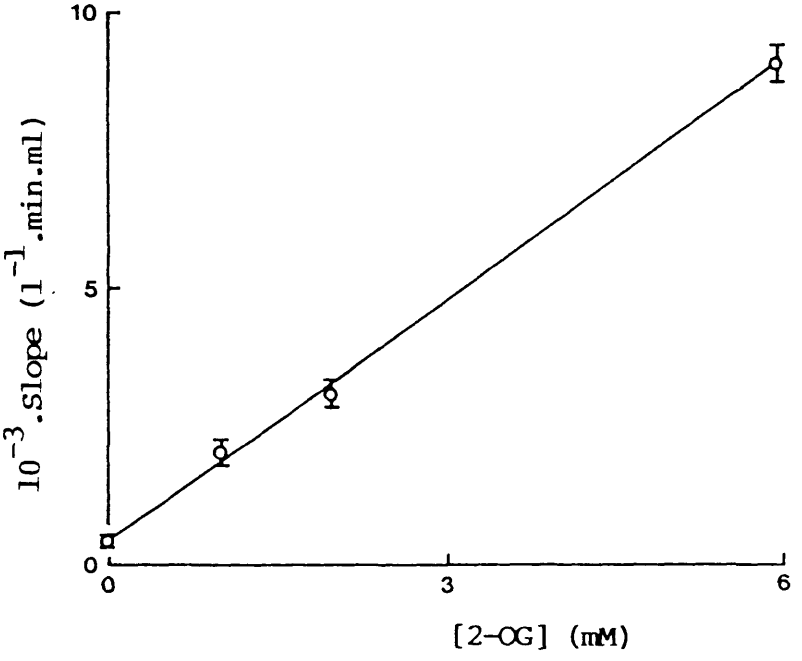


Figure 40

The Effect of 2-OG on the Slope of Double Reciprocal Plots of Dependence of M. methylotrophus CS on OAA



Points are the slopes of lines from Figure 39. The bars are ± S.E. calculated from least squares regression analysis of the data in Figure 39.

Figure 41

Double Reciprocal Plot of Dependence of M. methylotrophus CS on Acetyl-CoA at Various Concentrations of 2-OG

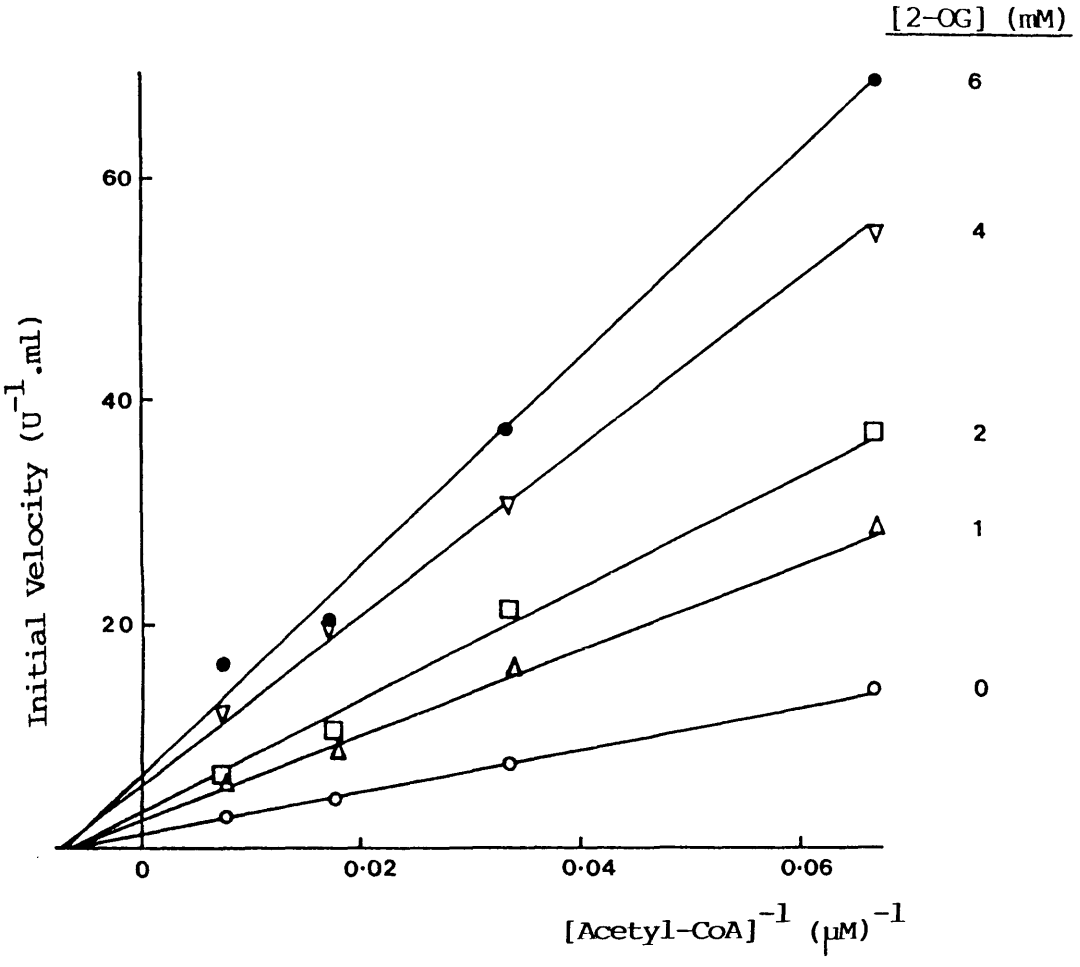


Table 22

Inhibition of M. methylotrophus CS by Succinyl-CoA

No KCl				+ 0.1 M-KCl			
[Acetyl-CoA] (μM)	[Acetyl-CoA] / K_m*	[Succinyl-CoA] (μM)	Inhibition (%)	[Acetyl-CoA] (μM)	[Acetyl-CoA] / K_m*	[Succinyl-CoA] (μM)	Inhibition (%)
155	1.3	168	0	155	7.9	420	10.2
77.5	0.7	168	0	38.8	1.94	420	27.5
38.8	0.33	168	0	6.3	0.31	420	48.6
38.8	0.33	420	0	-	-	-	-

*: The K_m values for acetyl-CoA were taken to be 119.2 μM in the absence of KCl and 19 μM in the presence of 0.1 M-KCl.

concentration which suggested that inhibition was of a competitive nature. The maximum level of inhibition observed was 49% at a [succinyl-CoA]:[acetyl-CoA] ratio of 66.7.

4.2.7.3. The Effects of Adenine Nucleotides. As there is a close positive correlation between the affinity of CS for acetyl-CoA and sensitivity to ATP-inhibition (**1.12.6.1.1.**), this experiment was conducted at a variety of acetyl-CoA concentrations. Further, as it was shown that the acetyl-CoA K_m of M. methylotrophus CS was decreased by increasing ionic strength (**4.2.6.1.**), the effects of adenine nucleotides were also investigated in the presence of 0.1 M-KCl.

Regardless of the acetyl-CoA concentration, without KCl, ATP, ADP and AMP (all at 1 mM) failed to exert more than 8% inhibition (Table 23). However, when the experiment was repeated in 0.1 M-KCl, there was a general increase in inhibition by the adenine nucleotides (Table 23). ATP in the presence of KCl was the most inhibitory nucleotide where the highest acetyl-CoA concentration tested appeared to prevent such inhibition. However, this effect was not generally observed (Table 23) probably as a result of the generally low level of inhibition.

$MgCl_2$ has been found to reduce ATP-inhibition (**1.12.6.1.1.**). This effect was also studied. Using T-8.6 in place of TE-8.6, the inhibition of CS by ATP was examined. Certainly at the two lower concentrations of acetyl-CoA, $MgCl_2$ reduced ATP-inhibition in the presence of KCl relative to that found in the absence of $MgCl_2$ (Table 23). However, again no judgement was possible in the absence of KCl because of the small amount of inhibition that occurred.

4.2.7.4. Nicotinamide Nucleotides. Under the conditions used to study the effects of adenine nucleotides, raised ionic strength (0.1 M-KCl) generally increased the inhibitory potency of all nicotinamide nucleotides (1 mM; Table 24). Even so, the effects of NAD^+ under any conditions were minimal (< 9% inhibition). From the effects of $NADP^+$, NADH and NADPH in the presence of KCl, it was clear that inhibition by nicotinamide nucleotides was inversely related to the concentration of acetyl-CoA, indicating that inhibition was competitive with acetyl-

Table 23

Inhibition of M. methylotrophus CS by Adenine Nucleotides

No KCl				+ 0.1 M-KCl			
Adenine* Nucleotide	[Acetyl-CoA] (μM)	[Acetyl-CoA]$ / K_m	Inhibition (%)	Adenine* Nucleotide	[Acetyl-CoA] (μM)	[Acetyl-CoA]$ / K_m	Inhibition (%)
ATP	54.6	0.5	5.8	ATP	10.1	0.5	18.7
	118	1	0		20.2	1	29.6
	505	4.2	0.4		505	26.6	0
ADP	54.6	0.5	2.9	ADP	10.1	0.5	17.7
	118	1	0.3		20.2	1	0
	505	4.2	0		505	26.6	17.4
AMP	54.6	0.5	6.1	AMP	10.1	0.5	0.1
	118	1	4.8		20.2	1	0
	505	4.2	8.0		505	26.6	21.7
Mg:ATP*	54.6	0.5	1.8	Mg:ATP	10.1	0.5	1.5
	118	1	0.8		20.2	1	8
	505	4.2	6.9		505	26.6	8.3

$: The K_m values for acetyl-CoA were taken to be 119.2 μM in the absence of KCl and 19 μM in the presence of 0.1 M-KCl.
*: The Adenine nucleotide concentrations were 1 mM; MgATP = Assays carried out in T-8.6 + 1 mM-$MgCl_2$.

Table 24

Inhibition of M. methylotrophus CS by Nicotinamide Nucleotides

No KCl				+ 0.1 M-KCl			
Nicotinamide* Nucleotide	[Acetyl-CoA] (μM)	[Acetyl-CoA]$ / K_m	Inhibition (%)	Nicotinamide* Nucleotide	[Acetyl-CoA] (μM)	[Acetyl-CoA]$ / K_m	Inhibition (%)
NAD^+	54.6	0.5	0	NAD^+	10.1	0.5	8.3
	118	1	4.0		20.2	1	0
	505	4.2	0		505	26.6	8.7
$NADP^+$	54.6	0.5	0	$NADP^+$	10.1	0.5	13.2
	118	1	1.9		20.2	1	0
	505	4.2	4.1		505	26.6	1.4
NADH	54.6	0.5	0	NADH	10.1	0.5	18.3
	118	1	0		20.2	1	11.5
	505	4.2	1.8		505	26.6	2.8
NADPH	54.6	0.5	29.2	NADPH	10.1	0.5	51.1
	118	1	11		20.2	1	23.5
	505	4.2	14.8		505	26.6	0

*: The Nicotinamide nucleotide concentrations were 1 mM.
$: The K_m values for acetyl-CoA were taken to be 119.2 μM in the absence of KCl and 19 μM in the presence of 0.1 M-KCl.

CoA. It also appeared that the general order of inhibitory potency was probably NADPH> NADH> $NADP^+$> NAD^+ (Table 24) and thus was positively correlated with the negative charge on the inhibitor.

The effects of the reduced nicotinamide nucleotides were particularly marked - 1 mM-NADPH exerted about 50% inhibition in the presence of KCl at a half K_m concentration of acetyl-CoA. NADH also inhibited CS in the presence of KCl (Table 24). In view of the significance of NADH to the regulation of bacterial CSs, the effects of NADH on the M. methylotrophus CS were studied further.

4.2.7.5. Possibility of Desensitisation of M. methylotrophus CS. Although NADH inhibits M. methylotrophus CS, the effect differs in two respects from that commonly seen with other CSs. First, if M. methylotrophus CS was sensitive to NADH, comparison with other CSs suggests that this effect should have been negated by KCl (**1.12.9.1.**). However, here, KCl increased NADH-inhibition (Table 24). Secondly, CSs from heterotrophically-growing Gram-negative organisms are totally inhibited by the level of NADH used here to detect inhibition of M. methylotrophus CS. However, this CS was no more than 18% inhibited under any of the tested conditions. There are many examples that show the sensitivity of CS towards NADH-inhibition [see references in Weitzman and Danson (1976) and Weitzman (1981)]; however, particularly relevant here are the NADH-sensitive CSs from facultative methylotrophs such as Pseudomonas 3A2 and Organism 5B1 which are completely inhibited by 0.1 mM-NADH (Colby and Zatman, 1975c).

The relative insensitivity of M. methylotrophus CS to NADH in the absence of KCl may be an authentic property or it may be artefactual, arising from desensitisation. This could be brought about by the high pH of the assay medium (pH 8.6) or an extreme lability of NADH-inhibition towards desensitisation by the assay chromogen - DTNB (**1.12.9.1.**). These possibilities were examined. Attention has been drawn to the pH-dependence of the reaction of DTNB (**2.3.3.1.13.**). Thus, under the conditions used in **4.2.4.** the polarographic and DTNP-linked assays were used to study the response of M. methylotrophus CS to 1 mM-NADH at varying pH. The A. calcoaceticus CS was used as a positive control. Interestingly, the polarographic assay showed a

consistent but small degree of NADH-inhibition of M. methylotrophus CS that amounted to no more than 20%. However, no inhibition was found with the DTNP-linked assay (Figure 42). The CS from Acinetobacter was inhibited by 90% between pHs 6.2 and 8.6 by NADH in the DTNP-linked assay; however, above this pH, the enzyme became rapidly desensitised to NADH (Figure 42). Thus the inability of 1 mM-NADH to exert the complete inhibition of M. methylotrophus CS that would normally be expected of a large CS is not a result of desensitisation.

4.2.8. Use of M. methylotrophus CS as an NADH-Insensitive Control

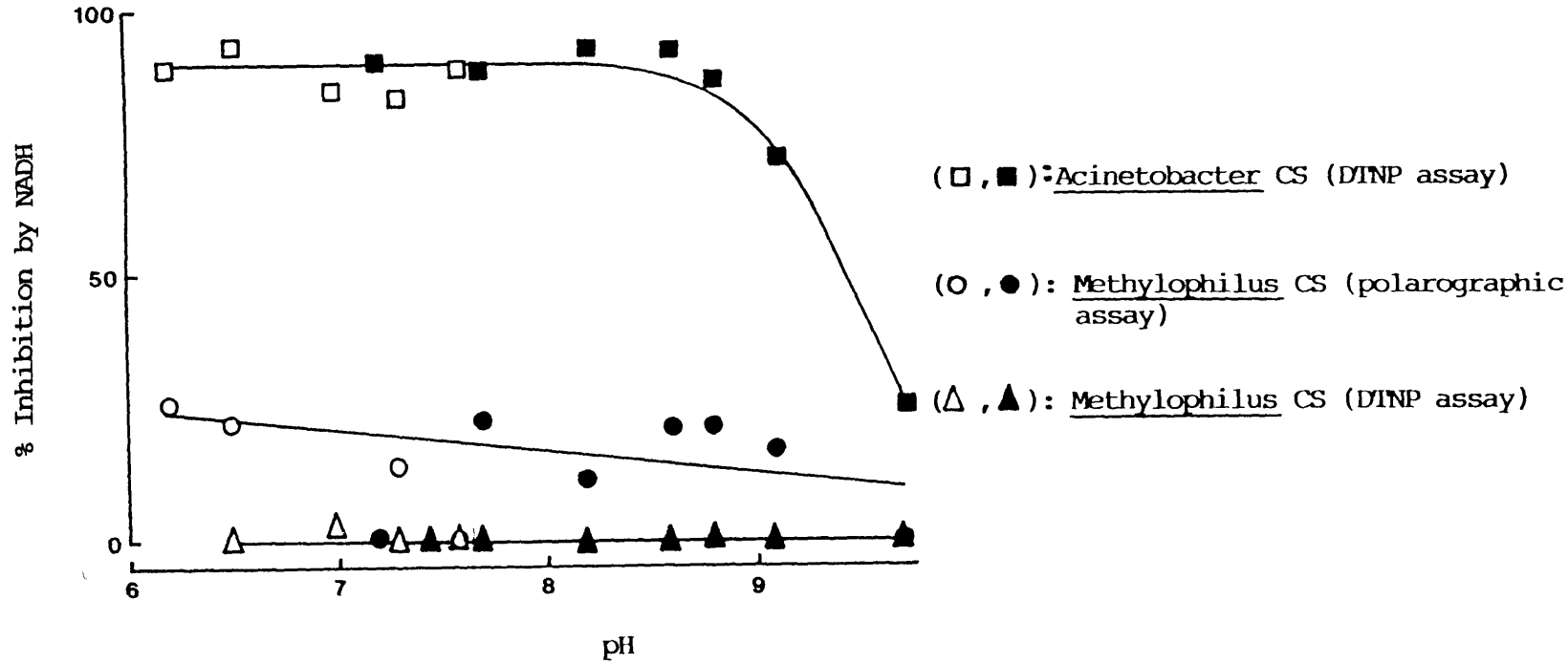
Thus far, it appears that M. methylotrophus CS is relatively NADH-insensitive but has the quaternary structure of a large NADH-sensitive CS (**4.2.2.**). The role of conformational change in the response of NADH-sensitive CS to NADH has been inferred from effects of NADH on thermal stability or gross morphology of CS as studied by electron microscopy (**1.12.9.2.**). It may now be possible to assess the relevance of such effects to NADH-inhibition by direct comparison with the behaviour of M. methylotrophus CS in the presence of NADH. The NADH-sensitive CS used here was the purified A. calcoaceticus CS.

4.2.8.1. The Effects of NADH on Thermal Stability of CS. T_{50} for 5 min was determined for the A. calcoaceticus and M. methylotrophus CSs desalted into TE-8.0. 0.15 ml of TE-8.0 was pre-warmed to between 55°C and 65°C for 2 min. 50 µl of CS was added to give concentrations of 11.7 $\mu g.ml^{-1}$ and 12.5 $\mu g.ml^{-1}$ of Acinetobacter and M. methylotrophus CS respectively. After 5 min, the enzyme was chilled and remaining activity was determined. As can be seen in Figure 43, the T_{50} values for M. methylotrophus and Acinetobacter for 5 min were 60°C and 62°C respectively. At these temperatures, the time course of inactivation was determined for both enzymes over 10 min. Incubations were carried out as before and samples were removed every 2 min. The experiment was repeated after addition of 1 mM-NADH to the incubation. All samples were assayed with 1 mM-AMP to nullify any effects of NADH. The data were plotted semi-logarithmically in Figure 44.

NADH protected the NADH-sensitive Acinetobacter CS by reducing

Figure 42

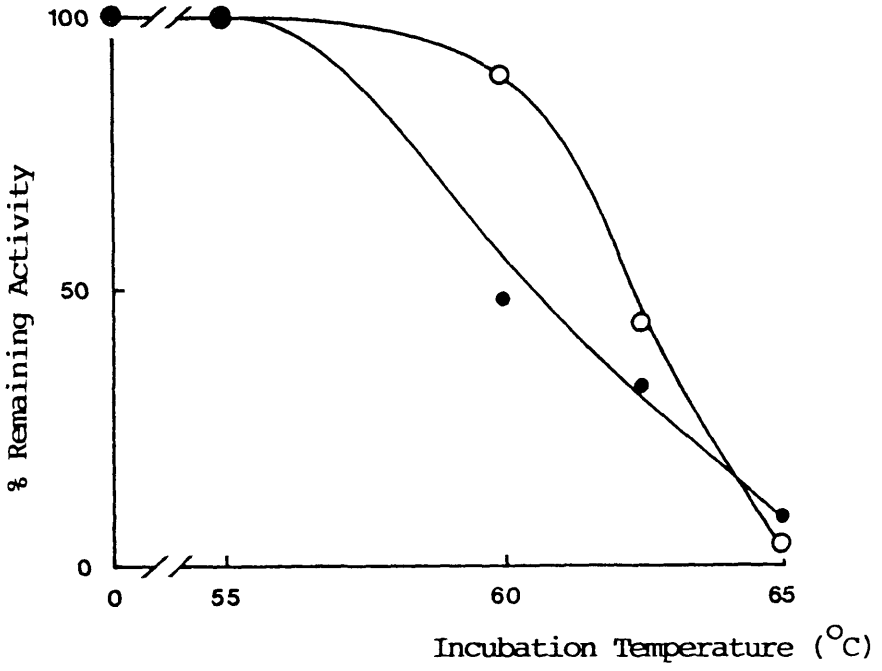
The Effect of pH on the Response of Acinetobacter and Methylophilus CSs to NADH



Filled symbols represent data derived from assays carried out in 20 mM-Tris and 1 mM-EDTA. Open symbols represent data derived from assays carried out in 20 mM-Mops and 1 mM-EDTA. The NADH concentration was 1 mM.

Figure 43

The Effect of Increasing Temperature on the Activity of A. calcoaceticus and M. methylotrophus CSs



●—●: M. methylotrophus CS activity

○—○: A. calcoaceticus CS activity

A. calcoaceticus CS and M. methylotrophus CS were incubated in TE-8.0 at final concentrations of 11.7 and 12.5 $\mu g.ml^{-1}$ respectively. Incubations were for 5 min at the specified temperature.

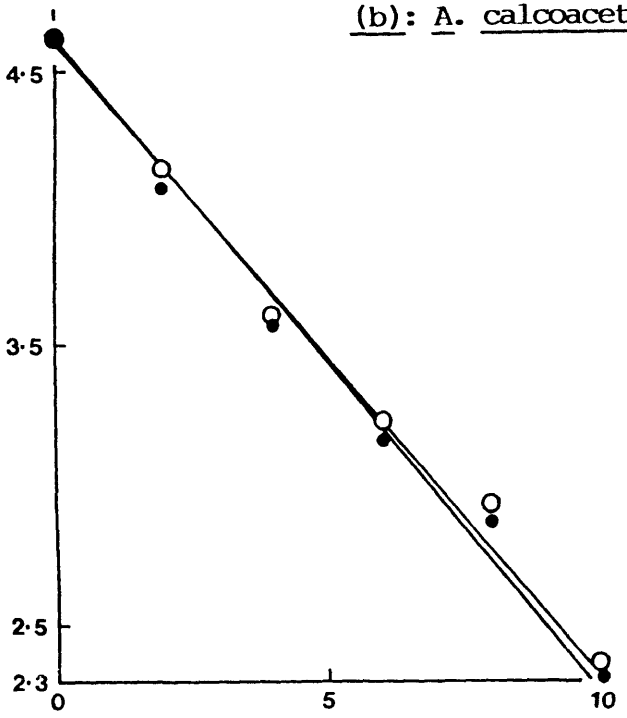
Figure 44

Time Courses of Thermal Inactivation of CSs from M. methylotrophus and A. calcoaceticus in the Presence and Absence of NADH

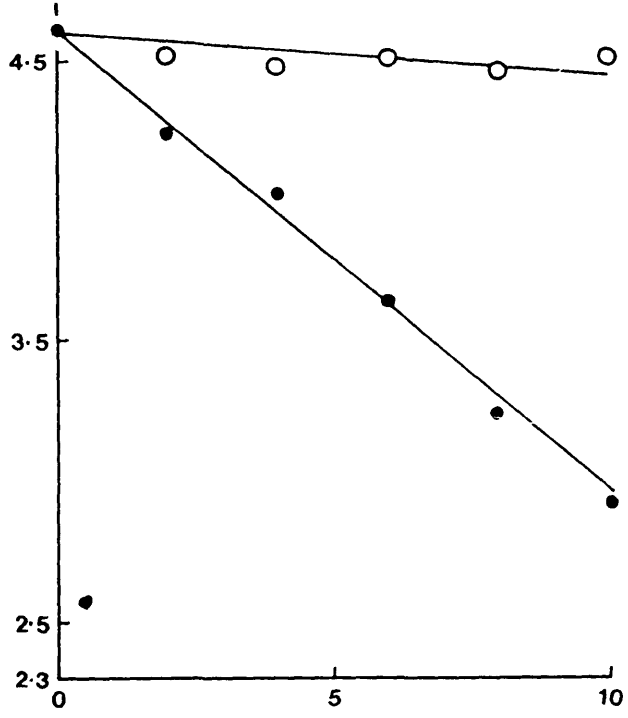
●——●: Incubation in TE-8.0 alone

○——○: Incubation in TE-8.0 and 1 mM-NADH

(a): M. methylotrophus CS



(b): A. calcoaceticus CS



A. calcoaceticus CS and M. methylotrophus CS were incubated at final protein concentrations of 11.7 $\mu g.ml^{-1}$ and 12.5 $\mu g.ml^{-1}$ respectively.

its rate constant of inactivation 15-fold (Figure 44, Table 25). NADH did not affect inactivation of the NADH-insensitive M. methylotrophus CS. Evidence from crosslinking studies suggests that protection of Acinetobacter CS by NADH against thermal inactivation results from a change in conformation induced by NADH (**1.12.9.2.**). Thus the behaviour of the CS from M. methylotrophus implies that NADH is unable to influence the conformation of this enzyme and indirectly confirms the role of conformational change in the response of the Acinetobacter CS to NADH. The inability or otherwise of M. methylotrophus to undergo a conformational change in the presence of NADH was examined directly by electron microscopy.

4.2.8.2. Electron Microscopy. Rowe and Weitzman (1969) showed that NADH causes a significant increase in the size of Acinetobacter CS. This was demonstrable with two negative stains and metal shadowing and was taken to show that NADH caused a conformational change gross enough to be measured by electron microscopy. However, on such data alone, Rowe and Weitzman could not reject the following hypothesis. Size changes might have been caused because NADH increased the susceptibility of CS to deformation during preparation for electron microscopy and did not reflect responses of the conformation of CS in solution towards NADH. To counter this argument, Rowe and Weitzman (1969) showed an NADH-induced change in sedimentation in Acinetobacter CS but, unfortunately, it was impossible to quantify the confidence of this result. M. methylotrophus CS has a quaternary structure similar to the Acinetobacter CS and may have a large degree of sequence homology with the latter but, unlike the Acinetobacter CS, is NADH-insensitive. Thus M. methylotrophus CS may provide an opportunity to re-evaluate the conclusions of Rowe and Weitzman (1969).

Samples of pure CS were incubated in 1 mM of NADH, NADPH, NAD^+, AMP or NADH + AMP. Incubations were also carried out with 0.2 M-KCl and 0.2 M-KCl + 1 mM-NADH. All incubations were for 10 min prior to application to microscope grids. Particle widths were assessed from the negatives by measuring maximum particle widths in a direction parallel to the longest dimension of the negative with a travelling microscope. All widths are the mean of 200 measurements and are given

Table 25

Effect of 1 mM-NADH on the First Order Rate Constant of Thermal Inactivation of CSs from M. methylotrophus and A. calcoaceticus

M. methylotrophus			A. calcoaceticus		
$k_{[-NADH]}$ (min^{-1})	$k_{[+NADH]}$ (min^{-1})	$\frac{k_{[-NADH]}}{k_{[+NADH]}}$	$k_{[-NADH]}$ (min^{-1})	$k_{[+NADH]}$ (min^{-1})	$\frac{k_{[-NADH]}}{k_{[+NADH]}}$
0.236	0.226	1.0	0.165	0.011	15.0

± S.E.M. Results are presented as: micrographs (Figures 45, 46, 47, 49, 50 and 51), histograms of particle widths (Figure 48) and as a table of the mean values of particle widths (Table 26). Statistical comparisons were made with Student's t-test. Prior to its application, it was established that the sample variances were indistinguishable at the 97.5% confidence level using the F-test (Pollard, 1979).

4.2.8.2.1. A. calcoaceticus CS. Figure 45 shows a field of CS molecules. Although the main objective of this investigation was not a structural investigation of the CSs, it seems that a number of the particles from the Acinetobacter preparation are triangular (have a definite three-fold symmetry). This has been also been observed by Rowe and Weitzman (1969) and is discussed later. Measurement of particle widths revealed a distribution of sizes (Figure 48). This is not unexpected, as there is no reason to expect all particles to adopt a single orientation on the carbon substrate film. The mean width was 88 ± 0.77 Å. This value is within 11 % of that (98.7 ± 0.6 Å; n = 100) observed by Rowe and Weitzman (1969). A possible cause for this small discrepancy may be due to differing calibrations of magnification.

The effect of NADH on the Acinetobacter CS molecule was then examined. It seemed that NADH may have added a further complication to interpretation. This was that NADH caused 'clumping' of particles (Figure 46) and reduced the single particle density on the grid. The cause of this was unknown, and so it had to be assumed that this effect was irrelevant to any effects seen with single particles, and that the clumping and not the presence of a single particle was spurious. Widths of single particles seen with NADH again showed a distribution of sizes (Figure 48). Now the mean particle width was 112.4 ± 0.78 Å. This change in width (+27%) is not readily perceptible by eye from the images in Figures 45 and 46 but represented a very significant increase in size ($p < 0.001$).

When the experiment was repeated with NADH in the presence of AMP, the particle width dropped from the value it had with NADH to 85.7 ± 0.84 Å ($p < 0.001$) and the particle width distribution was correspondingly skewed towards this value (Figure 47, 48). There was no statistical difference between this population and that in the

Figure Legend to Figures 45, 46 and 47

A 0.16 $mg.ml^{-1}$ solution of A. calcoaceticus CS in TE-8.0 buffer with or without additions was applied to the grid, left for 1 min, blotted dry and then stained with uranyl acetate.

Micrograph negatives were printed onto Ilford Multigrade III paper. The scale bar is 500 Å. The final magnification is X 228,000.

Figure 45

An Electron Micrograph of a Field of Particles of A. calcoaceticus CS

Triangular aspects shown by the particles are circled. The inner diameter of each circle is equivalent to 330 Å.

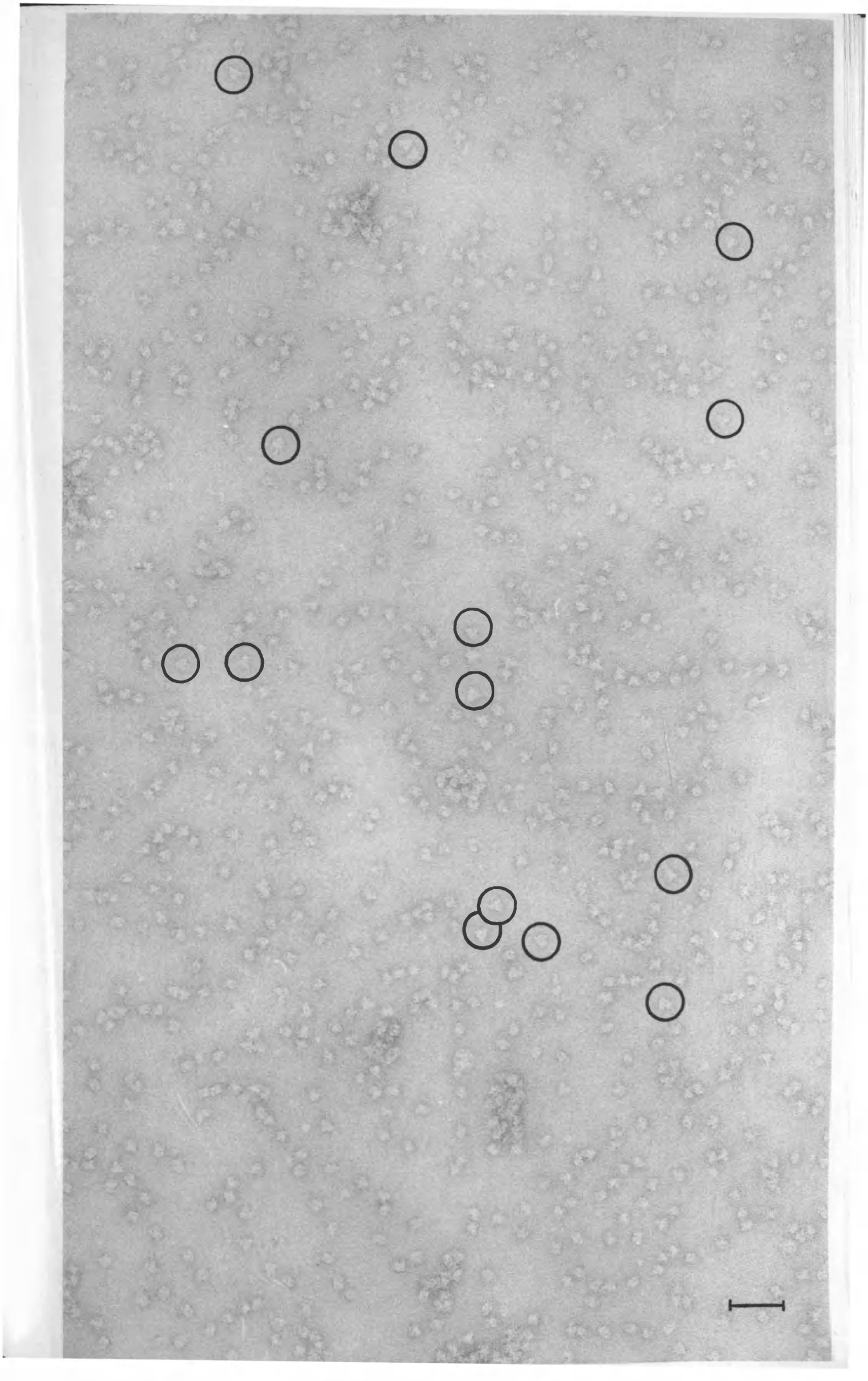


Figure 46

An Electron Micrograph of a Field of Particles of A. calcoaceticus CS Incubated in the Presence of 1 mM-NADH

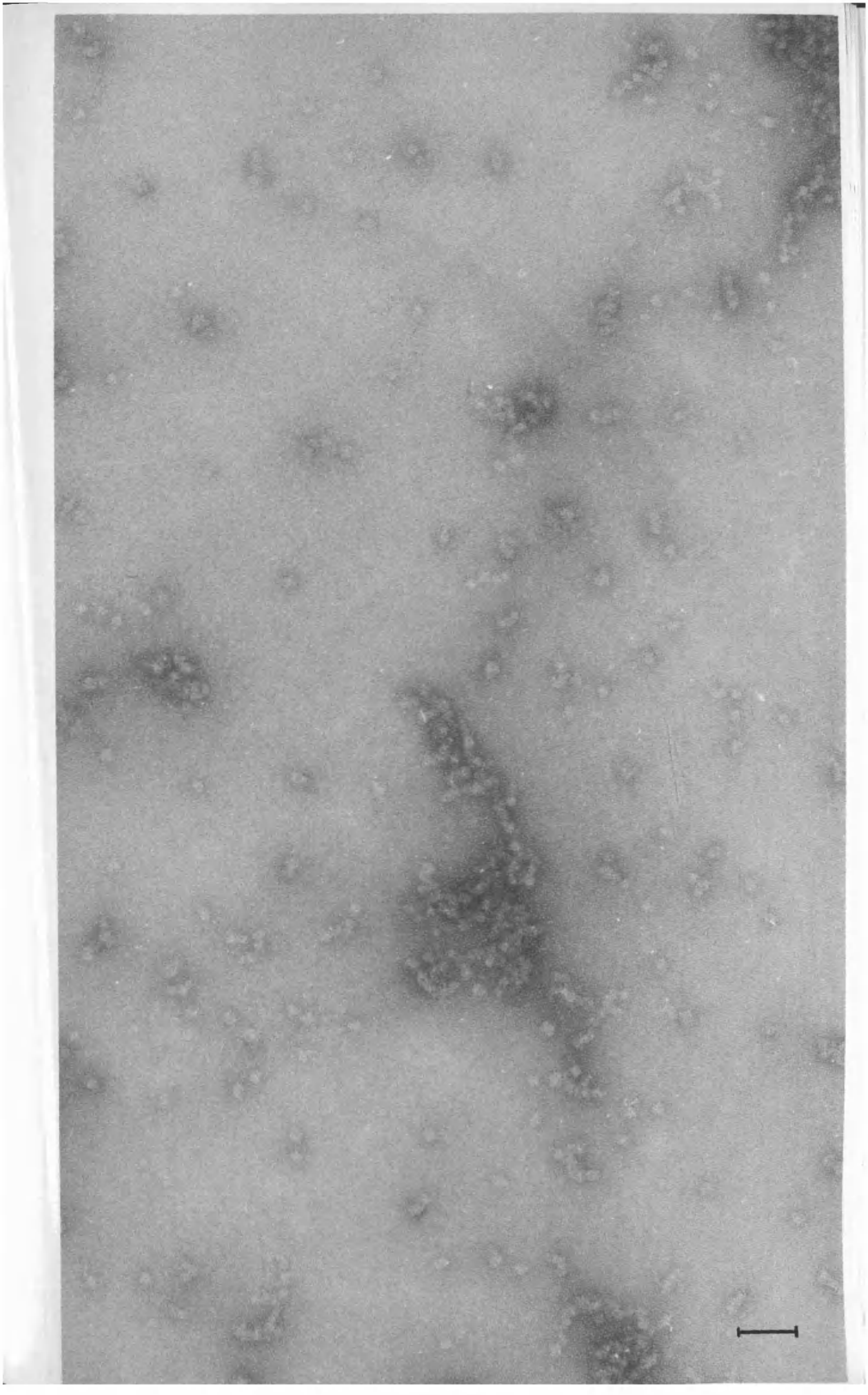


Figure 47

An Electron Micrograph of a Field of Particles of A. calcoaceticus CS Incubated in the Presence of 1 mM-NADH and 1 mM-AMP

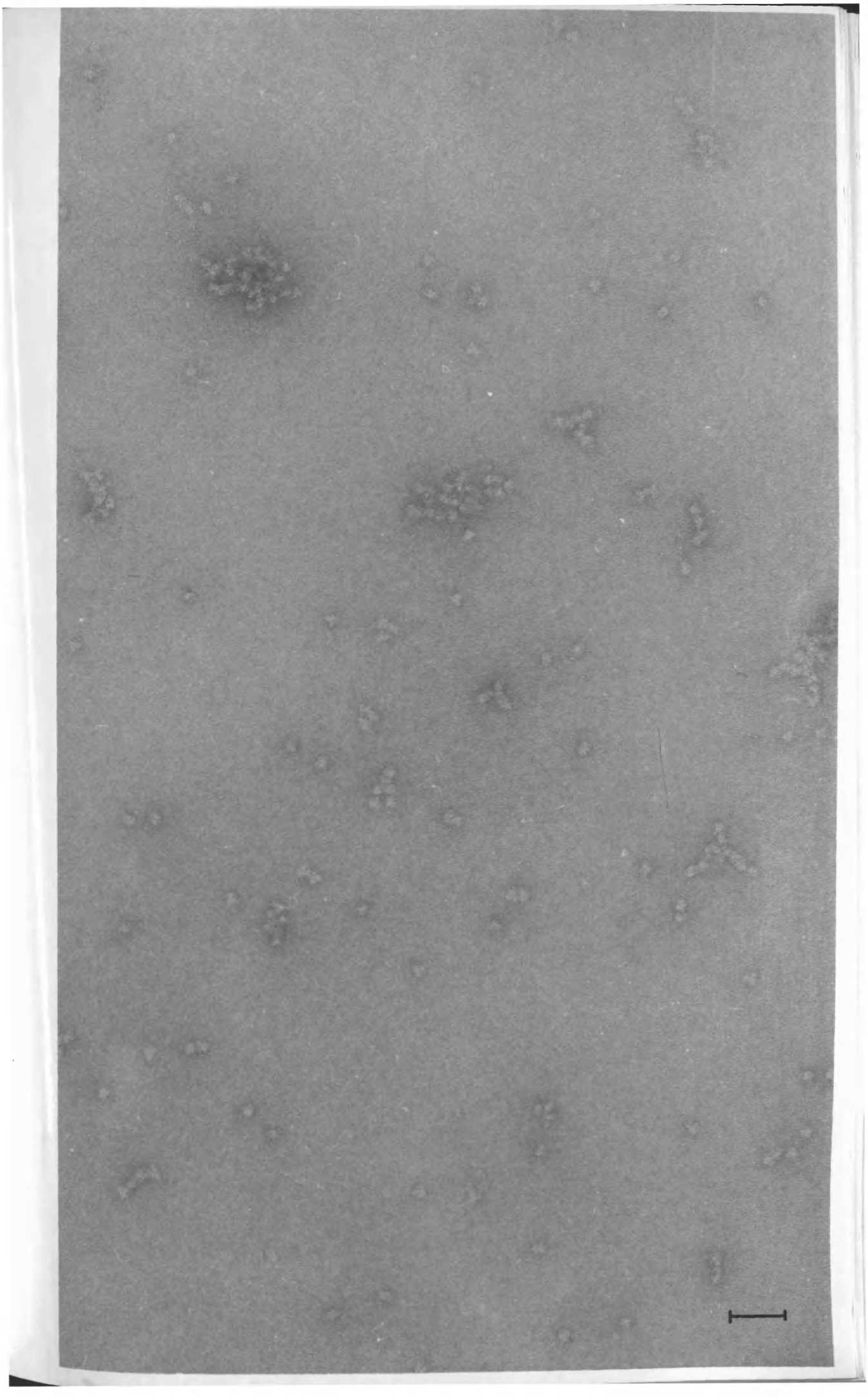
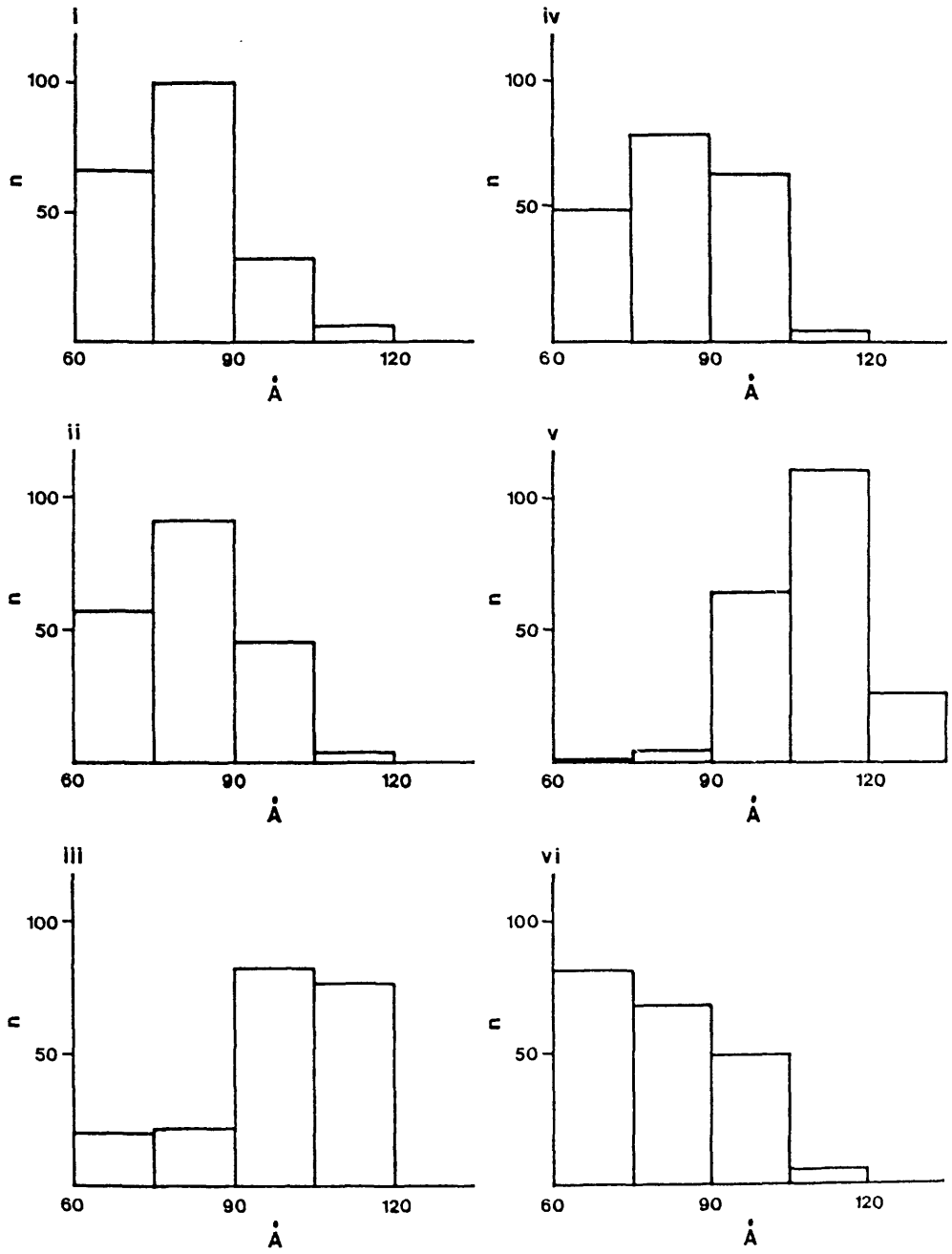


Figure 48

Histograms of Width Distributions of CSs from M. methylotrophus and A. calcoaceticus



(i)-(iii): M. methylotrophus CS; (iv)-(vi): A. calcoaceticus CS. (i) and (iv) are CS alone; (ii) and (v) are CS treated with 1 mM-NADH; (iii) CS treated with 0.2 M-KCl; (vi) CS treated with 1 mM-NADH and 1 mM-AMP. Each Histogram is composed of 200 measurements.

absence of both NADH and AMP. The effect of KCl was similar to that of AMP, 0.2 M-KCl decreased the width of CS in the presence of NADH ($p < 0.001$) to a value identical to that of the enzyme prepared from buffer alone (88 ± 0.69 Å). KCl and AMP alone exerted no effect on the width of the enzyme (Table 26). Further, as can be seen from Table 26, neither NAD^+ or NADPH affected the width of Acinetobacter CS, thus emphasising the specificity of the effects of NADH on the size of CS.

Apart from the failure here to find any effect of KCl on the size of the Acinetobacter CS, the data were identical to those reported by Rowe and Weitzman (1969). It was now of interest to determine the effects of NADH, AMP and KCl on the M. methylotrophus CS.

4.2.8.2.2. M. methylotrophus CS. Although there was no easily recognisable shape (Figure 49), particle widths were distributed (Figure 48) around a mean of 85.7 ± 0.74 Å, a value very similar to that displayed by the Acinetobacter CS. When M. methylotrophus CS was exposed to NADH, (Figure 50), the particle widths were distributed (Figure 48) about a mean of 87.4 ± 0.72 Å. This was statistically identical to the value found without NADH. Similarly, no significant change in the width of M. methylotrophus CS was observable if, prior to grid preparation, the enzyme was incubated with NADPH, NAD^+, AMP or AMP + NADH (widths were between 85.2 ± 0.69 Å and 87.6 ± 0.67 Å; Table 26). Thus, it appeared that NADH-insensitivity of M. methylotrophus CS activity was reflected in its inability to increase its size on examination by electron microscopy. This supports the conclusion that the increase in particle width shown by the Acinetobacter CS in the presence of NADH reflects a related event in solution when the enzyme is inhibited by NADH.

0.2 M-KCl increased the width of M. methylotrophus CS (Figures 48, 51) by 19.7% to 102.6 ± 0.91 Å. This increase was significant ($p < 0.001$). NADH did not modify the KCl effect (100.4 ± 0.82 Å; $p < 0.001$ relative to CS in the absence of both KCl and NADH). This result suggests that KCl may exert some form of conformational alteration on the M. methylotrophus CS, one of the results of which is an increased sensitivity for its substrate - acetyl-CoA. However, it should be pointed out that this conclusion suffers from the type of criticisms

Figure Legend to Figures 49, 50 and 51

A 0.22 $mg.ml^{-1}$ solution of M. methylotrophus CS in TE-8.0 buffer with or without additions was applied to the grid, left for 1 min, blotted dry and then stained with uranyl acetate.

Micrograph negatives were printed onto Ilford Multigrade III paper. The scale bar is 500 A. The final magnification is X 228,000.

Figure 49

An Electron Micrograph of a Field of Particles of M. methylotrophus CS

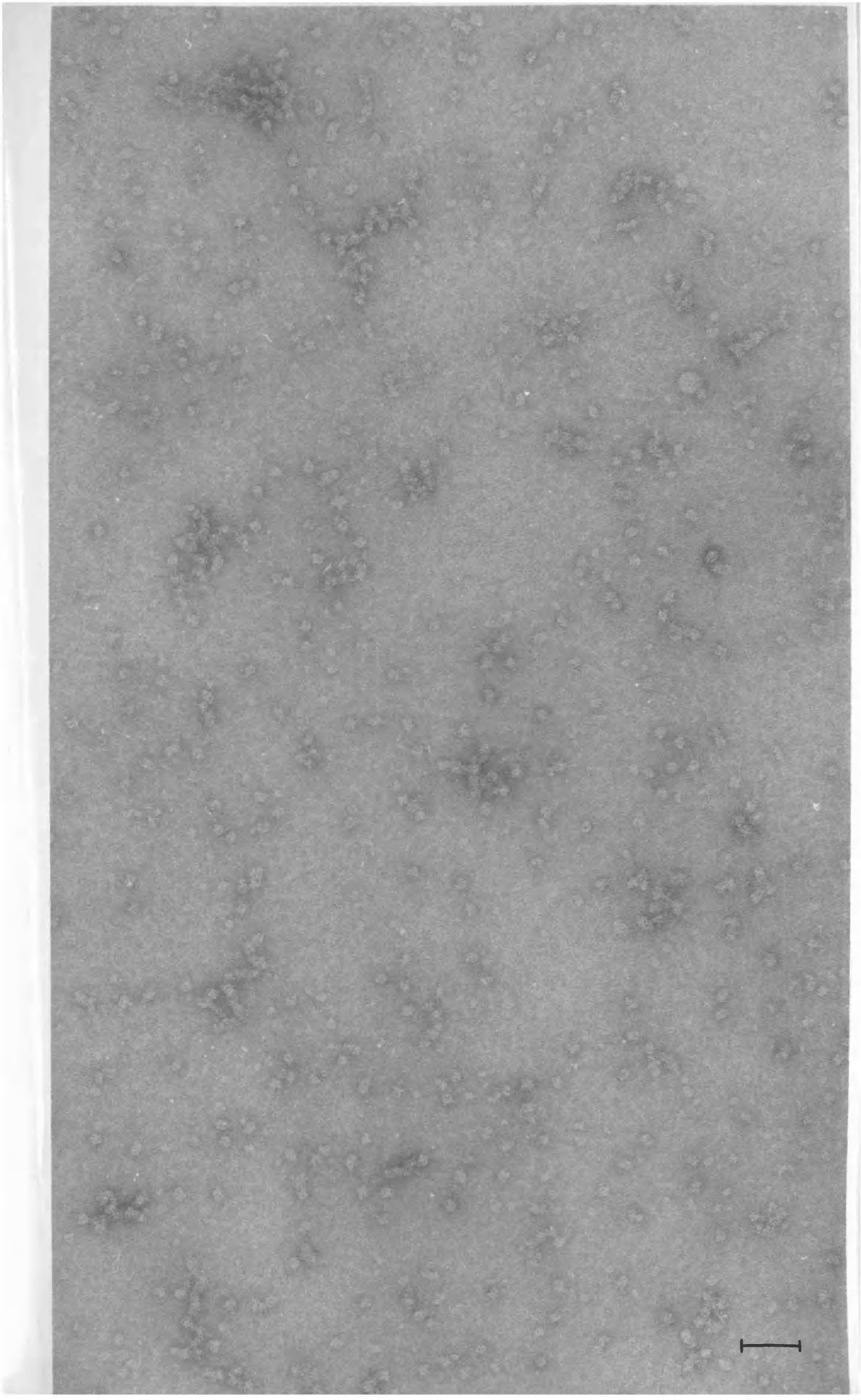


Figure 50

An Electron Micrograph of a Field of Particles of M. methylotrophus CS Incubated in the Presence of 1 mM-NADH

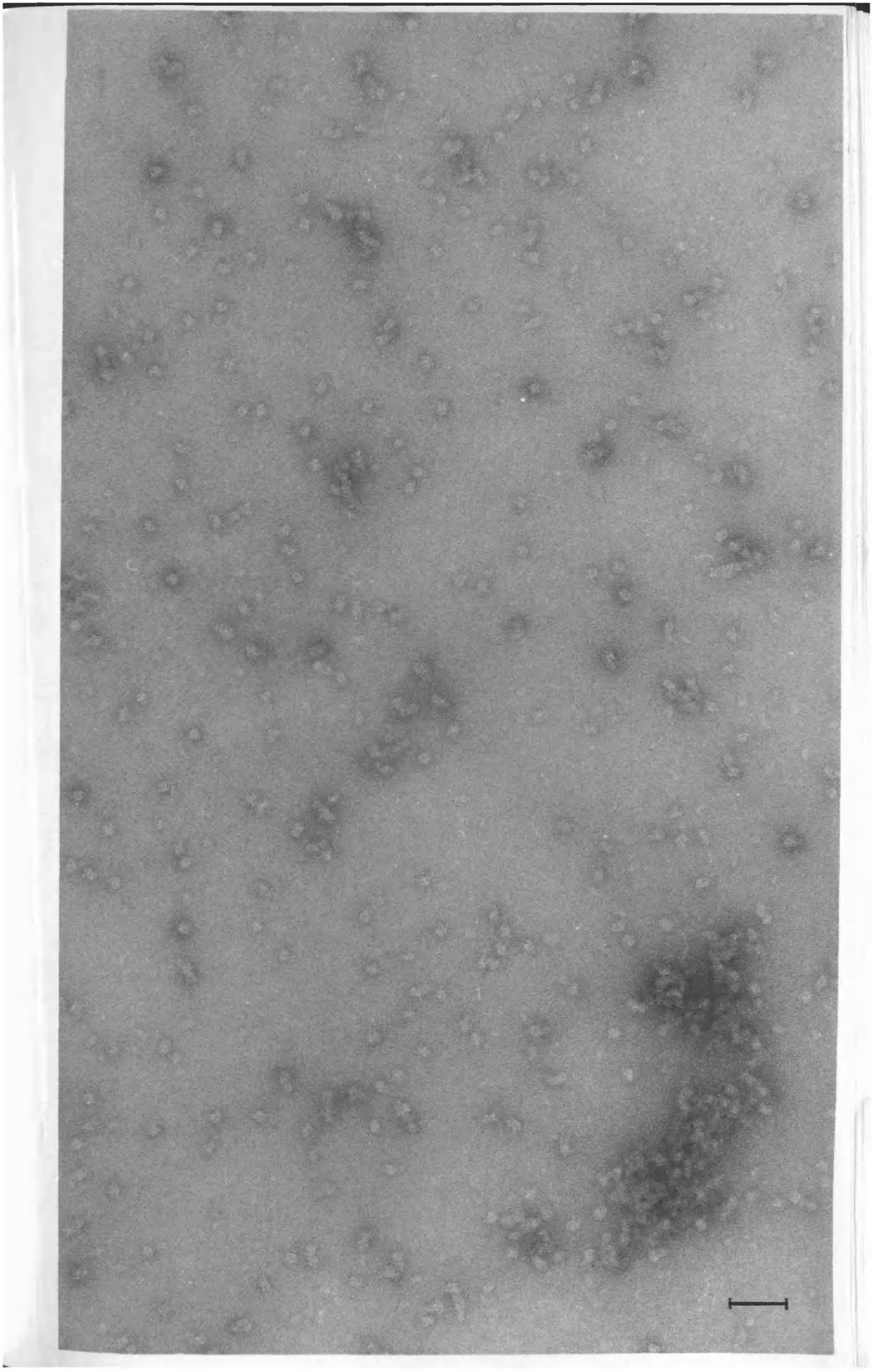


Figure 51

An Electron Micrograph of a Field of Particles of M. methylotrophus CS Incubated in the Presence of 0.2 M-KCl

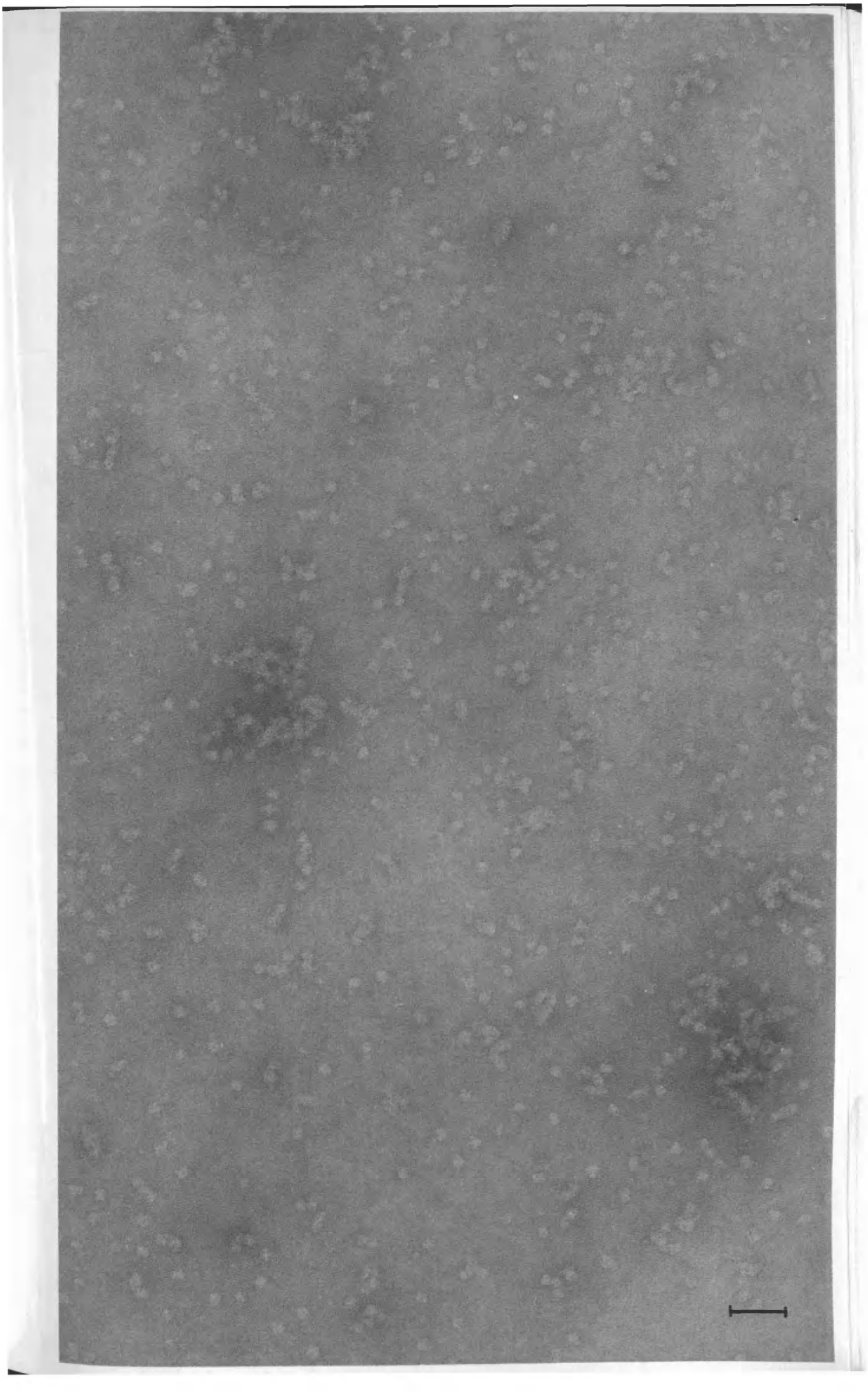


Table 26

Variation of Molecular Widths of CSs from M. methylotrophus and A. calcoaceticus in the Presence of Effectors

Addition (n = 200)	M. methylotrophus		A. calcoaceticus	
	Molecular Width (Å ± S.E.M.)	Significance Relative to CS in Absence of Additions	Molecular Width (Å ± S.E.M.)	Significance Relative to CS in Absence of Additions
None	85.7 ± 0.7	-	88.0 ± 0.8	-
+ 1 mM-NADH	87.4 ± 0.7	None ($p > 0.05$)	112.4 ± 0.8	Significant ($p < 0.001$)
+ 1 mM-NADH and 1 mM-AMP	85.2 ± 0.7	None ($p > 0.05$)	85.2 ± 0.8	None ($p > 0.05$)
+ 0.2 M-KCl	102.6 ± 0.9	Significant ($p < 0.001$)	89.5 ± 0.7	None ($p > 0.05$)
+ 0.2 M-KCl and 1 mM-NADH	100.4 ± 0.8	Significant ($p < 0.001$)	88.0 ± 0.7	None ($p > 0.05$)
+ 1 mM-NAD^+	85.6 ± 0.6	None ($p > 0.05$)	87.0 ± 0.7	None ($p > 0.05$)
+ 1 mM-NADPH	86.1 ± 0.6	None ($p > 0.05$)	87.4 ± 0.7	None ($p > 0.05$)
+ 1 mM-AMP	87.6 ± 0.6	None ($p > 0.05$)	86.8 ± 0.6	None ($p > 0.05$)

that these experiments were designed to answer. Namely, KCl may have increased the susceptibility of M. methylotrophus CS to deformation during sample preparation resulting in an apparent change in size. The pitfalls that are involved in the interpretation of these results are further considered in **4.3.1.** and **4.3.3.8.**

4.3. Discussion

This Chapter reports the first purification of CS from a methylotroph. This result is also significant as it is the first purification of a novel type of CS - one that has a large M_r (> 200,000) but is unusual in that it is NADH-insensitive

Tables 19 and 20 suggest that in M. methylotrophus, CS is only 0.04% (w/w) of the soluble cell protein, whereas A. calcoaceticus possesses ten times as much CS. This may be accounted for (at least in part) by the observation that the latter enzyme is part of a CAC that fulfills demands made on it by biosynthesis and energy conservation. However, the M. methylotrophus CS only needs to supply the citrate needed for biosynthetic purposes (**1.8.1.**; **1.8.2.1.**; **1.8.3.**; **1.10.1.**).

4.3.1. Structural Aspects

The native M_r of M. methylotrophus CS is 266,000 (**4.2.2.2.**). This is consistent with the Gram-stain of the organism and the data of Otto (1986) which suggest a native M_r greater than that of lactate dehydrogenase for this CS. The hexameric quaternary structure of M. methylotrophus CS is shared by CSs from other Gram-negative sources: A. calcoaceticus, Azotobacter vinelandii and a marine Pseudomonas sp. (Srere, 1972; Higa et al., 1978; Mitchell and Weitzman, 1983b).

M. methylotrophus CS also has a similar subunit stoichiometry to that of E. coli CS in the presence of 0.1 M-KCl (Tong and Duckworth, 1975). In the absence of KCl, E. coli CS is a mixture of oligomers (**1.12.3.**). As estimates of the native M_r of M. methylotrophus CS were done in 0.1 M-KCl, it is still possible that this CS is like E. coli CS without KCl. However, analysis of M. methylotrophus CS by non-denaturing PAGE (Figure 23) showed that it migrated as one species

while, under similar conditions, E. coli CS migrated as a number of species (Wright and Sanwal, 1971; Danson and Weitzman, 1973; Morse and Duckworth, 1980). This suggests that M. methylotrophus CS (unlike the E. coli CS) has a solely hexameric structure. In this regard, the M. methylotrophus enzyme resembles the Acinetobacter anitratum CS (Johnson and Hanson, 1974; Morse and Duckworth, 1980).

That the M. methylotrophus CS is similar in size to the large CSs from other Gram-negative sources is also shown by electron microscopy data (Table 26). The M. methylotrophus CS has an apparent molecular width of 88 Å which is within 3 Å of the value for A. calcoaceticus CS. Before further consideration of the electron microscopy results, the following points on interpretation of electron microscopy data should be noted (Haschemeyer, 1970; Dubochet et al., 1982):

a) Images from negatively stained preparations will be dependent on the extent to which the molecule is embedded in, and penetrated by the stain. If the stain thickness is variable, contributions made by different parts of the molecule to the image will vary (this may contribute to the distribution of molecular widths in Figure 48).

b) The orientation of molecules relative to the electron beam may vary depending on the protein and its preparation. Further, interaction of the protein with the support film will be influential in determining the molecule's orientation. This interaction may be influenced by factors such as the hydrophobicity, hydrophilicity or the charge of the support film on exposure to the aqueous sample.

c) Structural damage may result from flattening of particles by forces of surface tension when drying down on the electron microscope grid. Also, any solvent-protein interactions that contribute to protein structure will be lost. Further, extreme changes in concentrations of solute on drying down may affect the protein. However, it must be assumed that the carbon-film support maintains protein conformation.

d) There is no a priori way of knowing the shape of subunits and how they contribute to images of an oligomer.

Although a) to d) seem to undermine the validity of applying electron microscopy to analysis of proteins, the technique is capable of providing useful data. Two examples in particular show this to be the case: a) Scraba et al. (1988) show that the images of rabbit

muscle glycogen phosphorylase b depicted by electron microscopy accurately represent the shape of the molecule as deduced by X-ray crystallography; b) the structure of enteric glutamine synthase sugested by the electron microscopy data of Valentine et al. (1968) has been corroborated by the recent elucidation of the structure of the enzyme by X-ray crystallography (see review by Perutz, 1989).

Inspection of A calcoaceticus CS particles (Figure 45) suggests that some particles are triangular. This has also been noted by Rowe and Weitzman (1969). As the Acinetobacter CS is a hexamer, probably of identical subunits, there are three ways the subunits may be arranged: planar hexagon (Figure 52a), two layers of trimers staggered (octahedral) (Figure 52c) or two layers of trimers eclipsed (trigonal prism) (Figure 52b). Of these, the latter seems consistent with Figure 45. This proposal is consistent with crosslinking studies showing that the dimer is a structrual unit of Acinetobacter CS (Mitchell and Weitzman, 1983b) where this CS can be regarded as a trimer of dimers.

This is not the only possible orientation of CS on the electron microscope grid and it is possible that some of the images in Figure 45 could be other orientations of a trigonal prism (Figure 52b).

For M. methylotrophus CS, there is no easily discernible shape to the particles (Figure 49). This may represent a genuine difference in structure from that of Acinetobacter CS. However, the non-descript shape of the M. methylotrophus CS could result from distortion of its structure during preparation for microscopy. Otherwise, it may be that M. methylotrophus CS can be described as a trigonal prism like Acinetobacter CS but the orientation of M. methylotrophus CS that shows this is not favoured. Thus all that may be concluded from these data is that the M. methylotrophus and A. calcoaceticus CSs are different (because they respond to electron microscopy procedures differently), but not how they differ on a structural level.

The subunit M_r of M. methylotrophus CS is in the range of values quoted for the enzyme from other sources (Higa et al., 1978; Robinson et al., 1983b), i.e. 53,000 (subunit M_r of a marine Pseudomonas sp CS) to 40,300 (M_r value of the Bacillus megaterium CS subunit). Further, the M. methylotrophus CS subunit M_r value is identical to that of the model heterotrophic CS from A. calcoaceticus used in these studies

Figure Legend to Figure 52

In this figure, each sphere is meant to represent a CS subunit of a hexameric arrangement of the latter. Each of the panels 1) to 3) shows a different orientation of three basic subunit arrangements as they may appear lying on the carbon film of an electron microscope grid.

The basic subunit arrangements are:

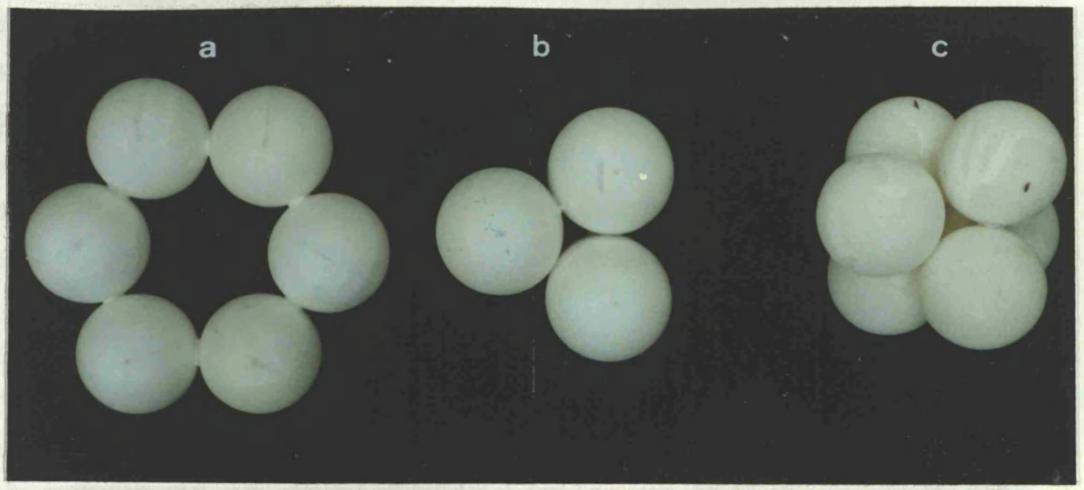
a: Planar hexagon

b: Trigonal prism (two layers of trimers eclipsed)

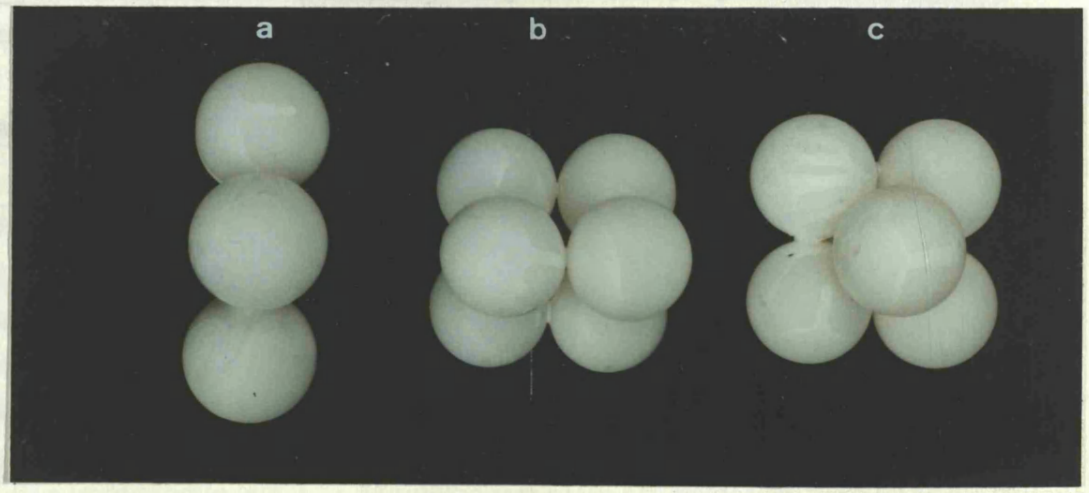
c: Octahedron (two layers of trimers staggered)

Figure 52

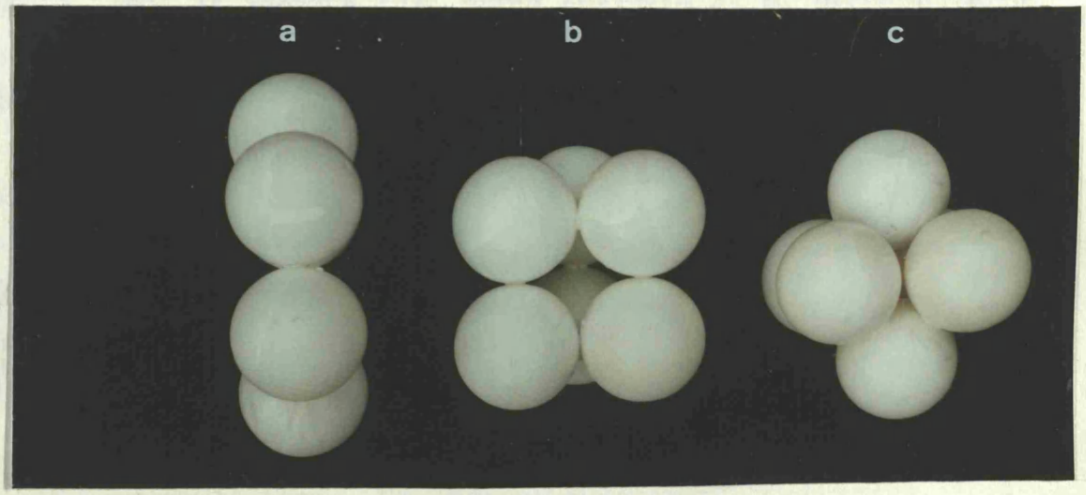
Models of Possible Subunit Arrangements of a Hexameric CS



1)



2)



3)

(1.3.1.). Thus far then, the M. methylotrophus CS appears to have a structure similar to that of CSs from Gram-negative heterotrophs.

The amino acid compositions (Table 21) suggests significant homology between the CSs from M. methylotrophus and A. calcoaceticus, despite the variability of the glycine estimations. Further, there is reason to suspect significant sequence identity between the CSs from M. methylotrophus and E. coli because the latter CS can be mutated to an enzyme with of very similar size and properties (e.g. hyperbolic substrate dependence and NADH-insensitivity) to those possessed by the enzyme from M. methylotrophus (Harford and Weitzman, 1978).

However, comparison of these results with the data of other workers suggests that the former is inconsistent with the latter. The amino acid sequence of Acinetobacter anitratum CS is known (Donald and Duckworth, 1987). It may be expected that A. calcoaceticus CS may have an amino acid sequence that is similar to that of Acinetobacter anitratum CS as both enzymes have very similar properties and are from the same bacterial genus. However, $S\Delta n$ for the amino acid composition of A. calcoaceticus CS and the amino acid sequence of Acinetobacter anitratum CS gives a value of 1164.31 for the number of differences in amino acids between the two proteins. This result is surprising as it suggests that there is no similarity at all between the primary structure of the two CSs. In reality, this is unlikely, for even between diverse sources of CS such as E. coli and pig heart, there is conservation of up to about 30% of the amino acid sequence (Bhayana and Duckworth, 1984). Thus, although Table 21 suggests that the M. methylotrophus and A. calcoaceticus CSs are related, the inconsistency of these data with published data indicate that the former may be unreliable. Thus it appears that amino analysis of M. methylotrophus and A. calcoaceticus CSs will have to be repeated before comparisons with other CSs can be made or further conclusions can be drawn.

4.3.2. Substrate Dependences and Ionic Effects

M. methylotrophus CS probably obeys Michaelis-Menten kinetics in TE-8.6 (Figures 29-31). The trend between obligately aerobic or facultatively anaerobic respiration and hyperbolic or sigmoid

substrate dependences has been noted previously (**1.12.8.**). M. methylotrophus is obligately aerobic and fits the aforementioned trend by possessing a CS with hyperbolic substrate dependences.

4.3.2.1. Effects of Monovalent Cations on CS Activity. A feature of the acetyl-CoA dependence of M. methylotrophus CS is its response to ionic strength. A rise in the latter increases the affinity of CS for acetyl-CoA, which is seen as an activation of CS at sub-saturating acetyl-CoA (Figures 33, 34). KCl has similar effects on CSs from the Gram-negative organisms Azotobacter vinelandii, Acinetobacter anitratum, E. coli and Rhodospirillum rubrum (Flechtner and Hanson, 1970; Faloona and Srere, 1969; Wright and Sanwal, 1971; Johnson and Hanson, 1974; Massarini et al., 1976; Morse and Duckworth, 1980). The Salmonella typhimurium, Pseudomonas fluorescens and Aphanocapsa sp. CSs are also activated by KCl although the basis of the effect was not established for these CSs (Flechtner and Hanson, 1970; Taylor, 1973).

The affinity of M. methylotrophus CS for OAA was not modified by KCl (Figure 33). This feature is also shared by CSs from other Gram-negative organisms like E. coli, Azotobacter vinelandii, Acinetobacter anitratum, Rhodospirillum rubrum and a marine pseudomonad (Faloona and Srere, 1969; Flechtner and Hanson, 1970; Johnson and Hanson, 1974; Massarini et al., 1976; Higa et al., 1978).

Although there is some variability in the extent of activation by KCl of CS from the above organisms, it appears that activation by 0.1 M-KCl of E. coli CS at sub-saturating acetyl-CoA (0.1 mM) is far greater (39- to 90-fold) than that of other CSs: Acinetobacter anitratum (1.65-fold), Azotobacter vinelandii (1.38-fold), Pseudomonas fluorescens (1.7-fold), Rhodospirillum rubrum (4.2-fold) and a marine Pseudomonas sp. (2.5- to 6-fold) (Flechtner and Hanson, 1970; Johnson and Hanson, 1974; Massarini and Cazzulo, 1974; Massarini et al., 1976; Talgoy et al., 1979; Morse and Duckworth, 1980; Anderson and Duckworth, 1988). From values of V_{max} and K_m for acetyl-CoA with and without KCl (**1.7.1.**), M. methylotrophus has a CS that is activated 1.7-fold at 0.1 mM-acetyl-CoA by 0.1 M-KCl in TE-8.6. Thus the sensitivity of this CS to KCl is 23- to 53-fold less than that of E. coli CS but is similar to those of the other CSs mentioned above.

The disparity between the sensitivities of E. coli CS and, for example, M. methylotrophus and Acinetobacter anitratum CSs to KCl activation is a result of the dependence of E. coli CS on acetyl-CoA. This is sigmoid without KCl but becomes hyperbolic with it (Faloona and Srere, 1969; Wright and Sanwal, 1971; Weitzman and Danson, 1976). Thus, at sub-saturating acetyl-CoA, KCl activation is more than it would be if the dependence on acetyl-CoA were hyperbolic without the salt. As this latter condition occurs in M. methylotrophus CS (**4.2.5.**), Acinetobacter anitratum CS (Morse and Duckworth, 1980), and (excepting E. coli CS) the CSs quoted in the previous paragraph (see refs. above), this may explain the disparity in the sensitivities to activation by KCl displayed by the bacterial CSs.

The kinetic and regulatory properties of Gram-negative CSs can be described in terms of the model of allostery proposed by Monod et al. (1965; **1.12.8.**). In this, an equilibrium is proposed between two conformations of an enzyme, one with a high affinity for substrate ("R" state), the other with a low affinity for substrate ("T" state). As well as substrate binding, the equilibrium can be shifted by activators which bind most avidly to the R state or inhibitors which bind most avidly to the T state. Each binding site within the T or R state binds ligands identically. The model accounts for co-operativity as binding of one molecule of ligand to a protein causes all ligand binding sites of that protein to change conformation concurrently. The equilibrium between T and R is defined as $L = [T]/[R]$.

The sigmoidal dependence of E. coli CS on substrates suggest that its value of L is large. If KCl is envisaged to shift the equilibrium in favour of the R state (i.e., reduce L), it would have a more marked effect on E. coli CS than on a CS whose value of L is far lower. That this interpretation of the state of E. coli CS may be accurate has been shown recently by Duckworth and Anderson (1988). These workers mutated Arg 319 of E. coli CS to leu. The mutant enzyme was less sensitive to NADH and had a higher affinity for acetyl-CoA than the wild type enzyme. This suggested a shift in L towards the R state. The reduced sensitivity to KCl that would be predicted to accompany this fall in the value of L was also observed in the mutant CS.

If CS from M. methylotrophus also exists in a R and T state, its

hyperbolic acetyl-CoA dependence without KCl (Figures 29, 30) suggests that it would have a lower value of L than the wild type E. coli CS. This would be consistant with the lower KCl activation of M. methylotrophus CS (relative to E. coli CS). Thus it is proposed that KCl displaces the R-T equilibrium for E. coli and M. methylotrophus CSs towards the R state. However, as, without KCl, M. methylotrophus CS may be markedly more in the R state than E. coli CS, the activation of M. methylotrophus CS by KCl that can take place is far lower than for the E. coli CS.

The point of this line of reasoning is that it leads to a framework for a possible explanation of the relative insensitivity of M. methylotrophus CS to NADH-inhibition (see **4.3.3.6.**).

If raised KCl reduces the value of L for the M. methylotrophus CS, then the reverse effect should have been caused by reducing the ionic strength of the medium. Thus the 120-fold activation of M. methylotrophus CS by KCl in 1/20.TE-8.6 (Figure 34) may show that L is now much larger. Unfortunately, this was not confirmed as the behaviour of M. methylotrophus CS under these conditions predicted by such an argument (sigmoid acetyl-CoA dependences) was not examined.

4.3.2.2. Other Effects of Monovalent Cations. KCl protected CS against thermal inactivation in a concentration-dependent manner (Figures 19, 20) and increased its molecular width by 20% as estimated by electron microscopy (Figure 51; Table 26). The latter observation may suggest a conformational change induced by KCl in M. methylotrophus CS. Also, the KCl-enhanced thermal stability may reflect an increased stability of a conformation that this CS has at raised ionic strength, but here there may be other explanations, e.g. unfavourable interactions between amino acids of identical charge on the surface of M. methylotrophus CS may be nullified by the presence of ions of opposite charge, thus stabilising the enzyme (Pace and Grimsley, 1988).

Observations of conformational change have also been made in the CSs from heterotrophic sources. E. coli CS undergoes KCl-induced changes in its ultraviolet absorbance spectrum. This has been interpreted by Faloona and Srere (1969) as a change in the solvating or electrostatic environment of enzyme chromophores. KCl stabilises

Acinetobacter anitratum and E. coli CSs against inactivation by urea and increases the thermal stability of E. coli CS (Faloona and Srere, 1969; Morse and Duckworth, 1980). KCl also modifies the reaction of DTNB with thiol groups on E. coli CS (Danson and Weitzman, 1977; Talgoy et al., 1979). Finally, it has been possible to crosslink A. calcoaceticus CS in 0.1 M-KCl with 3,3'-dithiobispropionimidate, remove the salt and demonstrate preservation of the desensitising effects of KCl on the crosslinked CS towards NADH. Cleavage of the crosslinks of the enzyme so-treated restores the ability of the enzyme to respond to NADH (Lloyd and Weitzman, 1987).

Together, these results suggest that KCl-induced conformational changes are a feature of bacterial CSs irrespective of source.

4.3.2.3. Divalent Cations. $MgCl_2$-inhibition of CSs from Gram-negative sources - Azotobacter vinelandii, E. coli, Salmonella typhimurium, Rhodospirillum rubrum and Pseudomonas fluorescens; Gram-positive sources: Corynebacterium lilium, Bacillus polymyxa and mammalian sources like pig heart and rat kidney has been observed (Kosicki and Lee, 1966; Lee and Kosicki, 1967; Jangaard et al., 1968; Faloona and Srere, 1969; Flechtner and Hanson, 1970; Srere et al., 1973; Tanaka and Hanson, 1975). Where studied (in mammalian CSs), inhibition is competitive with acetyl-CoA (Kosicki and Lee, 1966; Lee and Kosicki, 1967). Conversely, those CSs from anaerobic Clostridia that catalyse the attack of the re face of OAA by acetyl-CoA have a requirement for a divalent metal ion (Srere, 1972).

M. methylotrophus has no obvious requirement for divalent cations and at high concentrations is, like other CSs, inhibited by them (Figure 35). However, at lower concentrations (< 40 mM), $MgCl_2$ activates M. methylotrophus CS. This has also been observed in E. coli CS by Weitzman and Danson (1976) who attributed it to polarisation of the OAA carbonyl, causing a reduction of the OAA K_m. However, as $MgCl_2$ concentrations that maximally stimulate M. methylotrophus CS have no effect on the V_{max} or K_m for OAA, and as saturating levels of acetyl-CoA almost eliminate this activation (Figures 36, 37), it seems that $MgCl_2$ activation is an ionic strength effect. There is no explanation for this discrepancy, except to note that the conditions used by

Weitzman and Danson (1976) would have ensured E. coli CS was in the T state, while (if applicable to M. methylotrophus CS) M. methylotrophus CS is probably mostly in the R state, and such a difference may have contributed to the differing response to $MgCl_2$ between the two CSs.

4.3.3. Regulatory Properties

Depending on the in vivo environment of CS in M. methylotrophus, this topic may have been partly discussed. If M. methylotrophus has a means of varying the ionic strength of the in vivo environment of CS, then this could control the entry of acetyl-CoA into the CAC and thus control synthesis of C_5 skeletons. However, more conventional candidates for regulation of CS activity - 2-OG, succinyl-CoA, adenine nucleotides and nicotinamide nucleotides - were also examined.

4.3.3.1. 2-OG. A consequence of an OGDH lesion is that 2-OG is an end product of the CAC. In such cases, CS is often subject to feedback inhibition by 2-OG (**1.12.6.2.1.**; **1.12.10.**).

The absence of OGDH from M. methylotrophus (**3.2.2.1.**) suggested that its CAC only operated biosynthetically. Similar observations and conclusions have been made for other obligately methylotrophic RuMP cycle utilisers and type M RFMs (**1.8.1.**; **1.8.2.1.**; **1.8.3.**; **1.10.1.**). Thus, it seems that the CSs from these methylotrophs may also be subject to 2-OG-inhibition. Colby and Zatman (1975c) have shown that CSs from RuMP cycle utilising obligate methylotrophs such as organisms 4B6 and C2A1 and the type M RFM W3A1 are inhibited by 2-OG while methylotrophs with a complete CAC such as the obligate serine pathway-utiliser Methylosinus trichosporium OB3B, the type L RFM Bacillus PM6 and the facultative organisms 5B1 and Pseudomonas 3A2 are not.

That M. methylotrophus CS was also inhibited by 2-OG was first shown by Aperghis (1981). The results here confirm and extend this work. Inhibition of M. methylotrophus CS was competitive with OAA and non-competitive with acetyl-CoA (Figure 39, 41) and thus resembled inhibition of E. coli CS by 2-OG (Wright et al., 1967).

K_i values for 2-OG-inhibition of CS (at varied OAA) have been reported for CSs from CACs that can operate biosynthetically, e.g.

the E. coli CS, which has a K_i of 0.060–0.093 mM (Wright et al., 1967; Anderson and Duckworth, 1988). Much higher values are shown by CSs from CACs that function catabolically, e.g. rat kidney and A. calcoaceticus CSs, [both 10 mM (Srere et al., 1973; Harford, 1977)]. These results show the insensitivity to 2-OG of CSs from CACs whose primary function is not biosynthetic. M. methylotrophus CS has a K_i for 2-OG of 0.31 mM (Figure 40). Thus, it is clear that sensitivity of M. methylotrophus CS to 2-OG-inhibition is more similar to that shown by E. coli CS, than to that of CSs from catabolically-orientated CACs. This is borne out on comparison of the responses of M. methylotrophus and porcine heart CSs to 2-OG (Figure 38).

Although these observations are consistent with the biosynthetic role of CS in M. methylotrophus, other obligate RuMP cycle utilisers and type M RFMs, Colby and Zatman (1975c) considered that these CSs are relatively insensitive to 2-OG. Thus, significant inhibition (44 to 64%) of CSs from C2A1, 4B6 and W3A1 was only achieved by large amounts (10 mM) of 2-OG. Thus Colby and Zatman (1975c) questioned the significance of 2-OG-inhibition. It can be calculated using the 2-OG K_i and OAA K_m values for M. methylotrophus CS that, in conditions used by Colby and Zatman (1975c), M. methylotrophus CS would have been as sensitive to 2-OG as the CSs studied by Colby and Zatman (1975c). This may cast doubt on the contention that 2-OG may be an effector of CS in vivo. However, if, in vivo, OAA approaches the K_m of M. methylotrophus CS for OAA (12.2 μM; one sixteenth that used by Colby and Zatman, 1975c), the effect of low millimolar concentrations of 2-OG may be very significant indeed (c.f. Figure 38). Thus, to assess relevance of 2-OG-inhibition to regulation of M. methylotrophus CS, a knowledge of the in vivo OAA and 2-OG concentrations are required. In this context, it has been found that the intracellular OAA concentration of E. coli may be about 20 μM (Walsh et al., 1987). If this value is similar in M. methylotrophus, 2-OG-inhibition may be important.

4.3.3.2. Succinyl-CoA. The CAC is modified in cyanobacteria (Figure 9) such that succinyl-CoA is an end product of a pathway initiated by CS. Consistent with this, the cyanobacterial CSs are subject to feedback inhibition by succinyl-CoA (Lucas and Weitzman, 1977). If the presence

of ICL activity is confirmed in M. methylotrophus (3.2.2.2.6.) by further work, then M. methylotrophus may also be able to make succinyl-CoA as an end product of the CAC. In anticipation that this might be so, the sensitivity of M. methylotrophus CS to succinyl-CoA was studied. Without KCl, succinyl-CoA-inhibition of M. methylotrophus CS was not detected (Table 22) even at the highest [succinyl-CoA]/[acetyl-CoA] ratio tested. However, succinyl-CoA was inhibitory if 0.1 M-KCl was present and, under these conditions, inhibition was competitive with acetyl-CoA.

For CSs, the ATP K_i increases linearly with the K_m for acetyl-CoA (Weitzman, 1981). It seems an analogous situation may exist here. Thus conditions (0.1 M-KCl) that increase the affinity of M. methylotrophus CS for acetyl-CoA also increases its affinity for succinyl-CoA and so enhance its sensitivity to inhibition by the C_4-thioester.

The above highlights the first difference between the responses of the M. methylotrophus and cyanobacterial CSs to succinyl-CoA. KCl sensitises M. methylotrophus CS to succinyl-CoA but has the reverse effect on cyanobacterial CSs (Lucas and Weitzman, 1977). The effect of KCl on cyanobacterial CSs has been thought to show that succinyl-CoA acts allosterically; however, the opposite effect of KCl on the enzyme from M. methylotrophus suggests a different mechanism of inhibition.

Assuming the inhibition is linear, it can be calculated (Wharton and Eisenthal, 1981) from Table 22 that the M. methylotrophus CS K_i for succinyl-CoA may be 0.33 mM to 0.40 mM. This is at least ten-fold higher than any cyanobacterial CS K_i (Lucas and Weitzman, 1977). This highlights the second difference between the responses of the M. methylotrophus and cyanobacterial CSs - M. methylotrophus CS was far less sensitive to succinyl-CoA than the cyanobacterial CSs. Indeed, the succinyl-CoA K_i of M. methylotrophus CS may be 16- to 22-fold greater than its acetyl-CoA K_m, while the K_i values of the cyanobacterial CSs were less than their corresponding K_m values (Lucas and Weitzman, 1977). These facts make the physiological relevance of succinyl-CoA inhibition untenable. That enzymes producing succinyl-CoA (ICL and STK) are at low activity in M. methylotrophus (less than 1 $mU.mg^{-1}$; Table 12) reduces the likelihood that succinyl-CoA would accumulate to levels that would significantly inhibit CS. However, at

risk of repetition, the sensitivity to a competitive inhibitor will depend partly on the amount of the competing substrate (acetyl-CoA) *in vivo*, for which values have yet to be produced for *M. methylotrophus*.

4.3.3.3. The Effects of Adenine Nucleotides. CSs from methylotrophs irrespective of the state of their CAC are inhibited by ATP. Thus organisms with an incomplete CAC - type M RFMs and obligate RuMP cycle utilisers (W3A1, 4B6, C2A1, *Methylomonas albus*) and organisms with a complete CAC - obligate serine pathway utilisers (*Methylosinus trichosporium* OB3B), facultative serine pathway utilisers (5B1, 3A2) and facultative RuMP cycle utilisers (e.g. *Arthrobacter* 2B2) - are all moderately inhibited (15-65%) by high levels (5-10 mM) of ATP (Colby and Zatman, 1975c; Paddon *et al.*, 1985). As noted in **1.12.6.1.1.**, inhibition is competitive with acetyl-CoA (Paddon *et al.*, 1985).

The *M. methylotrophus* CS is no exception to the above and has been found to be inhibited by ATP (Aperghis, 1981). This was repeated here; Table 23 shows that no inhibition above 8% was exerted by any adenine nucleotide at 1 mM without KCl. However, with 0.1 M-KCl, up to 30% inhibition by 1 mM-ATP was observed. The KCl-induced change in behaviour may be due to an increase in the affinity of CS for acetyl-CoA and thus inhibitors competitive with acetyl-CoA (c.f. **4.3.3.2.**).

In addition to the variation in K_m for acetyl-CoA that may occur on extraction of CS (**1.12.6.1.1.**), there are reasons to believe that ATP-inhibition of *M. methylotrophus* CS may not be significant *in vivo*. First, the physiologically active form of ATP is probably the Mg^{2+} chelate. However, when $MgCl_2$ and ATP were added to CS in the presence of KCl, inhibition was reduced at the lower concentrations of acetyl-CoA (Table 23). This probably resulted from negation by Mg^{2+} chelation of the negative charges of ATP phosphates that otherwise interacted at the acetyl-CoA binding site (Kosicki and Lee, 1966; Lee and Kosicki, 1967; Tanaka and Hanson, 1975; Robinson, 1984). Secondly; ATP-inhibition is found among CSs from *M. methylotrophus*, other obligate RuMP cycle C_1-utilisers and type M RFMs. These organisms have biosynthetically orientated CACs and so have no need of ATP-sensitive CSs. Thus, ATP-inhibition may result as *M. methylotrophus* CS cannot dicriminate 5'-ATP from the 3-phospho, 5'-ADP moiety of acetyl-CoA.

4.3.3.4. The Effects of Nicotinamide Nucleotides in General. It has been shown that nicotinamide nucleotides other than NADH at 10 mM had no effect on CSs from organisms 4B6, C2A1, W3A1, PM6, 5B1, 3A2, Methylomonas albus or Methylosinus trichosporium OB3B (Colby and Zatman, 1975c). However, Aperghis (1981) found 10 mM-NADPH inhibited M. methylotrophus CS by 33% in the presence of 0.15-0.3 mM-acetyl-CoA. It has been shown that inhibition by NAD^+, $NADP^+$, NADH and NADPH (in order of potency) is weakly competitive with acetyl-CoA for pig heart CS (Lee and Kosicki, 1967). Thus, a range of concentrations of acetyl-CoA was used to study the effects of nicotinamide nucleotides on M. methylotrophus CS. The greatest effects on this CS were exerted by the reduced coenzymes in the presence of 0.1 M-KCl (Table 24). This, and the fact that NADPH was more inhibitory than NADH (Table 24), distinguished the inhibition discussed here from the more potent allosteric NADH-inhibition of other Gram-negative CSs (Weitzman, 1966a, b; Talgoy and Duckworth, 1979). With KCl, the most marked effects were exerted by 1 mM-NADPH which inhibited M. methylotrophus CS by over 50%. Inhibition fell with increasing acetyl-CoA, suggesting that, like succinyl-CoA and ATP-inhibition, NADPH-inhibition was competitive (Table 24). The extent of NADPH-inhibition is greater than reported previously with this or any other methylotrophic CS (Colby and Zatman, 1975c; Aperghis, 1981). However, this is probably a result of judicious selection of conditions.

Again, it is difficult to assess the physiological significance of the NADPH-inhibiton of CS. The effect is incompatible with the biosynthetic nature of the CAC. Also, the effects of NADPH may be reduced in vivo depending upon the in vivo affinity of CS for acetyl-CoA (**1.12.6.1.1.**) and the [NADPH]/[acetyl-CoA] ratio.

4.3.3.5. The Effects of NADH in Particular. In the absence of KCl, M. methylotrophus CS was virtually insensitive to NADH (Table 24); however, in comparable conditions, the NADH-sensitive CS from A. calcoaceticus was over 90% inhibited by a similar concentration of NADH (Figure 42). This situation is consistent with the respective catabolic and anabolic natures of the CACs of A. calcoaceticus and M. methylotrophus. The apparent NADH insensitivity of M. methylotrophus

CS agrees with the observations of Taylor (1977), Aperghis (1981) and Otto (1986) on this CS and with the NADH insensitivity of CSs from other Gram negative RuMP-cycle utilising obligate methylotrophs and type M RFMs (Colby and Zatman, 1975c). Again, like M. methylotrophus, these other methylotrophs have an incomplete, biosynthetically-orientated CAC.

Although NADH insensitivity of CS is consistent with current thinking on CAC function in methylotrophs, all reports on regulation of methylotrophic CSs used the DTNB-linked assay at pH 8.0 (Colby and Zatman 1975c; Aperghis, 1981; Babel and Müller-Kraft, 1979; Paddon et al., 1985; Otto, 1986). Thus, inability to show NADH-inhibition may have been due to an exceptional lability of the NADH effect to raised pH or DTNB. Both of these have been known to desensitise NADH-sensitive CSs (**1.12.9.1.**). This possibility was examined by using the polarographic and DTNP-linked CS assays. This is the first time that DTNP has been used in the assay of CS; however, other dithiobis pyridines have been used to modify thiol groups in CS (Wright and Sanwal, 1971; Talgoy et al., 1979; Morse and Duckworth, 1980).

In the absence of KCl, the DTNP-linked assay showed that CS from M. methylotrophus was insensitive to NADH between pH 6.2 and 9.7 (Figure 42). However, the behaviour of NADH-sensitive A. calcoaceticus CS indicated that the normal assay pH (8.6) was not the cause of the NADH-insensitivity of M. methylotrophus CS. However, there was still a possibility that the DTNP itself desensitised M. methylotrophus CS. Thus the polarographic assay was used. In the absence of KCl, this showed a consistent, progressive but small increase in NADH-inhibition of M. methylotrophus CS with decreasing pH, rising to 25% at pH 6.2. Most importantly, this shows that, as previously surmised, M. methylotrophus CS is far less sensitive to NADH than the CSs from most Gram-negative heterotrophs. Thus, relative to these latter organisms, it is far less likely that NADH would be an effective inhibitor in vivo of M. methylotrophus CS. This probably supports the conclusions drawn by other authors regarding the NADH-insensitivity of CSs from similar methylotrophs.

4.3.3.6. Why is M. methylotrophus CS Not Very Sensitive to NADH ?

Although probably insignificant in vivo, the slight inhibition of M. methylotrophus CS by NADH in the absence of KCl (Figure 42) was intriguing as it was observed with the polarographic assay but not the DTNP/B-linked assay. This effect (Figure 42) may not have been related to the weak competitive inhibition shown in Table 24 as the latter effect was mainly observed in the presence of KCl, while the results in Figure 42 were obtained without KCl. It seems tempting to infer that M. methylotrophus CS might have been desensitised to NADH by DTNP or DTNB. If this is so, it may be that M. methylotrophus CS may be very slightly prone to low levels of allosteric inhibition by NADH.

It was argued (**4.3.2.1.**) that KCl-activation of M. methylotrophus CS may result from displacement of an R-T equilibrium. If this was so, the behaviour of M. methylotrophus CS relative to that of other CSs suggested it would lie very much in favour of the R state. Such a situation may provide two causes of the NADH-insensitivity of the CS from M. methylotrophus relative to that of other NADH-sensitive CSs:
a) The R-T equilibrium for M. methylotrophus CS is far more towards the R state than any other NADH-sensitive CS; i.e. L is near zero, or;
b) L for M. methylotrophus CS is far less responsive to NADH than for other NADH-sensitive CSs. The cause of this may be that the affinity of the T state of M. methylotrophus CS for NADH may itself be far less than that of the T states of NADH-sensitive Gram-negative CSs.

Of the two possibilities, b) is preferable as a) may be inconsistent with the quantitative similarity between activation of M. methylotrophus by KCl and activation of, e.g. the Acinetobacter anitratum enzyme by KCl. This latter CS behaves kinetically like a CS in the R state and is sensitive to NADH (Morse and Duckworth, 1980).

4.3.3.7. The Effect of NADH on Thermal Stability of CS. NADH protects A. calcoaceticus CS against thermal inactivation; however, this effect is eliminated on treatment with a crosslinking agent, but can be restored on cleavage of the crosslinks (Weitzman, 1981; Lloyd and Weitzman, 1987). Crosslinking also eliminates the sigmoidicity of the relationship between NADH-inhibition and NADH (Mitchell and Weitzman, 1983b). Thus, conformational changes associated with the co-operative response to NADH may be responsible for the thermal protection of

Acinetobacter CS.

The results support the notion that the relative insensitivity of M. methylotrophus CS to NADH is not an artefact of the assay method. That M. methylotrophus CS does not show the NADH-induced enhancement of thermal stability that was shown by A. calcoaceticus CS (Figure 44; Table 25) suggests that NADH cannot cause a conformational change of the type that stabilised the Acinetobacter CS. Thus, the results are consistent with the argument that processes that are responsible for the co-operative sensitivity of A. calcoaceticus CS to NADH-inhibition are those responsible for its NADH-enhanced thermal stability.

4.3.3.8. The Effect of NADH on the Size of CS. Conclusions that are similar to those drawn from the thermal inactivation data can be drawn from the electron microscopy results (**4.2.8.2.**). However, to do this, some assumptions have been made. These are not impervious to criticism and should be kept in mind on consideration of the results.

First, to make comparable estimates of widths of CS particles, it must be assumed that all A. calcoaceticus and M. methylotrophus CS conformations that were observed interacted with the support film in the same way(s). However, this is debatable. NADH caused Acinetobacter CS to aggregate on the electron microscope grid (Figure 46). NADPH also had this effect (not shown). The effect was less obvious with M. methylotrophus CS, as it aggregated to a small extent independently of any nucleotide (Figure 49). Possibly, the conditions allowed NAD(P)H to interfere with the surface film in a non-specific way which disrupted its interaction with the Acinetobacter CS. Dubochet et al. (1982) have noted aggregation may result if hydrophilic particles are dried onto a hydrophobic surface or if particle-particle cohesion is greater than particle-film cohesion. Such effects may partly undermine the confidence held in the change in size of the Acinetobacter CS.

Secondly, it has to be assumed that M. methylotrophus and Acinetobacter CSs are not affected by the changes in their environment or forces exerted on them during drying down. This may be so if the protein-surface film interaction was strong enough to nullify factors that cause distortion of the protein (**4.3.1.**). Otherwise, it must be assumed that sample preparation affects all samples identically.

4.3.4. Comparison of M. methylotrophus and Acetobacter xylinum CSs

Apart from generation of mutant enzymes from E. coli which are large and NADH-insensitive (Harford and Weitzman, 1978), the only detailed study of a large NADH-insensitive CS is that of Swissa and Benziman (1976) who studied Acetobacter xylinum CS. This enzyme strongly resembles M. methylotrophus CS. As well as identical native M_r values (M. methylotrophus: 266,000 ± 19,000; Acetobacter: 280,000), both CSs have similar pH optima (M. methylotrophus: pH 8.2-8.6; Acetobacter: pH 8.4), are more sensitive to competitive inhibition by NADPH than NADH, have similar substrate dependences and similar K_m values for OAA. However, the characteristics of the enzymes that are affected by ionic strength are difficult to compare as Swissa and Benziman (1976) used 0.1 M-glycine (pH 8.4) as an assay buffer. The characteristics of this buffer (pKa_1 = 2.4; pKa_2 = 9.8; Morris, 1974) meant that, at the assay pH, it would have been 96% zwitterionic. Thus, it is difficult to assess the contribution the buffer made to the ionic strength of the assay medium or the facets of CS behaviour that were sensitive to ionic strength.

Thus, consider the following: a) the acetyl-CoA K_m of the Acetobacter CS was identical to that of M. methylotrophus CS with 0.1 M-KCl and b) monovalent cations had no effect on Acetobacter CS even at low acetyl-CoA concentrations. a) and b) suggest that either the Acetobacter enzyme was extraordinary in that it was insensitive to ionic strength effects or the assay buffer had saturated the ionic strength effect shown by Acetobacter CS. Further, the lack of NADH-sensitivity of the Acetobacter CS is questionable because of the effect of ionic strength on the NADH-sensitivity of other CSs (**1.12.9.1.**). However Weitzman (1981) has shown that Acetobacter CS is indeed NADH-insensitive.

4.4. Further Work

In the long term, due to the low cellular abundance of M. methylotrophus CS, any future studies of this enzyme will benefit from the cloning and amplified expression of the gene encoding it. The

availability of a cosmid library of the M. methylotrophus genome and the cloned genes for the E. coli and Acinetobacter anitratum CSs from which suitable probes might be derived, would facilitate this approach (Duckworth and Bell, 1982; Ner et al., 1983; Donald and Duckworth 1986, 1987; Lyon et al., 1988). The site-directed mutagenesis approach to the study of E. coli CS that has been pioneered by Duckworth's group has paid great dividends (Duckworth, et al., 1987; Anderson and Duckworth, 1988, 1989). Such an approach would clearly benefit the study of M. methylotrophus CS. Duckworth and Anderson (1988) have identified residues in the E. coli CS primary structure whose substitution (Arg 319 to Leu) alters the R-T equilibrium in favour of the R state (as indicated by reduced sensitivity to NADH and KCl and an increased substrate affinity). Sequencing of the gene encoding M. methylotrophus CS followed by identification and site-directed mutagenesis of equivalent residues in M. methylotrophus CS may show whether, as proposed here, M. methylotrophus does indeed exist in an R-T equilibrium. Further, if, as is believed, the sequences of this NADH-insensitive CS and those of NADH-sensitive enzymes are very similar, comparison between the two may reveal those residues that are crucial to NADH-inhibition.

In lieu of the molecular biological/genetical approach the following issues thrown up by the results require attention:

1) The purification protocol for the M. methylotrophus CS needs to be re-examined, and its efficiency improved. This may be achieved by a wider screening of triazine dyes, using, e.g. those dyes used in purification of IDH (**5.1.1.**), or the use of other steps like 'hydrophobic chromatography' as was used by Smith et al.(1987). This step may allow an earlier use of ammonium sulphate fractionation.

2) The amino acid analyses of M. methylotrophus and A. calcoaceticus CSs require repetition to confirm that the two proteins are very closely related to each other, and to establish their relatedness to other CSs from Gram-negative sources.

3) The sensitivity to NADH and the Hill coefficient of M. methylotrophus CS for acetyl-CoA should be studied at a lower ionic strength than that of the assay buffer used here. If there is an R-T equilibrium whose position is sensitive to ionic strength, such a line

of enquiry may uncover it. Ligand binding studies with NADH may also contribute to an understanding of the NADH-insensitivity of this CS.
4) Most electron microscope based experiments of biological samples are flawed because the object is examined devoid of water, even though this is one of the most important constituents in the sample's environment. Thus it has to be assumed that this will not disqualify the conclusions drawn about the *in vivo* situation. This applies to the electron microscope studies that were conducted with CS. However, recently, developments have been made in preparation and examination of biological samples that may largely remove the aforementioned assumption and the criticisms that come with it.

It has been shown that if water is cooled fast enough it can vitrify. This has allowed preparation of rapidly frozen samples as a thin film between the spaces of an uncoated electron microscope grid, without the damage incurred by the formation of ice crystals. If the sample is studied at low enough temperatures ($< -150^{o}C$), images can be produced from such unstained grids due to the difference in density between the biological material and the vitrified medium, and the very low background signal (Dubochet *et al.*, 1985; Chiu, 1986). This procedure avoids sample preparation artefacts and interactions with a surface film. Further, the low temperature at which the observations are made reduces electron beam damage to the specimen (Dubochet *et al.*, 1981, 1985; Chiu, 1986). These features of cryo-electron microscopy may commend this technique to analysis of CS.

CHAPTER 5

Methylophilus methylotrophus Isocitrate Dehydrogenase

5.1. Results

Unless stated otherwise, assays were performed with NAD^+.

5.1.1. Purification of M. methylotrophus IDH

The specific activity of M. methylotrophus IDH in crude extracts approaches 1 % of that in some heterotrophic bacteria e.g. Bacillus KSM-1050 and glycerol-grown E. coli (Shikata et al., 1988; Borthwick et al., 1984). This and the purification of M. methylotrophus CS (Chapter 4) suggested IDH would be a very small fraction of cell protein. Large amounts of cells would be needed; so, as with CS, frozen cell pastes of methanol-grown M. methylotrophus supplied by ICI, Billingham, U.K. were used as source material for purification.

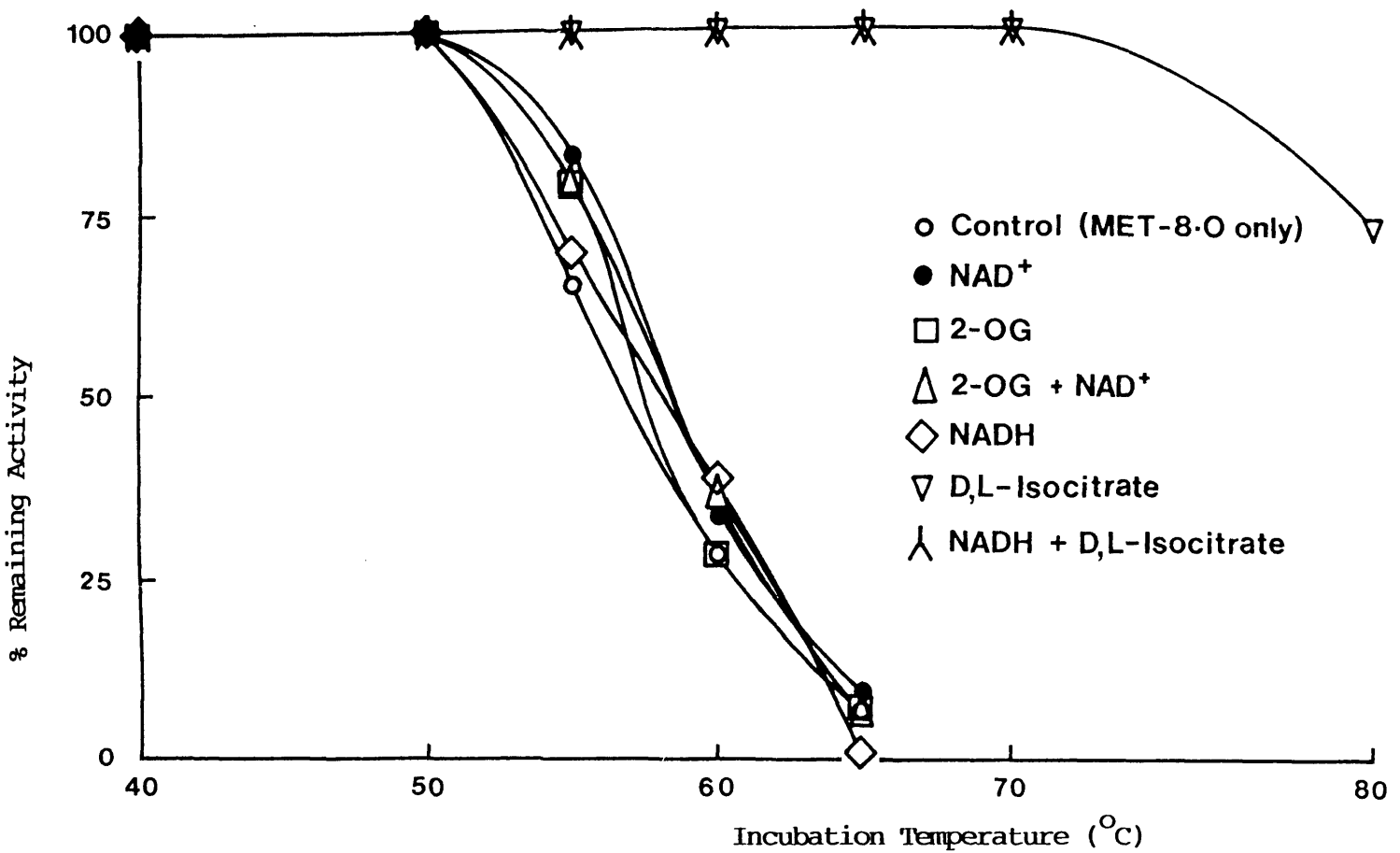
5.1.1.1. Thermal Fractionation. Mycobacterium phlei and Bacillus stearothermophilus NADP-IDHs are protected from thermal inactivation by isocitrate, Mn^{2+} or $NADP^+$ (Howard and Becker, 1970; Nagaoka et al., 1977; Dharwinal and Venkitasubramanian, 1987). Thus, the ability of substrates and products of M. methylotrophus IDH to prevent thermal inactivation was investigated as a means of purification.

0.3 ml of M. methylotrophus crude extract in MET-8.0 were added to 0.7 ml MET-8.0 that had been pre-warmed for 5 min in a water-bath. The mixture was incubated for 10 min between 40°C and 80°C at a protein concentration of 7.6 $mg.ml^{-1}$. Incubations were carried out alone or with 1 mM-NAD^+, 1 mM-NADH, 2 mM-D,L-isocitrate, 1 mM-2-OG, 1 mM-NAD^+ + 1mM-2-OG or 2 mM-D,L-isocitrate + 1 mM-NADH. After incubation, samples were chilled and centrifuged (**2.2.7.4.**). The supernatants were then assayed for activity.

In MET-8.0 alone, activity dropped to zero between 50°C and 65°C (Figure 53) with a T_{50} of 56.8°C. 2-OG with or without NAD^+, NAD^+ alone or NADH had little effect on inactivation (Figure 53). However, isocitrate, with or without NADH, dramatically increased the thermal stability of IDH - there was no loss of activity at all below 70°C and the T_{50} value increased to >80°C. Also, heat treatment of IDH at 70°C with isocitrate alone increased the specific activity of the enzyme

Figure 53

The Effect of Substrates and Coenzymes on the Response of M. methylotrophus IDH to Increasing Temperature



With the exception of isocitrate, the final concentration of all additions was 1 mM. D,L-isocitrate was added to a final concentration of 2 mM.

7.3-fold. The inactivation of IDH in MET-8.0 alone was followed at its T_{50}. Over 16 min, 66.7% of the activity was lost in a first-order process with a rate constant of 0.055 min^{-1}. No activity was lost if 2 mM-D,L-isocitrate was added to the extract (Figure 54).

5.1.1.1.1. Characterisation of the Effects of Isocitrate. The above had obvious application to the purification of M. methylotrophus IDH. To optimise conditions for purification, the effect of isocitrate on the thermal stability of the enzyme was characterised.

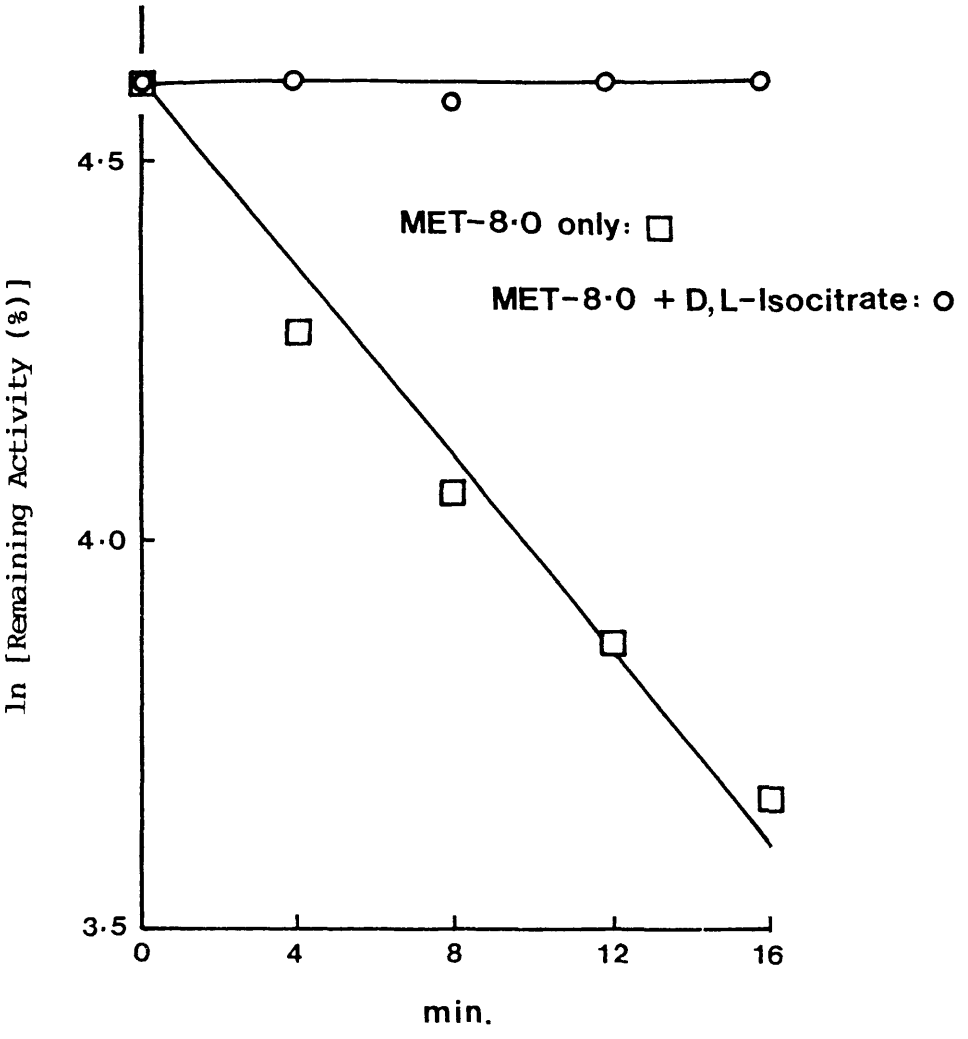
In these experiments, incubations were carried out as above. After incubation, enzyme and protein were then assayed in the supernatant. Extended incubation of the extract with isocitrate over 60 min at 70°C resulted in a negligible loss of activity. Most of the protein precipitated in the first 6 min (Figure 55); however, the optimum purification (8.2-fold) was not attained until after 30 min (Figure 55). The protection of IDH by isocitrate was concentration-dependent. Extract was incubated at 70°C at a protein concentration of 6.72 $mg.ml^{-1}$ with 10 µM-1.6 mM-isocitrate for 30 min. There was a sigmoid dependence of recovery and specific activity on isocitrate (Figure 56). Half-maximal protection was exerted by about 0.2 mM-D,L-isocitrate, and complete protection by 1 mM-D,L-isocitrate.

It was concluded that incubation of crude extract with 2 mM-D,L-isocitrate for 30 min at 70°C would be an effective first step. When applied as such, the extended stability of IDH over 1 h would allow large volumes of extract to be safely raised to the incubation temperature without additional loss of activity.

5.1.1.2. Immobilised Triazine Dye-Ligand Chromatography. Immobilised triazine dye-ligand chromatography has been used to purify NADP-IDH from a variety of sources (Nagaoka et al., 1977; Seelig and Colman, 1977; Vasquez and Reeves, 1979; Nimmo and Holms, 1980; Borthwick et al., 1984; Ishii et al., 1987). The application of triazine dye-ligand chromatography to purification of M. methylotrophus IDH was therefore attempted. The following approach was adopted. Fifteen triazine dyes immobilised on Sepharose 4B (**2.2.7.6.1.**) were screened for binding of IDH. Those that bound the enzyme were studied further.

Figure 54

Time Course of Thermal Inactivation of M. methylotrophus IDH at 57°C in the Presence and Absense of Isocitrate



Incubations were carried out + 2 mM-D,L-isocitrate. The final protein concentration after dilution of the crude extract into pre-warmed incubation medium was 7.6 $mg.ml^{-1}$.

Figure 55

Effect of Prolonged Incubation at 70°C in the Presence of 2 mM-D,L-Isocitrate on M. methylotrophus IDH Activity and Crude Extract Protein

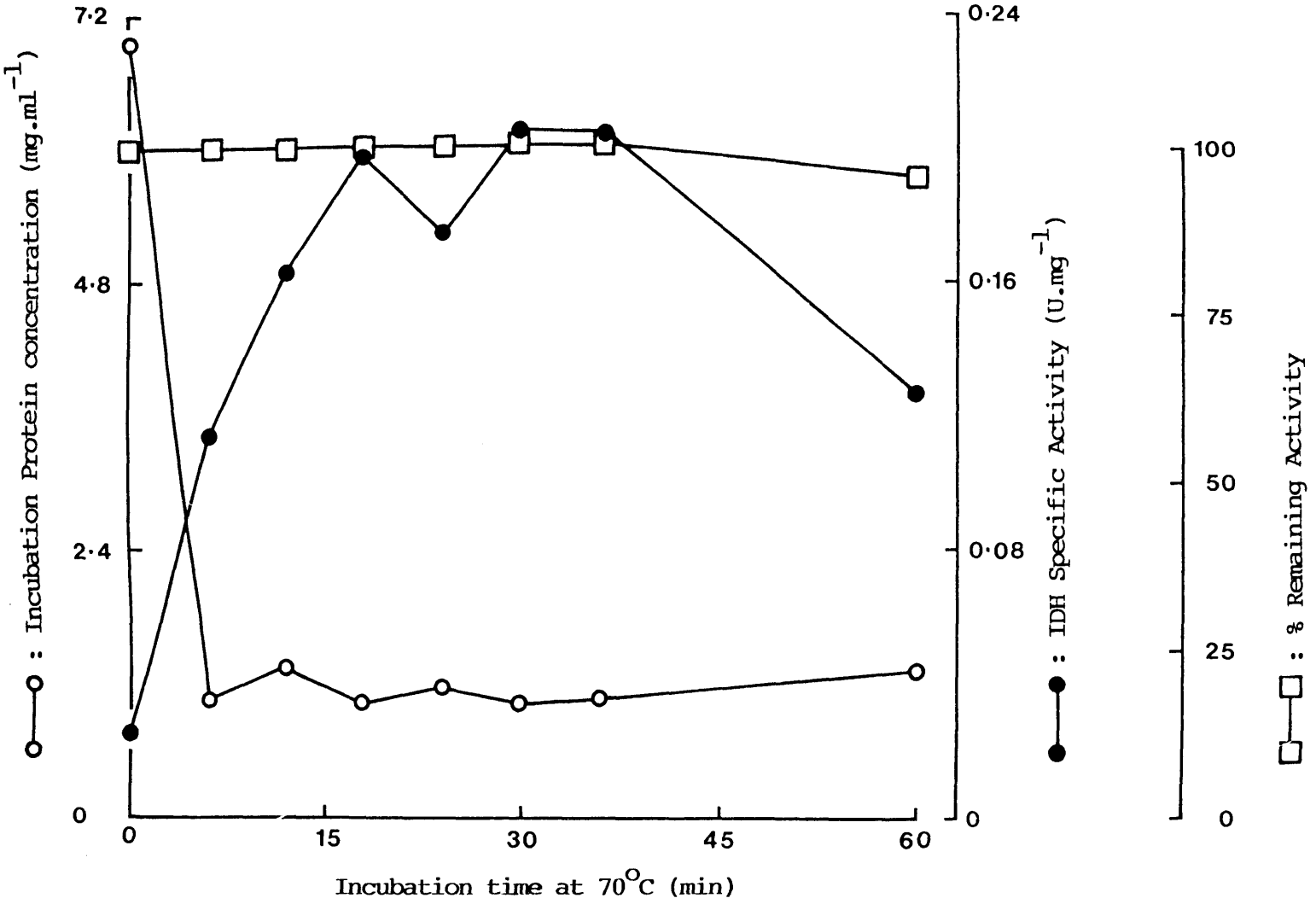
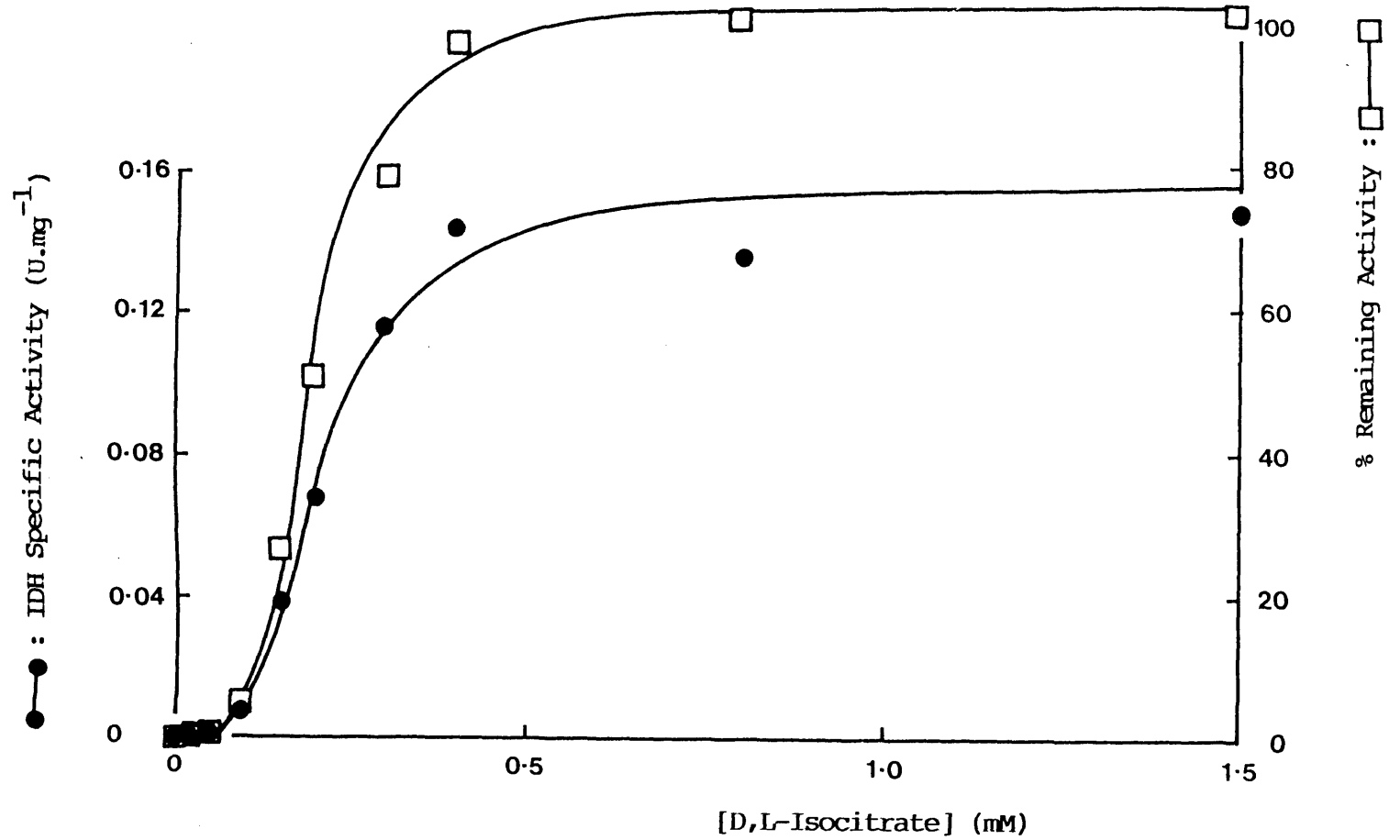


Figure 56

Dependence of the Thermal Stability of M. methylotrophus IDH on Isocitrate Concentration at 70°C



5.1.1.2.1. Initial Screening of Immobilised Dyes. As pH has marked effects upon the binding properties of dyes (Hey and Dean, 1981), screenings were carried out at pH 7.2 and pH 8.0. Crude extract that had been heat-treated at 70°C for 30 min with 2mM-D,L-isocitrate was dialysed at 4°C in 2 l of MET-8.0 and 2 mM-DTT or the same buffer adjusted at 4°C to pH 7.2 with HCl (MET-7.2 and 2 mM-DTT) for 2 h, and then in 5 l of fresh buffer at 4°C overnight.

Sepharose 4B-triazine dye conjugates were packed in 10-ml syringe barrels to form 5-ml beds. All steps were now carried out at 4°C. Columns were equilibrated with 50 ml of MET-8.0 and 2 mM-DTT or MET-7.2 and 2 mM-DTT. Columns were then loaded with IDH at the desired pH (load wash) and washed under gravity with 2 column volumes of MET-8.0 and 2 mM-DTT or MET-7.2 and 2 mM-DTT (buffer wash). Columns were then washed with buffer wash and 1 M-KCl (salt wash; 2 column volumes).

The columns of interest were those that bound IDH and released it in the salt wash. Two dye columns behaved like this. These were columns prepared with Blue HE-RD and Green HE-4BD (Table 27). Both bound IDH at both pHs. At pH 7.2, Blue HE-RD released 74.5% of the applied activity, while at pH 8.0, all of the activity that was loaded was eluted. With the Green HE-4BD column, all of the loaded activity at pH 7.2 and 83.7% of it at pH 8.0 was eluted by 1 M-KCl.

5.1.1.2.2. Biospecific Elution of IDH. Biospecific elution has the potential of being far more selective than elution by raised ionic strength. It may improve the purification factors obtained with Blue HE-RD and Green HE-4BD (Table 27). Thus the ability of substrates to elute IDH from immobilised Blue HE-RD and Green HE-4BD was studied.

Given that fewer enzymes bind isocitrate than NAD^+, initial work centred on the use of isocitrate as an eluant. All experiments here were performed at 4°C. 5-ml columns of Blue HE-RD and Green HE-4BD were equilibrated with MET-7.2 + 2 mM-DTT and loaded (as above) with 4.478 U IDH and 32.5 mg protein in 7.5 ml of MET-7.2 + 2 mM-DTT. This extract was prepared as for that used in the initial screening of the dyes (**5.1.1.2.1.**). Columns were eluted with a 10-ml buffer wash and then 10 ml of MET-7.2 + 2 mM-DTT + 4 mM-D,L-isocitrate (made up by addition of isocitrate prior to adjustment of pH at 4°C). Columns were

Table 27

Screening of Triazine Dye Columns for Binding of M. methylotrophus IDH

Triazine Dye	% Yield of Applied Activity in Salt Wash		Specific Activity ($U.mg^{-1}$) in Salt Wash		Purification Factor*		Total Activity recovered in all Column Washes	
	pH 7.2	pH 8.0	pH 7.2	pH 8.0	pH 7.2	pH 8.0	pH 7.2	pH 8.0
Red MX-50	0.0	0.0	-	-	-	-	104.1	106.6
Red HE-3B	35.9	0.0	0.030	-	0.26	-	123.1	97.1
Ramazol Red	9.6	12.9	0.178	0.181	1.55	1.31	91.2	104.7
Red R3BN	19.4	15.1	0.146	0.101	1.27	0.75	81.6	122.9
Scarlet MX-G	1.3	6.3	0.028	0.088	0.24	0.65	102.3	109.9
Brown H2G	0.8	0.0	0.018	-	0.16	-	107.6	95.9
Brown MX-5BR	1.8	0.0	0.035	-	0.30	-	98.8	83.8
Blue MX-3G	16.7	27.0	0.232	0.320	2.02	2.37	100.8	99.2
Blue HE-RD	74.5	102.6	0.407	0.312	3.54	2.31	76.3	102.8
Blue PGR	12.9	5.1	0.128	0.041	1.11	0.30	109.3	88.8
Turquoise H-A	5.8	0.0	0.383	-	1.44	-	120.1	66.5
Turquiose H-7G	2.5	0.0	0.167	-	1.45	-	117.7	63.2
Green HE-4BD	104.6	83.7	0.552	0.281	4.80	2.02	106.6	107.1
Yellow MX-G8	0.0	0.0	-	-	-	-	126.7	63.3
Orange MX-2R	17.7	1.6	0.184	0.028	1.60	0.21	114.4	55.3

*: Calculated from the specific activities of samples loaded onto columns in MET-7.2 + 2 mM-DTT and MET-8.0 + 2 mM-DTT: MET-7.2 + 2 mM-DTT: 0.115 $U.mg^{-1}$ and MET-8.0 + 2 mM-DTT: 0.135 $U.mg^{-1}$.

then washed with 1 mM-NAD+ and then 1 M-KCl in MET-7.2 (10 ml each).

4 mM-D,L-Isocitrate eluted M. methylotrophus IDH from Blue HE-RD (Figure 57a). Only 5.1% of the activity was found in the eluate from the load and buffer washes. Elution with MET-7.2 + 2-mM DTT + 4 mM-D,L-isocitrate displaced 57.8% of the applied activity. This purified IDH 13.4-fold which compares favourably with the purification (3.45-fold) achieved when the enzyme was eluted from Blue HE-RD by KCl (Table 27). Further elution with 10 ml of buffer wash followed by a 1 M-KCl salt wash eluted only a further 4% of activity.

Isocitrate only eluted 26.4% of the activity applied to Green HE-4BD (Figure 57b). The purification factor (5.7-fold) was only slightly better than that achieved by KCl elution (Table 27). Further, this purification factor was far less than that achieved by isocitrate elution from Blue HE-RD. Elution with a further 10 ml buffer wash followed by 1 mM-NAD^+ (10 ml) failed to elute any more IDH; however. the remaining activity could be recovered in the 1 M-KCl salt wash. Thus, Sepharose 4B-immobilised Blue HE-RD was studied further.

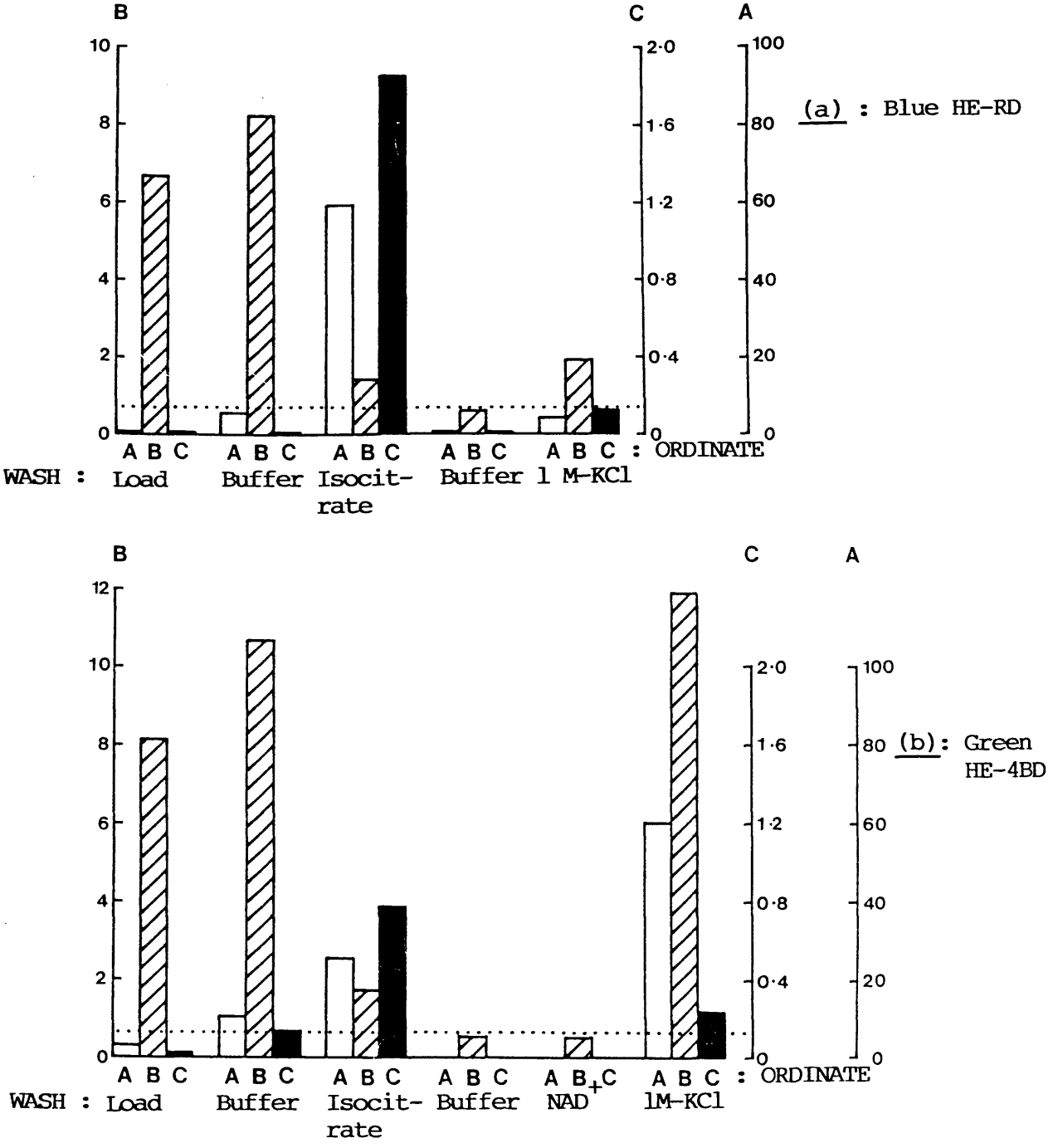
The ability of 2-OG, NAD^+ and NADH as well as isocitrate to elute IDH from Blue HE-RD was tested. Five 5-ml columns were equilibrated at 4°C with MET-7.2 + 2 mM-DTT. Each column was loaded with extract (0.65 ml; 2.425 U IDH and 7.22 mg protein) and eluted with 10 ml of the same buffer and 2 mM-NAD^+, 2 mM-2-OG, 2 mM-NADH, 2mM-2-OG + 2 mM-NAD^+ or 2 mM-NADH + 4 mM-D,L-isocitrate. Columns were then washed with 1 M-KCl. Apart from 1 M-KCl, the only successful eluant contained isocitrate (NADH + isocitrate; Table 28).

The ability of isocitrate to elute IDH from Blue HE-RD was studied at pH 8.0. A 5-ml column was equilibrated at 4°C with MET-8.0 + 2 mM-DTT. The column was loaded with a heat-treated extract that had been dialysed in MET-8.0 + 2 mM-DTT, and eluted with: 10 ml of MET-8.0 + 2 mM-DTT, 10 ml of 4 mM-D,L-isocitrate in MET-8.0 + 2 mM-DTT, a further buffer wash and 1 M-KCl in MET-8.0 + 2 mM-DTT. No IDH was eluted by isocitrate, in contrast to its effect at pH 7.2 (above). 66.9% of the IDH was eluted with 1 M-KCl. Thus, from now on, all work concerning elution of IDH from Blue HE-RD columns was done at pH 7.2.

5.1.1.2.3. Ionic Strength and Elution of IDH from Blue HE-RD. The

Figure 57

Elution of M. methylotrophus IDH Activity from Columns of Immobilised Blue HE-RD and Green HE-4BD with Isocitrate and NAD^+



The ordinate axes labelled A, B, and C are as follows. A: % activity applied to column; B: Protein (mg) eluted by column wash; C: IDH specific activity ($U.mg^{-1}$). The dotted line is the starting specific activity. Details regarding the precise composition of the washes are given in the text.

Table 28

Elution of IDH from Immobilised Blue HE-RD by Substrates and Coenzymes

Column Wash*	% Activity Applied to Column in Eluate* of Column No.:				
	1	2	3	4	5
Load Wash	0.0	0.0	0.0	0.0	0.0
Buffer Wash	0.0	0.0	0.0	0.0	0.0
2 mM-NAD^+	0.0	*N/A	N/A	N/A	N/A
2 mM-NADH	N/A	0.0	N/A	N/A	N/A
2 mM-2-OG	N/A	N/A	0.0	N/A	N/A
2 mM-(2-OG + NAD^+)	N/A	N/A	N/A	0.0	N/A
2 mM-NADH + 4 mM D,L-Isocitrate	N/A	N/A	N/A	N/A	73.2
1M-KCl	82.7	85.2	67.8	85.9	11.1

*: Material applied (Load Wash) was heat-treated crude extract that had been fractionated with ammonium sulphate (see **4.1.1.3.**) and dialysed in MET-7.2 + 2 mM-DTT. 2.425 U IDH and 7.22 mg protein were applied to each column in 0.65 ml.

The column washes containing KCl, coenzymes, substrates or combinations thereof were made up in buffer wash, i.e. MET-7.2 + 2 mM-DTT. Apart from the load wash, the volume of all column washes was 10 ml.

N/A = Column wash not applied.

ionic strength of MET-7.2 is about 0.072. This would be raised significantly by 4 mM-isocitrate (ionic strength for fully ionised D and L trisodium salts = 0.024). This shows that isocitrate may elute IDH by ionic strength. Thus, the ability of KCl to elute IDH from Blue HE-RD was assessed to test this hypothesis.

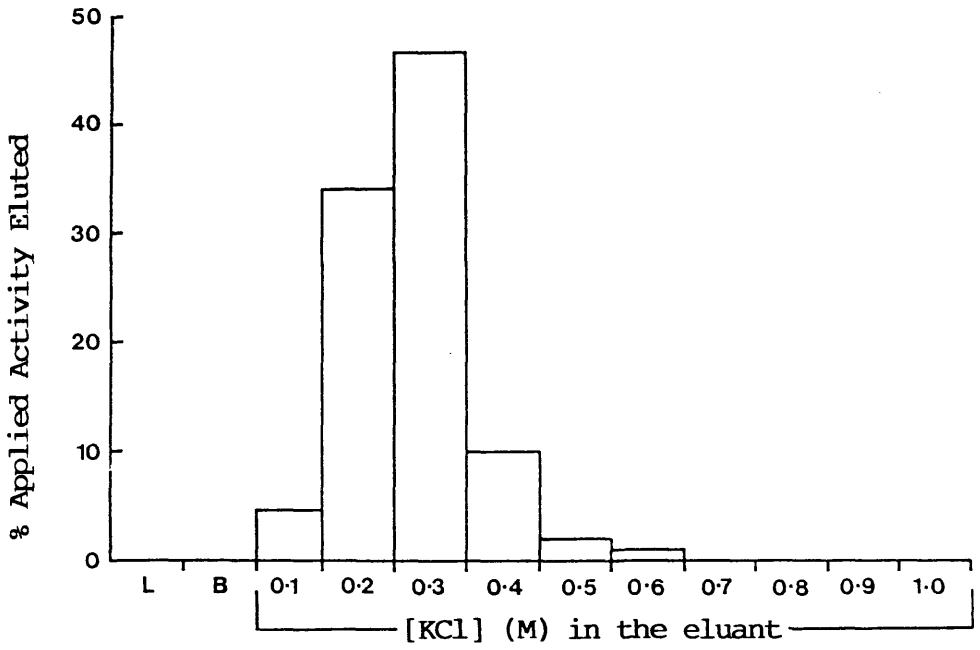
A pre-equilibrated 5-ml Blue HE-RD column was loaded with 16.48 U IDH and 59.2 mg protein that was a resuspended dialysed 55-70% $(NH_4)_2SO_4$ precipitate (see below) of heat-treated material. The column was eluted with a 10-ml buffer wash, and then 10 ml each of buffer containing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 M-KCl. Most IDH (80.7%) was eluted by 0.2 and 0.3 M-KCl (Figure 58). Thus it seemed that isocitrate would not elute IDH as the increase in ionic strength required was too great. This suggests that any contaminating protein that would have been eluted with IDH by ionic strength of the added isocitrate could be eluted with a low concentration of KCl (e.g. 0.1 M) prior to elution with isocitrate. Indeed, such a procedure was adopted in the final purification protocol (see **5.1.1.3.**).

5.1.1.3. A Purification Protocol for IDH from M. methylotrophus. Heat treatment in the presence of isocitrate followed by elution of IDH from Blue HE-RD with isocitrate was inconvenient as the small amounts of IDH in M. methylotrophus crude extract would necessitate processing of large amounts of protein after heat treatment. Thus, an ammonium sulphate precipitation was incorporated into the final protocol.

Crude extract was prepared in MET-8.0. This was made 2 mM in D,L-isocitrate. The extract was heated to 70°C and maintained for 30 min at that temperature. The precipitate was removed by centrifugation for 30 min at 4°C. The supernatant was brought to 55% saturation with respect to ammonium sulphate and the precipitate was discarded. The supernatant was then brought to 70% saturation with respect to ammonium sulphate and the precipitate was resuspended in MET-7.2 + 2 mM-DTT and dialysed against MET-7.2 + 2 mM-DTT (**5.1.1.2.1.**). The dialysate was then applied to 5-ml columns (up to 25 mg protein per column) of Blue HE-RD that were pre-equilibrated with MET-7.2 + 2 mM-DTT. Columns were then washed sequentially under gravity with 2 column volumes of: MET-7.2 + 2 mM-DTT, 2 column volumes of the same buffer +

Figure 58

Elution of M. methylotrophus IDH Activity from Blue HE-RD by Ionic Strength



L = Load Wash

B = Buffer Wash

0.1 M-KCl and then MET-7.2 + 2 mM-DTT until no protein could be detected. IDH was then eluted with 10 column volumes of the previous buffer and 8 mM-D,L-isocitrate. The column was then washed with 1 M-KCl (Figure 59). IDH was concentrated to > 0.1 $mg.ml^{-1}$ by ultrafiltration (**2.2.6.**) and stored at -20°C in the buffer in which it was eluted. Under these conditions IDH activity was stable for at least 9 months.

Discontinuous SDS-PAGE (**5.1.2.**) of the protein eluted from Blue HE-RD indicated that the enzyme was homogeneous (Figure 60, track 5). However, the effluent from the Blue HE-RD column had to be entirely free of protein before isocitrate was applied, if the purity of the final product was not to be compromised (Figure 60, track 6).

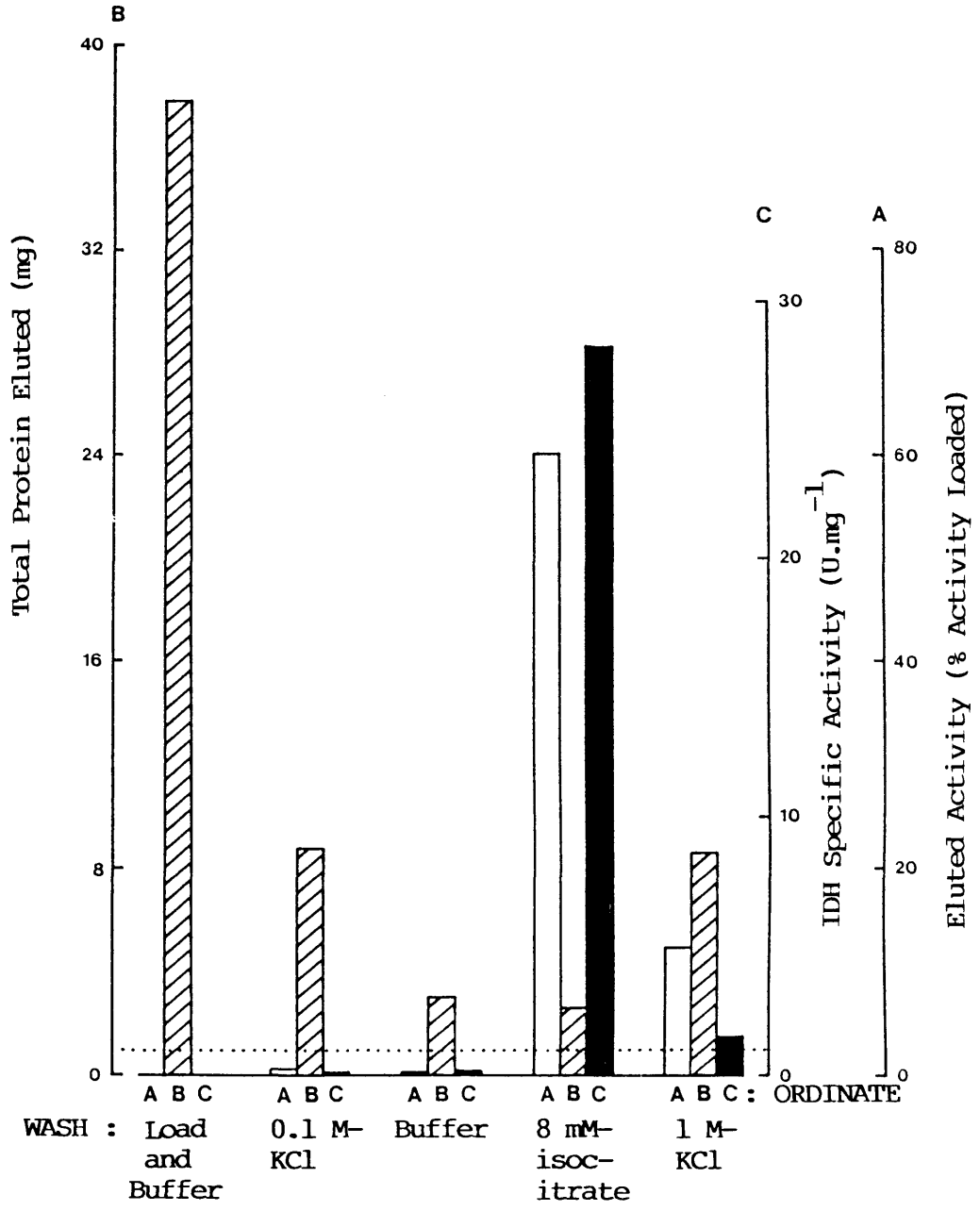
The purification procedure was rapid to perform; milligram amounts of enzyme could be obtained in 48 h. A typical purification from 300g (wet weight) cell paste is summarised in Table 29. IDH was purified over 1,400-fold. The Blue HE-RD step alone resulted in a 28.2-fold purification (Table 29). Yields were in the range of 30-40%. The specific activity of the electrophoretically homogeneous enzyme was 27.2 $\pm$ 3.8 $U.mg^{-1}$ (mean $\pm$ S.E.M., n = 3 purifications).

5.1.1.4. The Interaction of Blue HE-RD with M. methylotrophus IDH. Only isocitrate protected IDH from thermal inactivation and eluted it from Blue HE-RD. Thus it was speculated that interactions that prevented thermal inactivation were related to those between IDH and Blue HE-RD. To evaluate this proposal, the effect of Blue HE-RD on the thermal stability of IDH was examined.

Using a dye concentration that totally inhibited the enzyme (0.2 $mg.ml^{-1}$; see below), IDH (in crude extract) was incubated for 10 min at 70°C with those agents that eluted it (2 mM-D,L-isocitrate and/or 0.5 M-KCl) with or without Blue HE-RD itself. Blue HE-RD or KCl either alone or in combination had no effect on the stability of IDH (Table 30). Further, neither Blue HE-RD or KCl prevented isocitrate from protecting IDH. Thus it seems the ability of isocitrate to protect IDH and its ability to elute the enzyme from Blue HE-RD are unconnected. The inability of KCl to protect IDH from thermal inactivation suggests that protection by isocitrate is not an ionic strength effect.

Figure 59

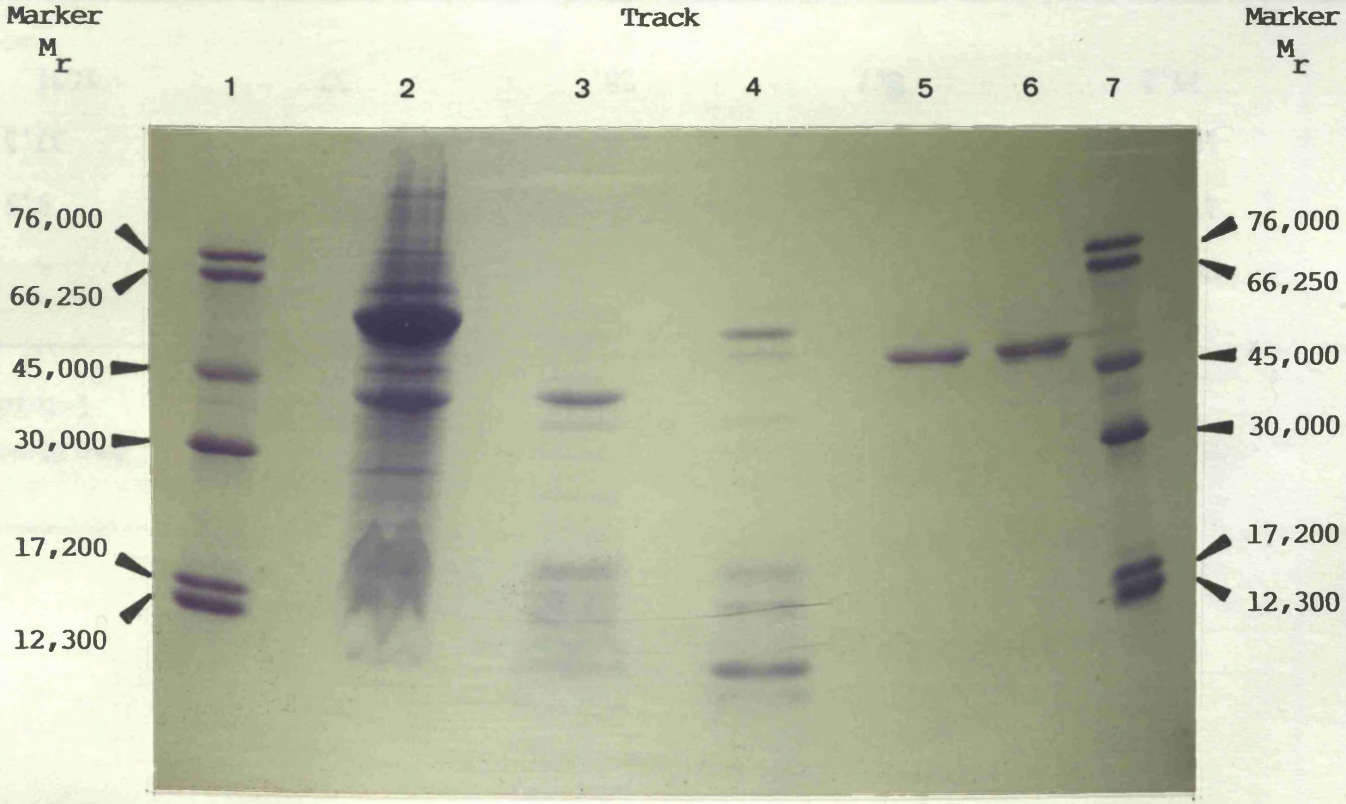
Purification of M. methylotrophus IDH on Blue HE-RD



The data represents the pooled results from the five columns used in the final step. The dotted line indicates the initial specific activity of IDH in the loaded material. In this instance, 125.3 U of IDH in 125.1 mg of protein were divided into five aliquots and loaded onto the Blue HE-RD columns.

Figure 60

Analysis of the Purification of M. methylotrophus IDH by SDS-PAGE



10 µg of each M_r marker was loaded. The following was loaded onto each track:

1 and 7: M_r markers

2: 20 µg Crude extract protein

3: 20 µg of protein from the thermal precipitation step

4: 20 µg of protein from the $(NH_4)_2SO_4$ step

5 and 6: 20 µg of IDH eluted from Blue HE-RD by isocitrate; samples in 5 and 6 were from separate purifications

Table 29

Purification of IDH from M. methylotrophus

Step	Volume (ml)	Total IDH (U)	Total Protein (mg)	Specific Activity ($U.mg^{-1}$)	Yield (%)	Purification (-fold)
Crude Extract	315	202.6	10316	0.020	100	-
Heat Treatment	270	179.7	941.5	0.191	89	9.5
55-70% $(NH_4)_2SO_4$	36	100.5	137.6	0.730	50	37.3
Blue HE-RD	264	75.2	2.7	28.1	37	1431

Starting material was 300g (wet weight) cell paste.

Table 30

The Effects of Combinations of Blue HE-RD, Isocitrate and KCl on the Thermal Stability of M. methylotrophus IDH

Addition to Crude Extract	Activity Remaining (%) After 10 min Incubation at 70°C*.
None	4.7
+ 2 mM-D,L-Isocitrate	100
+ 0.5M-KCl	4.2
+ 2 mM-D,L-Isocitrate + 0.5 M-KCl	81.5
+ 0.2 $mg.ml^{-1}$ Blue HE-RD	4.0
+ 0.2 $mg.ml^{-1}$ Blue HE-RD + 2 mM-D,L-Isocitrate	100
+ 0.2 $mg.ml^{-1}$ Blue HE-RD + 0.5 M-KCl	5.7
+ 0.2 $mg.ml^{-1}$ Blue HE-RD + 2 mM-D,L-isocitrate + 0.5 M-KCl	92.3

*: Extract prepared in MET-8.0 (0.75 ml) was added to 1.5 ml pre-warmed MET-8.0 containing Blue HE-RD, isocitrate or KCl as specified in the table. The final protein concentration was 8.7 $mg.ml^{-1}$.

The effect of Blue HE-RD on IDH activity was also studied. These experiments were conducted with MET-7.2 + 2 mM-DTT as the assay buffer. At 0.2 mM-NAD^+ and 4 mM-D,L-isocitrate, Blue HE-RD inhibited the purified IDH, half-maximal inhibition being effected by about 40 $\mu g.ml^{-1}$ Blue HE-RD (Figure 61). The kinetics of Blue HE-RD-inhibition were examined. For this purpose, IDH was desalted into MET-7.2 + 2 mM-DTT with a G-25 PD-10 column to remove isocitrate (**2.2.5.**). Fractions with no activity without added isocitrate were used. Cornish-Bowden plots ([isocitrate or NAD^+]/v vs. [Blue HE-RD]) at fixed NAD^+ (0.2 mM) or D,L-isocitrate (4 mM) and varying Blue HE-RD concentrations (Figures 62a,b) curved upwards. This suggested that binding of Blue HE-RD to IDH was co-operative or that there was more than one type of interaction between IDH and Blue HE-RD. From such data, it was impossible to suggest how IDH interacted with Blue HE-RD.

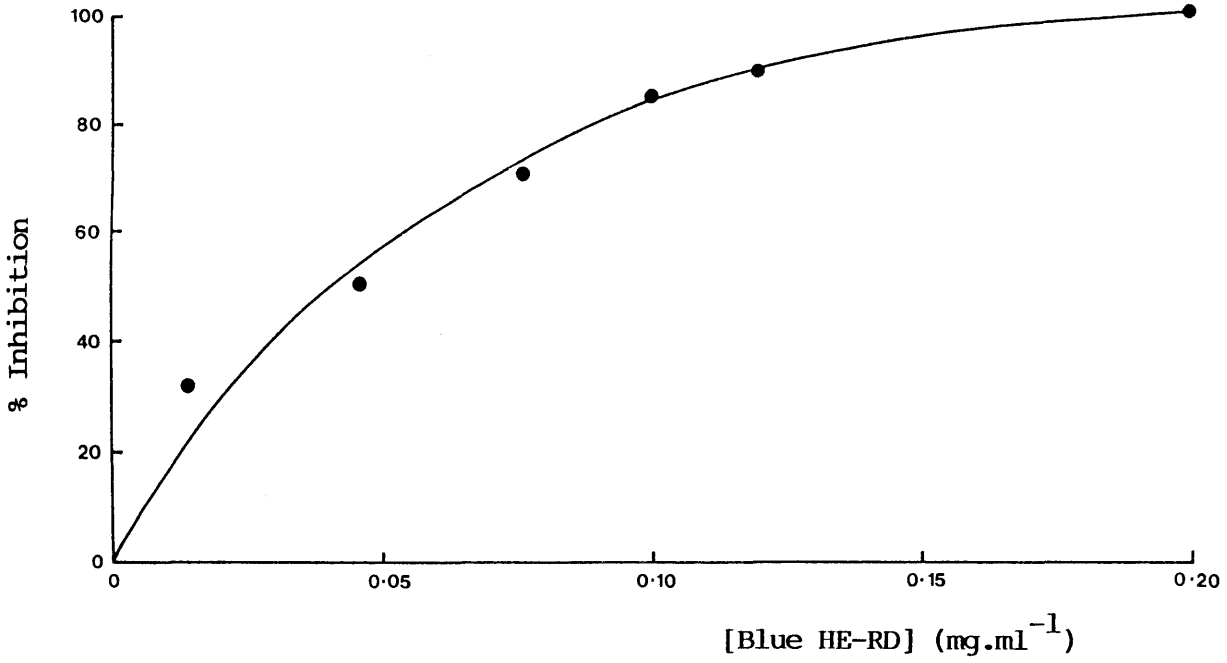
5.1.2. Quaternary Structure of M. methylotrophus IDH

5.1.2.1. Subunit M_r. IDH from the Blue HE-RD step was electrophoresed discontinuously (Figure 60) in gradient gels (**2.3.4.3.**) with the M_r markers used to size the CS subunit (**4.2.2.1.**). The mobilities of markers relative to bromophenol blue (R_f) are plotted vs. $\log_{10}$ of their M_r values in Figure 63. From these data, the subunit M_r value of M. methylotrophus IDH was 45,600 ± 1,200 (± S.E.M., n = 3).

5.1.2.2. Native M_r. The native M_r of purified IDH was estimated by analytical gel filtration in Sephacryl S-200 equilibrated in MET-8.0 + 0.1 M-KCl (**2.3.5.**). The void volume of the column was 177.5 ml. The column was calibrated with 4 mg each of the following M_r markers [native M_r values quoted by the supplier]: ferritin [450,000], catalase [240,000], aldolase [158,000], bovine serum albumin [68,000], chymotrypsinogen A [45,000] and cytochrome C [12,500], and their elution is shown in Figure 64. The relationship between log10 native M_r and Ve is shown in Figure 65. Ve for M. methylotrophus IDH activity was 63.4 ± 3.8 ml (± S.E.M., n = 3). From this, the IDH native M_r deduced from the data in Figure 65 was 94,400 ± 6,900 (± S.E.M., n = 3).

Figure 61

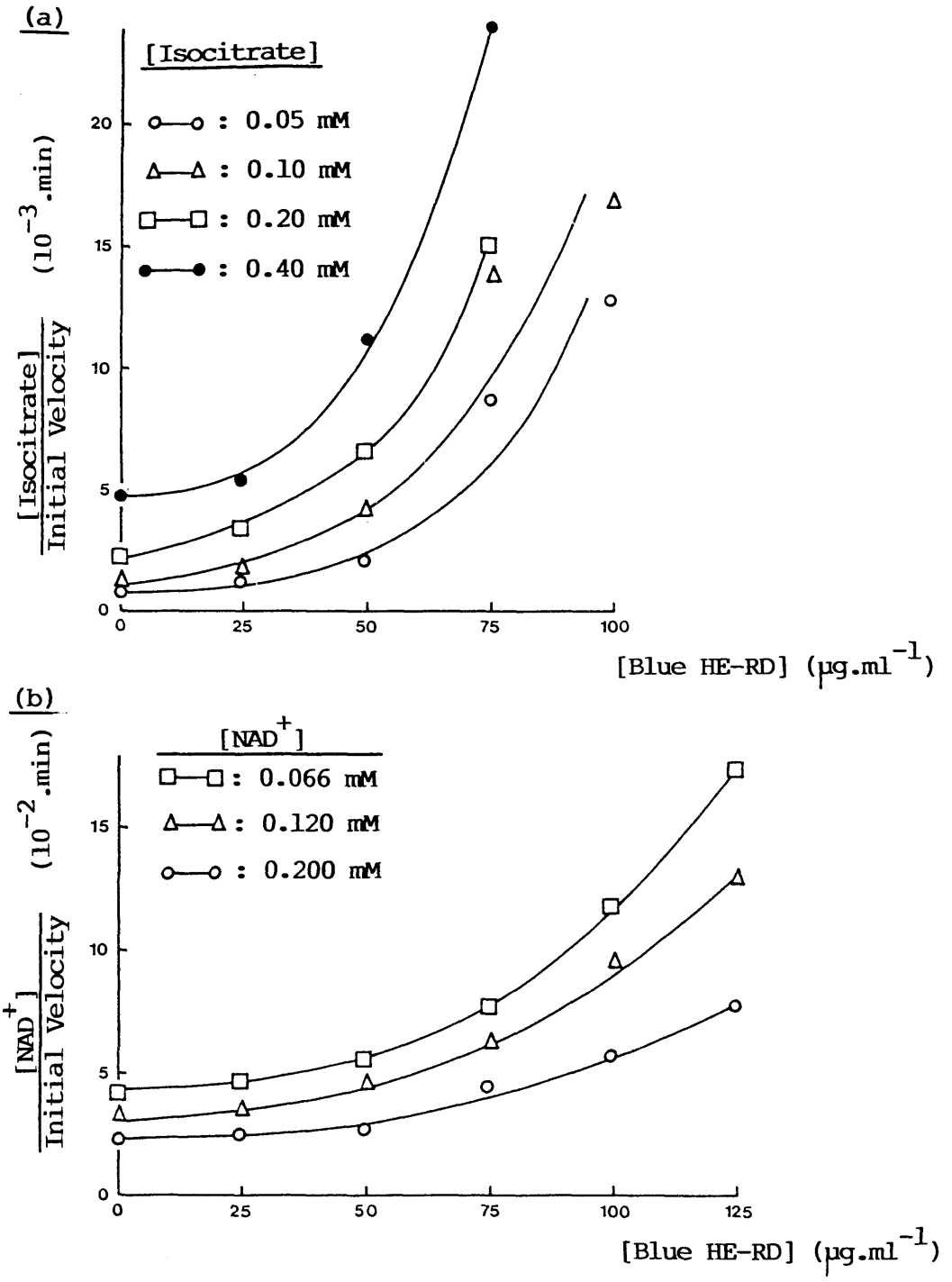
Inhibition of M. methylotrophus IDH by Blue HE-RD



Enzyme activity was assayed in MET-7.2 + 2 mM-DTT. [D,L-isocitrate] = 4 mM; [NAD^+] = 0.2 mM

Figure 62

Cornish-Bowden Plots of Inhibition of M. methylotrophus IDH by Blue HE-RD



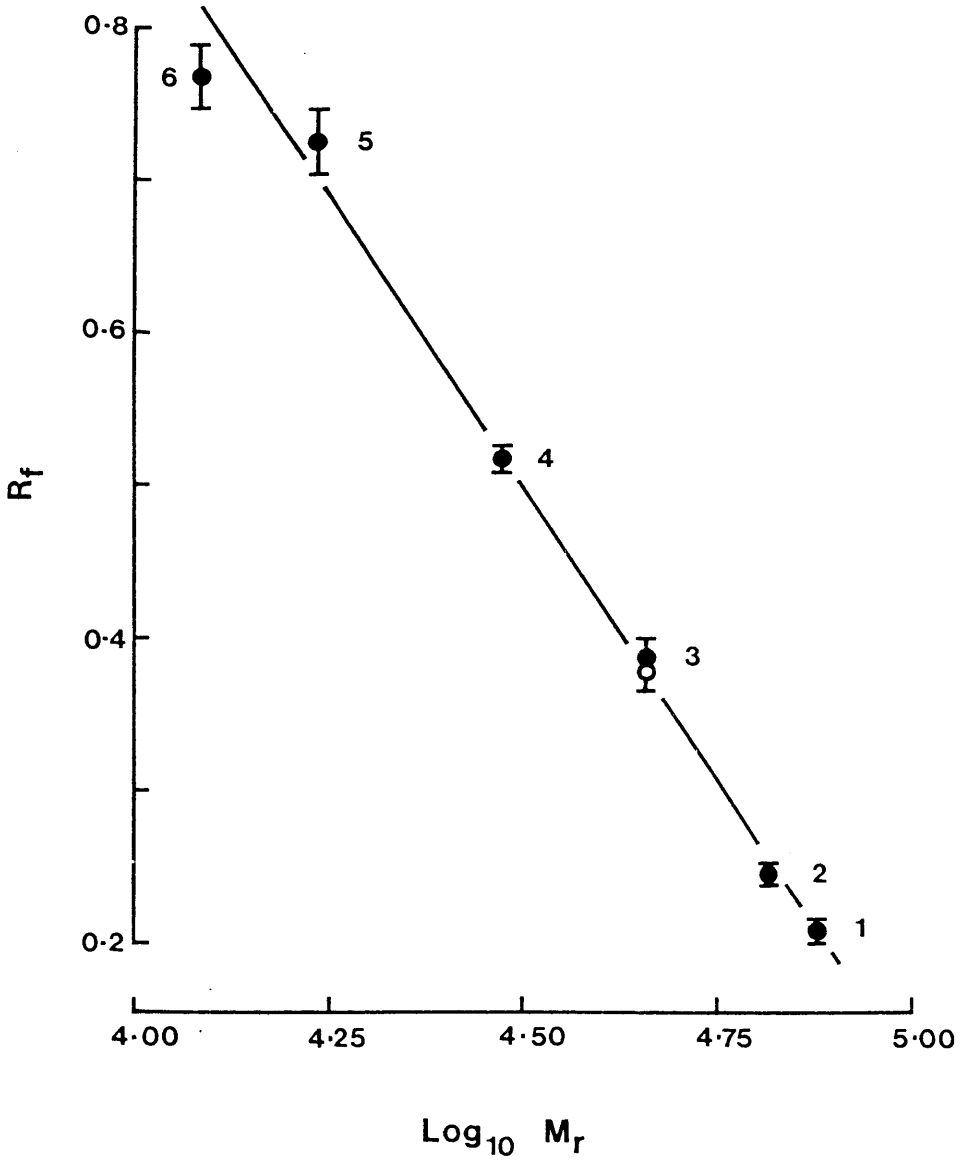
(a): $[NAD^+]$ = 0.2 mM, [D,L-isocitrate] varied

(b): [D,L-isocitrate] = 4 mM, $[NAD^+]$ varied.

Assay buffer was MET-7.2 + 2 mM-DTT.

Figure 63

Determination of the Subunit M_r Value of M. methylotrophus IDH by SDS-PAGE



Points 1-6 (the closed symbols) represent the positions of migration relative to bromophenol blue of [M_r values in square brackets] of:

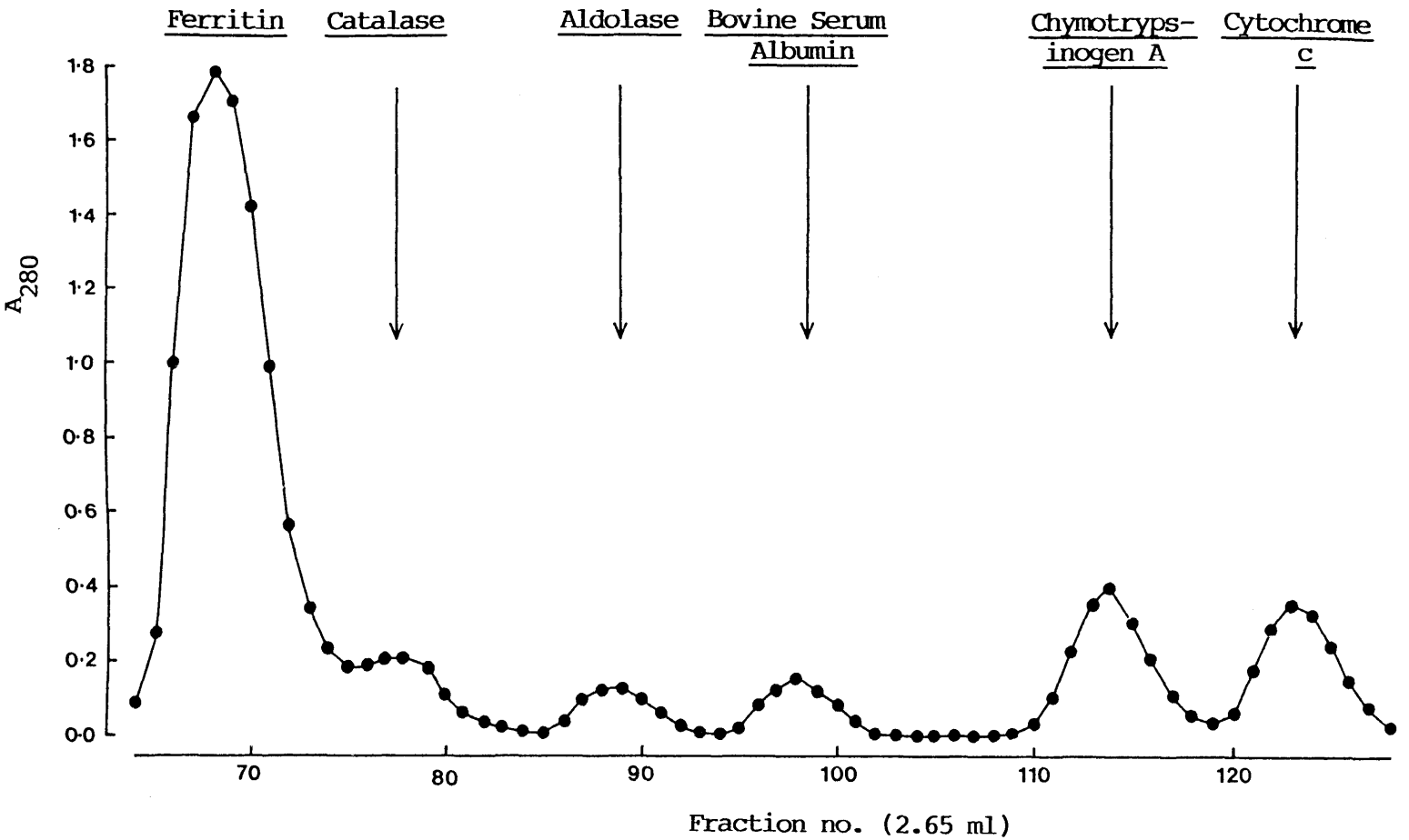
(1): ovotransferrin [76,000]
(2): albumin [66,250]
(3): ovalbumin [45,000]
(4): carbonic anhydrase [30,000]
(5): myoglobin [17,200]
(6): cytochrome c [12,300]

The open symbol below 3 is the migration position of M. methylotrophus IDH. All points are the mean values of three experiments.

Figure 64

Elution Profile of Native M_r Markers from Sephacryl-S200

4 mg of each protein was dissolved and loaded in 4 ml of MET-8.0 + 0.1 M-KCl. The column was equilibrated and developed in the same buffer



Legend to Figure 65

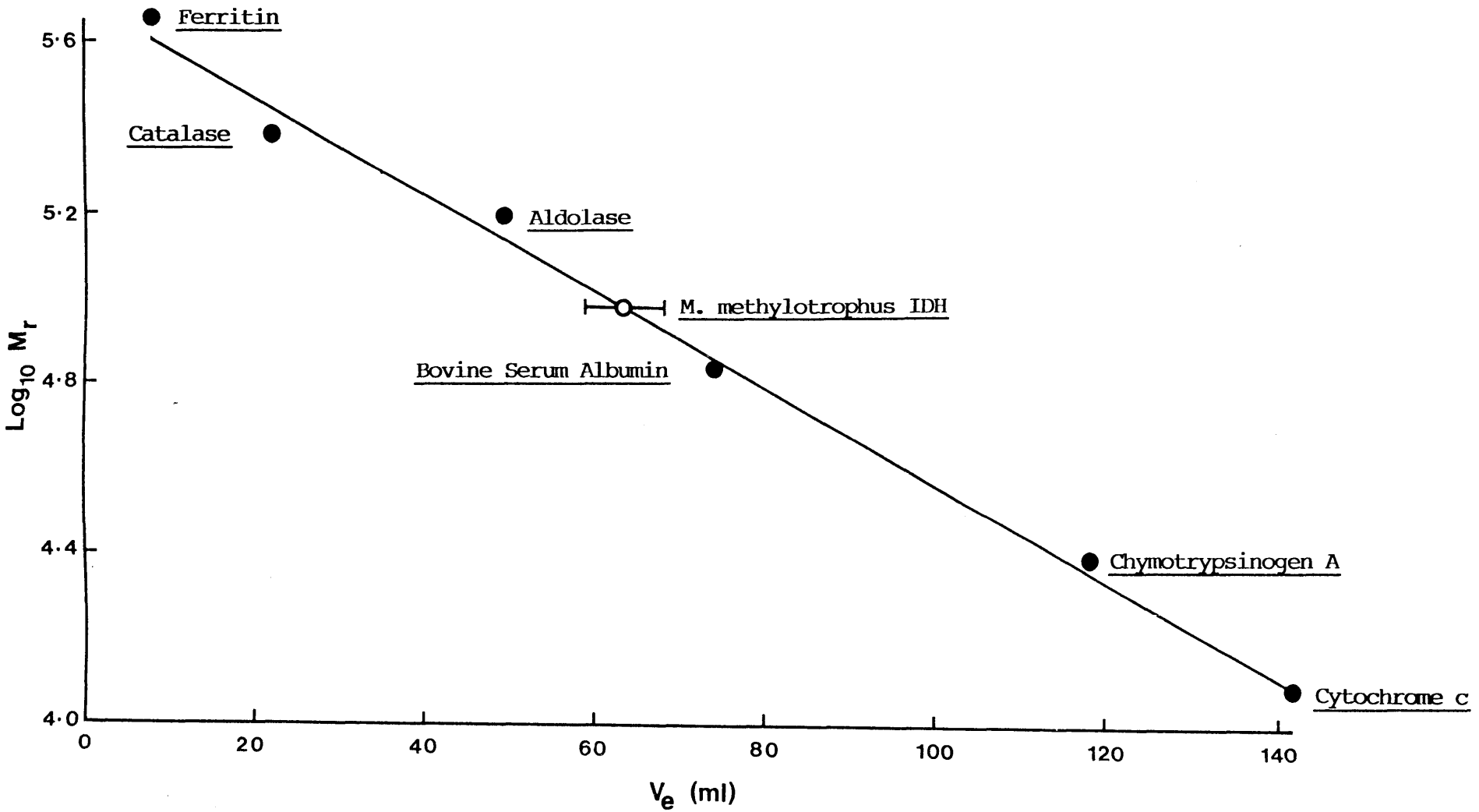
the native M_r values of the markers (closed symbols) was as follows:

Ferritin:	450,000
Catalase:	240,000
Aldolase:	158,000
Bovine Serum Albumin:	68,000
Chymotrypsinogen A	25,000
Cytochrome c	12,500

The elution position of M. methylotrophus IDH (open symbol) was detected by monitoring its activity. The error bar is ± S.E.M. for 3 determinations.

Figure 65

Determination of the Native M_r of M. methylotrophus IDH by Gel Filtration Through Sephacryl S-200



5.1.2.3. Subunit Stoichiometry. The data above suggest a native M_r: subunit M_r ratio of 2.07. Thus, M. methylotrophus IDH is dimeric.

5.1.3. Substrate, Coenzyme and pH Dependence and Metal ion Specificity

5.1.3.1. Isocitrate Dependence. This was determined with pure IDH after buffer exchange with MET-8.0 using a PD-10 G-25 column. Initial rate measurements were carried out at 0.5 mM-NAD^+.

The dependence of initial velocity on isocitrate was hyperbolic (Figure 66a). The D,L-isocitrate K_m was 3.9 μM and the V_{max} was 24.4 $U.mg^{-1}$ (Figure 66). The isocitrate concentrations needed to obtain data in the K_m region of the saturation curve were very low and the data here may be prone to error. Thus the accuracy of the kinetic constants may not be high. Unfortunately, this meant that the data could not be used to gain meaningful information from a Hill plot regarding co-operativity of isocitrate dependence because most of the points were beyond the linear portion of the plot (Figure 68a).

5.1.3.2. NAD^+ Dependence. Enzyme prepared similarly to that used in 5.1.3.1. was used to study the NAD^+ dependence of M. methylotrophus IDH. D,L-isocitrate was held at 4 mM and NAD^+ was varied from 3 μM to 0.4 mM. The dependence of the enzyme on NAD^+ was hyperbolic. The K_m value for NAD^+ was 45.2 μM and the V_{max} value was 16.8 $U.mg^{-1}$ (Figure 67). Replotting these results as a Hill plot (Figure 68b) gave a linear relationship between $\log_{10}[v/(V_{max}-v)]$ and $\log_{10}[NAD^+]$ from which a Hill coefficient of 0.96 ± 0.05 (± S.E.) was obtained. This figure suggests that binding of NAD^+ is not co-operative.

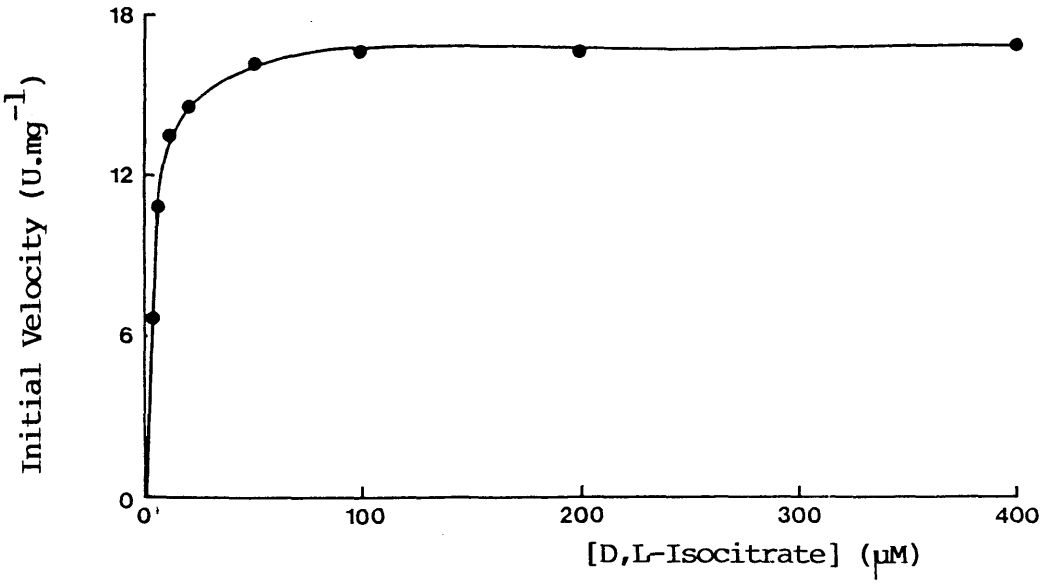
5.1.3.3. Coenzyme Specificity. The ability of M. methylotrophus IDH to use $NADP^+$ was examined. First, the coenzyme purity was established.

5.1.3.3.1. Separation of $NADP^+$ and NAD^+ by Reverse-Phase HPLC. HPLC resolved 0.1 mg of the $NADP^+$ from Boehringer-Mannheim (2.3.6.) into one major peak comprising 97.5% ± 0.8 (± S.E.M., n = 6) of the material with a retention time of 2.45 ± 0.05 min (± S.E.M., n = 6; Figure 69a). When 0.1 mg of the NAD^+ (Sigma) was analysed, it too

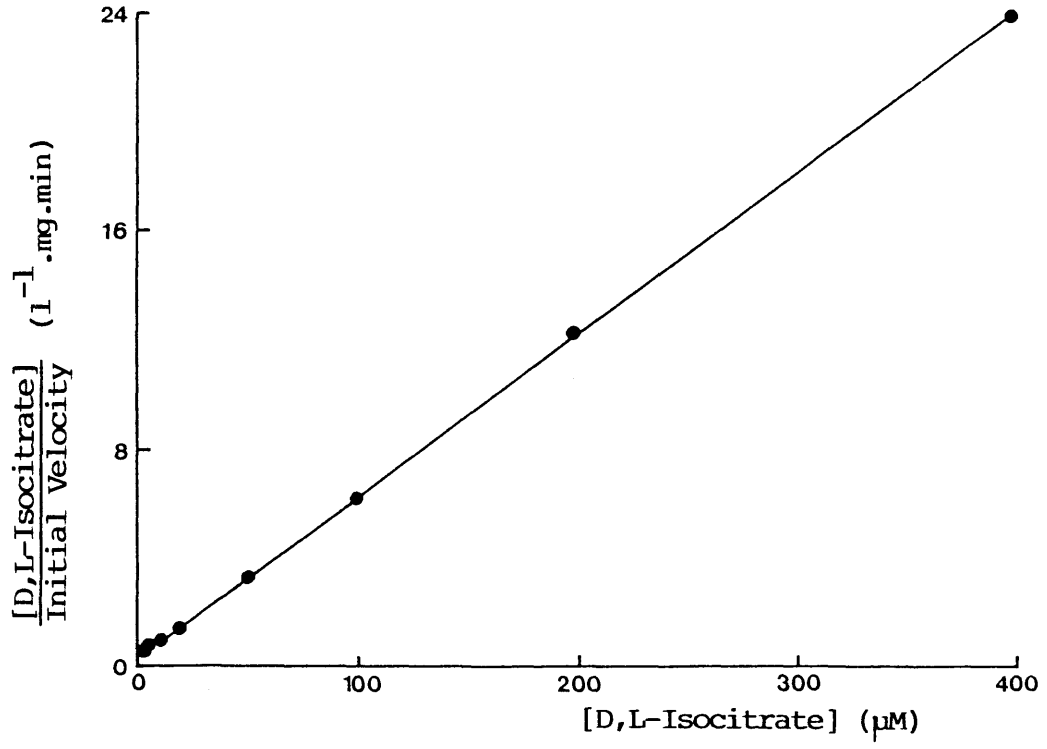
Figure 66

Relationship Between Isocitrate Concentration and M. methylotrophus IDH Activity

(a)



(b)

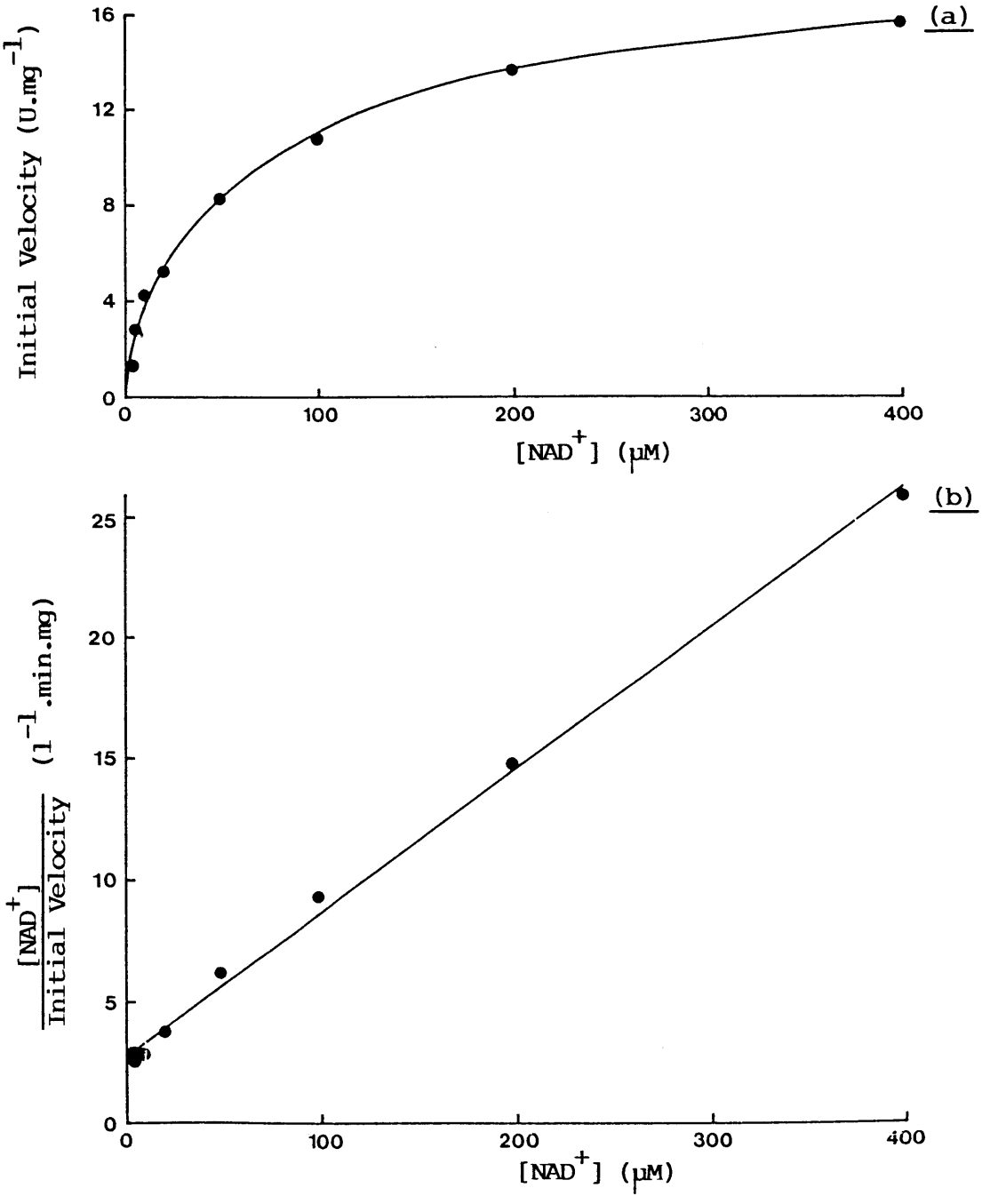


(a): Dependence of initial velocity on isocitrate concentration

(b): Half reciprocal plot of the data in (a)

Figure 67

Relationship Between NAD^+ Concentration and M. methylotrophus IDH Activity

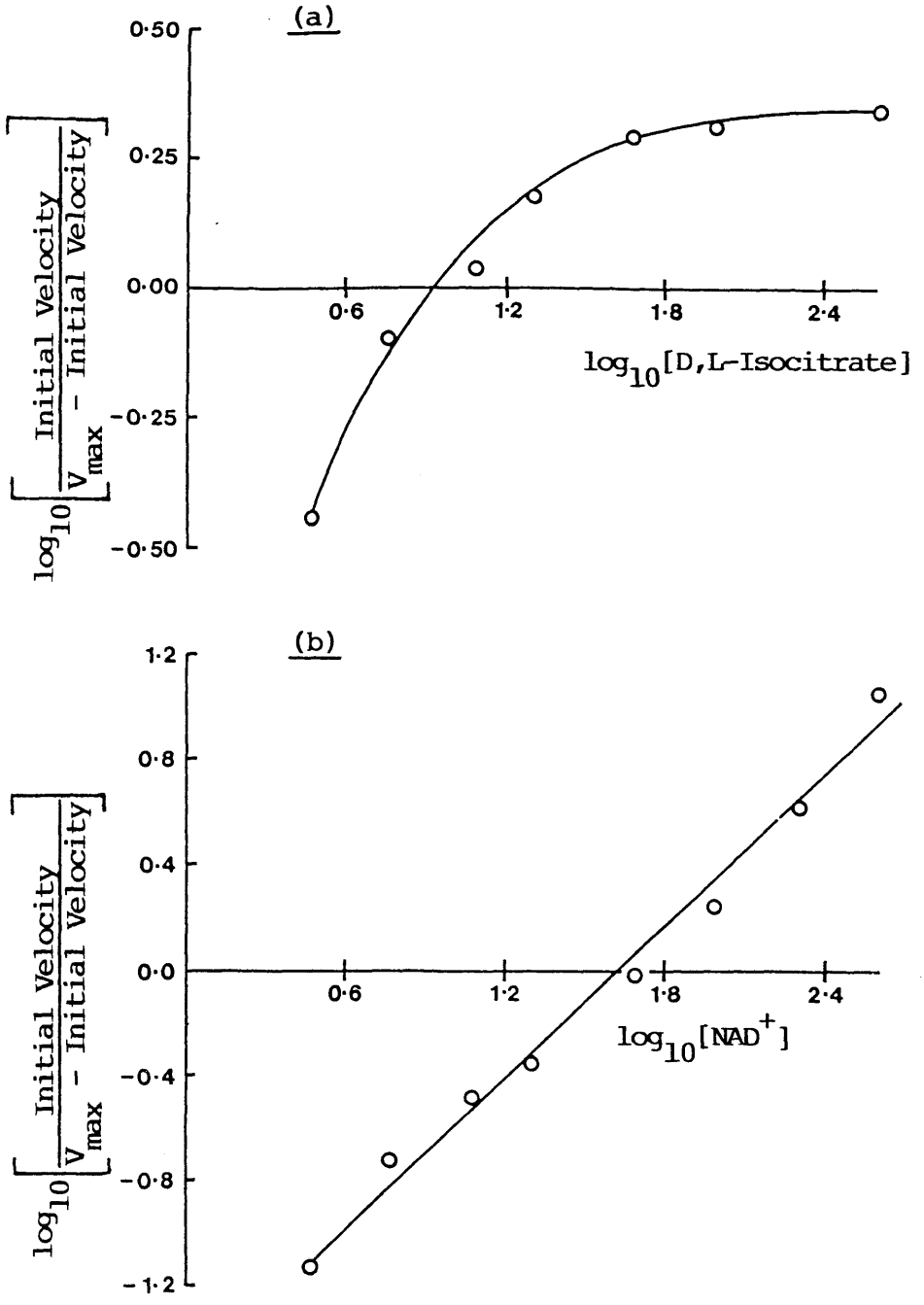


(a): Dependence of initial velocity of IDH on NAD^+ concentration.

(b): Half reciprocal plot of the data in (a).

Figure 68

Hill Plots of the Relationships shown by M. methylotrophus IDH Between Initial Velocity and NAD^+ and Isocitrate Concentration



(a): Hill plot of the data in Figure 66

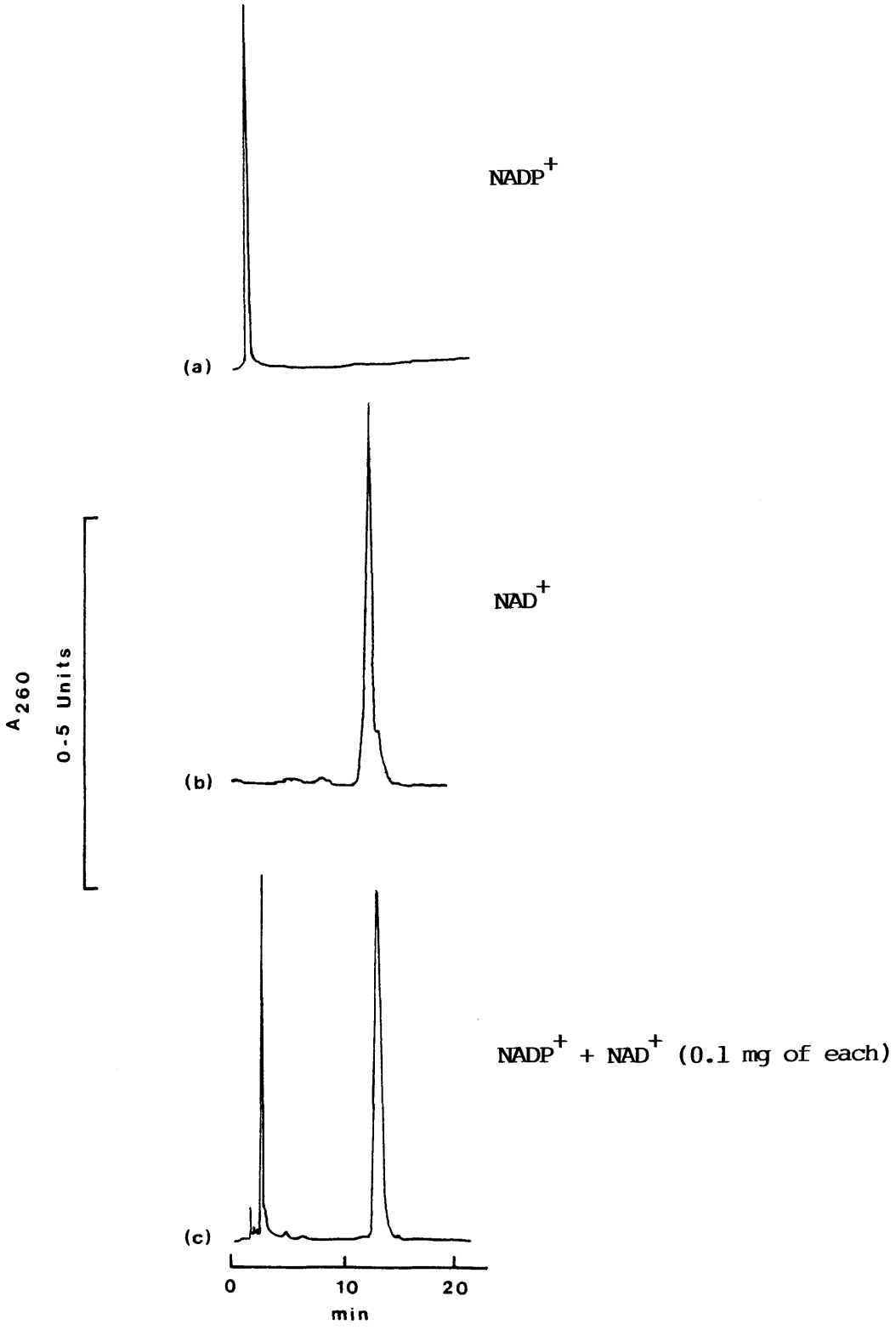
(b): Hill plot of the data in Figure 67

Figure Legend to Figure 69

0.1 mg of $NADP^+$ [trace (a)], 0.1 mg of NAD^+ [trace (b)] or 0.1 mg of $NADP^+$ and 0.1 mg of NAD^+ [trace (c)] were loaded onto a C-18 reverse phase column in 20 µl of 20 mM-potassium phosphate (pH 6.8). The column was eluted in the same buffer for 10 min, and then in the same buffer with a 0-40% gradient of methanol applied over 15 min. The flow rate was 2 $ml.min^{-1}$. $NAD(P)^+$ was detected spectrophotometrically at 260 nm.

Figure 69

Reverse Phase HPLC Analysis of Nicotinamide Nucleotides Used to Assay M. methylotrophus IDH



contained one major component (Figure 69b) which eluted after 11.5 ± 0.5 min (± S.E.M., n = 5) and accounted for 91.7% ± 2.1 (± S.E.M., n = 5) of the material applied. These degrees of purity are what was expected from the supplier's data: 98% for $NADP^+$ and 94% for NAD^+.

Assuming that the major peaks were the authentic nucleotides, it seems that no contaminants in $NADP^+$ were eluted after $NADP^+$ itself (Figure 69a), indicating that there was no contamination of $NADP^+$ by NAD^+. However, it was possible that contaminating nucleotide would be masked if its retention time was altered by the other nucleotide. Analysis of a mixture of NAD^+ and $NADP^+$ showed that either nucleotide did not significantly modify the retention time of the other (Figure 69c). Both NAD^+ and $NADP^+$ were well separated with retention times similar to those observed on HPLC of the nucleotides separately (2.34 min and 11.75 min for $NADP^+$ and NAD^+ respectively n = 2). Thus it seemed that the $NADP^+$ was not contaminated by NAD^+.

5.1.3.3.2. $NADP^+$ Usage by M. methylotrophus IDH. M. methylotrophus IDH could reduce $NADP^+$ with isocitrate. IDH was assayed from 0.2 to 10 mM-$NADP^+$ at 4 mM-D,L-isocitrate. The dependence on $NADP^+$ is shown in Figure 70a. Above 3 mM-$NADP^+$, IDH was inhibited. The relationship between rate and coenzyme ([S]) under these circumstances is:

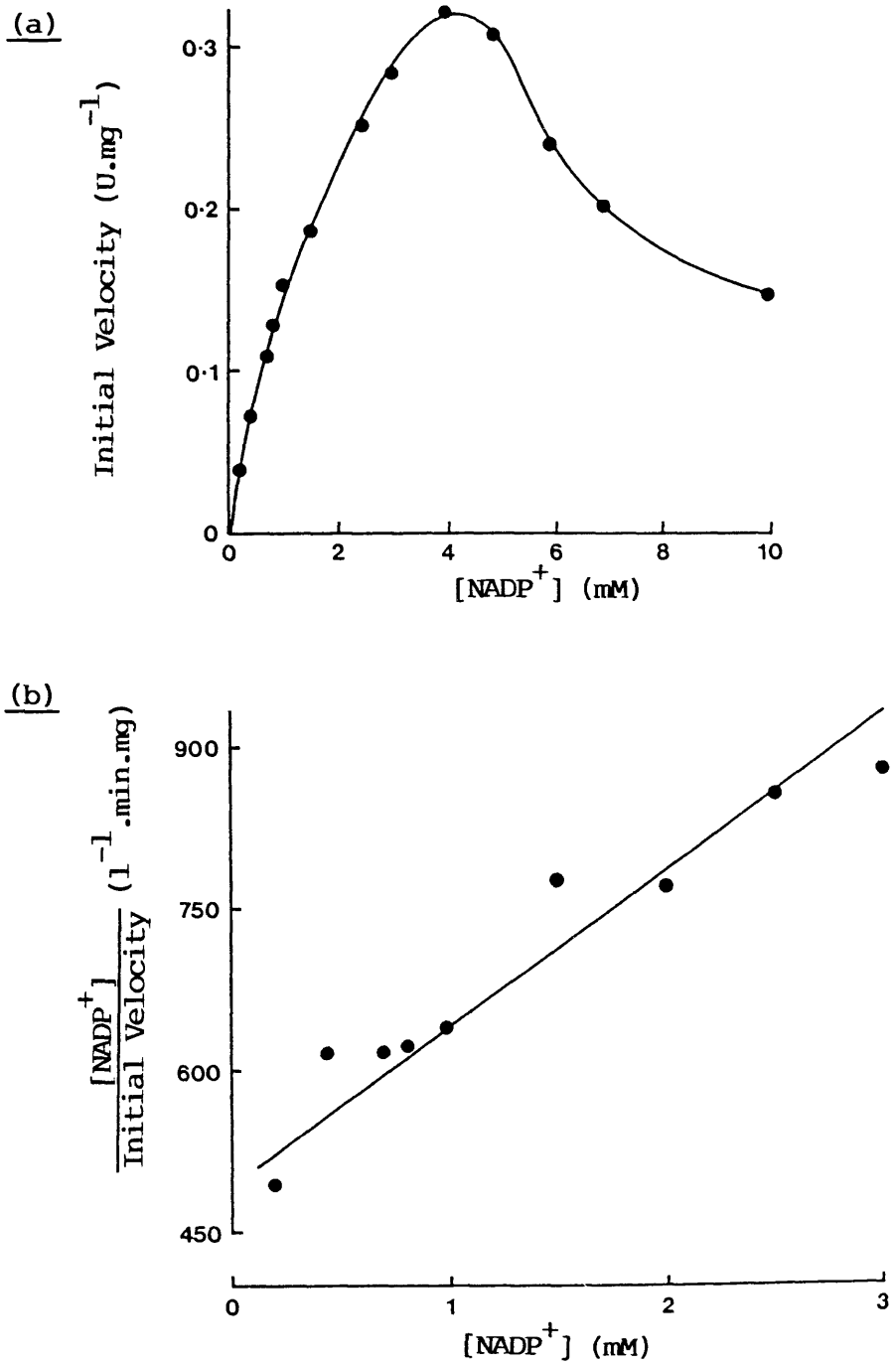
$$v = \frac{V_{max}}{\frac{K_m}{[S]} + 1 + \frac{[S]}{K_i}}$$

where K_i = inhibition constant. When [S] is small and $[S]/K_i$ is near zero, IDH is hyperbolically dependent on [S]. When [S] is large, $K_m/[S]$ approaches zero and $[S]/K_i$ becomes significant in the denominator. The decline in activity with [S] can be described by:

$$v = \frac{V_{max}}{1 + \frac{[S]}{K_i}}$$

Figure 70

Relationship Between $NADP^+$ Concentration and M. methylotrophus IDH Activity



(a): Effect of $[NADP^+]$ on the initial velocity

(b): Half reciprocal plot of initial velocity below 3 mM-$NADP^+$

The Km and V_{max} values from data obtained between 0.2 and 4 mM-$NADP^+$ were 3.55 mM and 7.36 $U.mg^{-1}$ respectively (Figure 70b). The K_i for $NADP^+$ was found from a plot of 1/v vs. [$NADP^+$] (Figure 71) of the data obtained above 2.5 mM-$NADP^+$. The K_i was 1.56 $\pm$ 0.17 mM ($\pm$ S.E.M.).

5.1.3.3.3. Relative Coenzyme Specificity of M. methylotrophus IDH. From the foregoing, the V_{max}/K_m values for NAD^+ or $NADP^+$ are 0.54 $min.mg^{-1}.1^{-1}$ and 0.0021 $min.mg^{-1}.1^{-1}$ respectively. The ratio of these two figures is a measure of the specificities of IDH for NAD^+ compared with $NADP^+$. Thus, as the ratio $V_{max}/K_m(NAD^+):V_{max}/K_m(NADP^+)$ = 257, this IDH is predominantly specific for NAD^+.

5.1.3.4. Metal Ion Specificity of M. methylotrophus IDH. The ability of various divalent cations to satisfy the metal ion requirement for M. methylotrophus IDH was tested. Purified IDH was used that was desalted into 20 mM-Tris (without EDTA) adjusted to pH 8.0 (T-8.0).

IDH was tested with Mn^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, Ca^{2+} and Fe^{2+}. Assays were carried out in T-8.0. All metal ions were present at 10 mM concentration and all except Cd^{2+} (which was the acetate salt) were used as the chloride. Activity was supported by (listed in decreasing order of preference) Mg^{2+}, Mn^{2+} and Co^{2+} (Table 31). Mn^{2+} was almost as effective as Mg^{2+}, and both were almost ten times more effective than Co^{2+}. Zn^{2+}, Cd^{2+}, Ca^{2+} and Fe^{2+} could not support IDH activity.

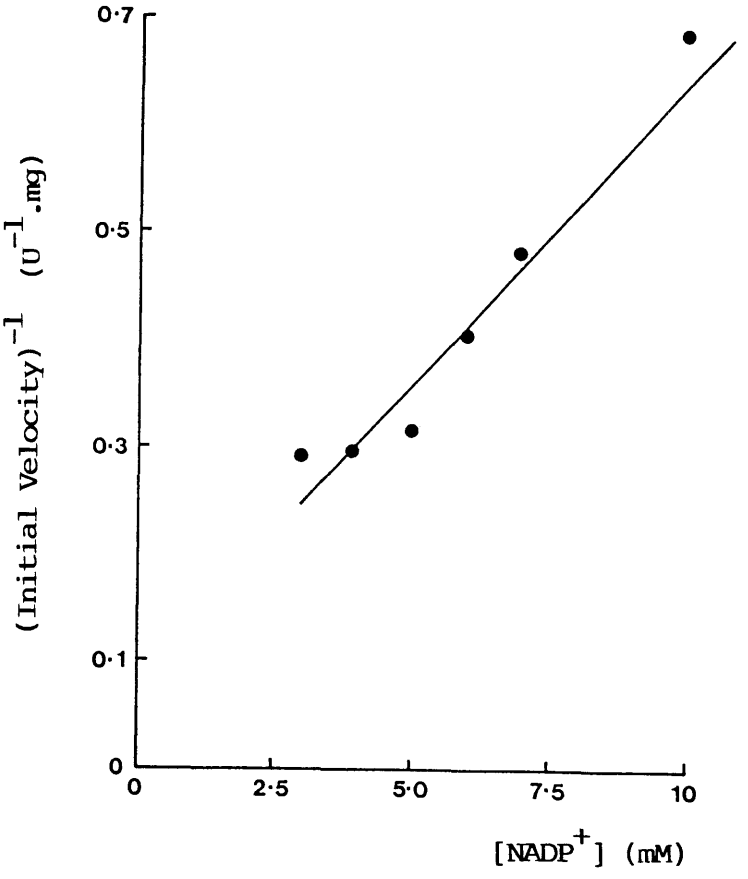
5.1.3.5. Dependence of M. methylotrophus IDH on pH. The effects of pH on activity were studied from pH 5.5 to pH 10.5. Assays in 10 mM-$MgCl_2$ and 2.4 mM-EDTA were buffered with bis-Tris (pKa=6.5); Tris (pKa=8.3) and Ches (pKa=9.3). The relationship between activity and pH was a bell-shaped curve. The pH optimum was 8.2 - 8.6 (Figure 72).

5.1.4. Effect of Various Metabolites on IDH Activity

The CAC in M. methylotrophus has an OGDH lesion and is likely to have a biosynthetic role (**1.8.1.**; **3.2.2.1.**), making C_5-skeletons for synthesis of glutamate, glutamine, arginine and proline. IDH was thus studied for responses to compounds that are related to carbon supply

Figure 71

Substrate Inhibition of M. methylotrophus IDH by $NADP^+$



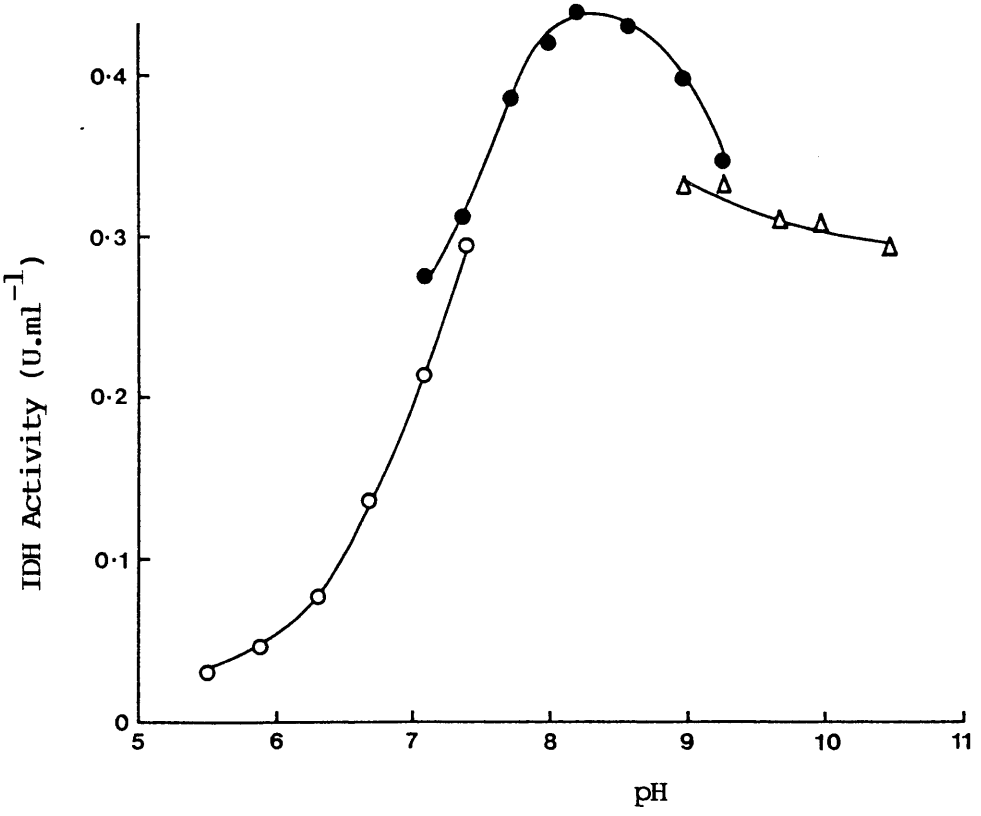
The data were obtained at $NADP^+$ concentrations between 3 and 10 mM. The above is a linear transformation of the rate equation:

$$\text{Initial velocity} = \frac{V_{max}}{1 + \frac{[NADP^+]}{K_i}}$$

The K_i value was obtained from the negative intercept on the abscissa.

Figure 72

Variation of M. methylotrophus IDH Activity with pH



All assays were carried out in the presence of 10 mM-$MgCl_2$, 2.4 mM-EDTA 4 mM-D,L-isocitrate and 0.2 mM-NAD^+

Assay buffers were: o—o : 20 mM-bis Tris

●—● : 20 mM-Tris

Δ—Δ : 20 mM-Ches

Table 31

Ability of Metal Ions to Support M. methylotrophus IDH Activity

Metal ion	Rate Relative to Mg^{2+} (%)
Mg^{2+}	100
Mn^{2+}	89.3
Co^{2+}	10.7
Zn^{2+}; Cd^{2+}; Ca^{2+} and Fe^{2+}	0

of the CAC (acetyl-CoA and CoA), indicative of energy availability (ATP, ADP AMP, NADH and NADPH) and are CAC end products (glutamate and glutamine) in M. methylotrophus.

Acetyl-CoA and CoA were practically without effect (Table 32). Of the adenine nucleotides, the potency of inhibition was ADP> ATP>> AMP; 2 mM-ADP caused nearly 60% inhibition of IDH, AMP being without effect. Increasing NAD^+ from ½ to 4½.Km did not greatly decrease the inhibition exerted by ATP or ADP. This suggested that the adenine nucleotides do not exert competitive inhibition with respect to NAD^+.

The effects of NADH and NADPH were also examined. Under standard assay conditions (0.2 mM-NAD^+, 4 mM-D,L-isocitrate), a low level of inhibition was exerted by 0.1 mM-NADH (9%) and 0.1 mM-NADPH (18.4%).

The effects of glutamate and glutamine (in crude extracts) on IDH were studied. IDH was only marginally inhibited by (% inhibition) 2 mM-D,L-glutamate (15.7%) and 2 mM-D,L-glutamine (20.3%).

5.1.5. Serological Relationships of M. methylotrophus IDH

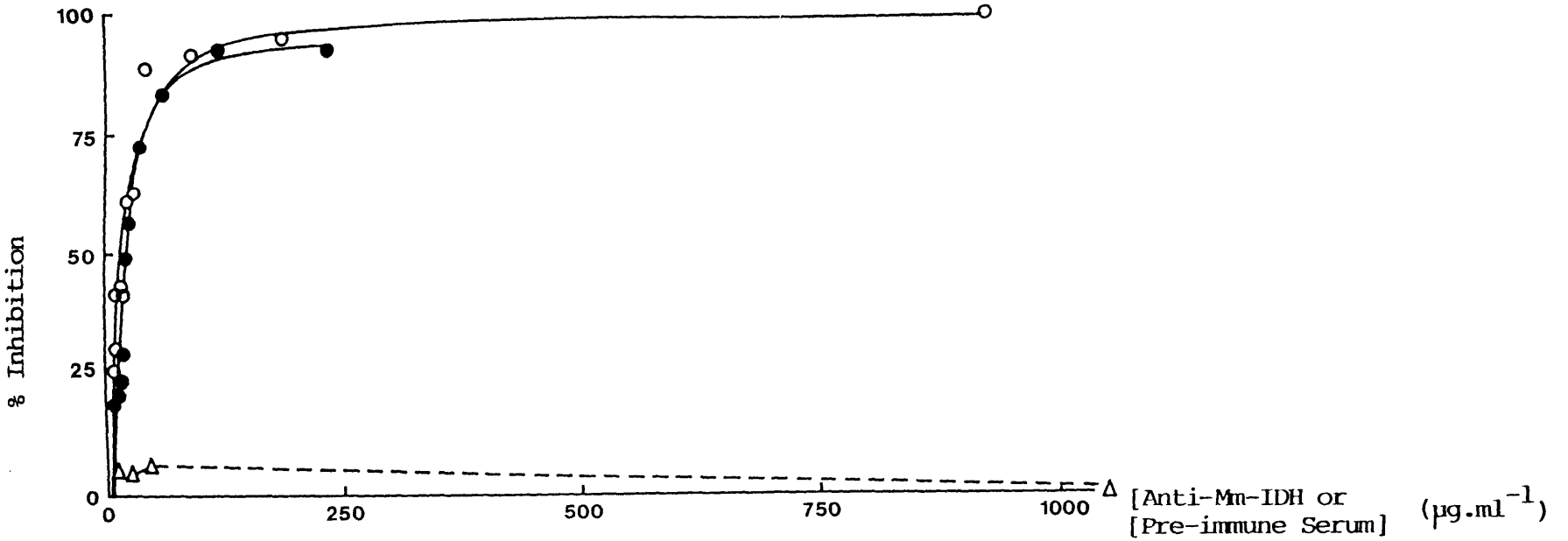
The effects of rabbit antiserum raised against M. methylotrophus IDH on the activity of other IDHs was used to examine the relatedness of the methylotrophic IDH to other IDHs. To achieve this, it was necessary to examine the effect of the antiserum on the activity of the antigen (i.e. M. methylotrophus IDH) against which it was raised.

Pure IDH and pre-immune serum were incubated in the cuvette at 25°C for 10 min. Isocitrate and NAD^+ were then added and the rate was followed. Under these conditions, activity was unchanged. When the experiment was repeated with antiserum (anti-M.mIDH), IDH was almost completely inhibited by 0.93 $mg.ml^{-1}$ of anti-Mm.IDH (Figure 73), a half-maximal effect being exerted by 20-30 $\mu g.ml^{-1}$. If IDH in crude extract prepared in MET-8.0 was used instead of pure enzyme, the effect of the antiserum was identical (Figure 73). This suggested that purification of M. methylotrophus IDH, (particularly the heat step) had not drastically altered the structure of the enzyme.

The effect of anti-M.mIDH on NADP-IDHs from E. coli, Bacillus megaterium, Pseudomonas aeruginosa, A. calcoaceticus, porcine heart, the dual-coenzyme specific IDH from Sulpholobus acidocaldarius, the

Figure 73

Inhibition of M. methylotrophus IDH by Anti-Mm-IDH Antiserum



●—●: Inhibition of IDH in crude extract prepared in MET-8.0

○—○: Inhibition of purified, electrophoretically homogeneous IDH

Δ—Δ: Effect of pre-immune serum (dashed line) on the activity of purified, electrophoretically homogeneous IDH

Table 32

The Effects of Nucleotides and Related Metabolites on IDH Activity

Effector	NAD^+ Concentration* relative to K_m for NAD^+	Effector Assay Concentration (mM)	% Inhibition
ATP	$\frac{1}{2}.K_m$	2.0	30.3
	K_m	2.0	33.1
	$4\frac{1}{2}.K_m$	2.0	39.0
ADP	$\frac{1}{2}K_m$	2.0	46.2
	K_m	2.0	49.7
	$4\frac{1}{2}.K_m$	2.0	59.9
AMP	$\frac{1}{2}.K_m$	2.0	0.0
CoA	$\frac{1}{2}.K_m$	2.0	5.0
Acetyl-CoA	$\frac{1}{2}.K_m$	2.0	0.0

*: Actual NAD^+ concentrations were: $\frac{1}{2}.K_m$: $[NAD^+]$ = 23 μM
K_m: $[NAD^+]$ = 45 μM
$4\frac{1}{2}.K_m$: $[NAD^+]$ = 180 μM

NAD-IDH from bovine heart mitochondria and the M. methylotrophus NADP-IDH activity was next investigated.

All bacteria were extracted into MET-8.0, except Bacillus megaterium which was extracted into MET-8.0 + 20% (v/v) glycerol. Extracts of A. calcoaceticus were fractionated according to O'Neil and Weitzman (1988) to separate isoenzymes I and II (**1.13.7.**). The porcine NADP-IDH was supplied by Sigma. Bovine NAD-IDH was from a supernatant of an extract of mitochondria prepared as described in **2.2.3.**

With the exception of the bovine NAD-IDH, enzyme was incubated in the cuvette with anti-M.mIDH and assayed. Bovine NAD-IDH was treated in the same way except that the buffer was 50 mM-Mops and 10 mM-$MgCl_2$ adjusted to pH 7.0. It was shown that the effects of the anti-M.mIDH on M. methylotrophus IDH were the same in this medium (Figure 74).

Anti-M.mIDH failed to exert more than 5% inhibition over any of the NADP-IDHs or the bovine mitochondrial NAD-IDH tested (Figure 74). The results with the dual-specific enzyme from Sulpholobus also suggested its NADP-IDH activity was not immuno-reactive towards anti-M.mIDH; however, there was a slight effect on its NAD-IDH activity amounting to, at most, 15% inhibition. However, the pre-immune serum at 0.10 $mg.ml^{-1}$ also exerted a small (14.7%) inhibition of NAD-IDH activity. This makes the results obtained with the Sulpholobus dual-specific IDH difficult to interpret.

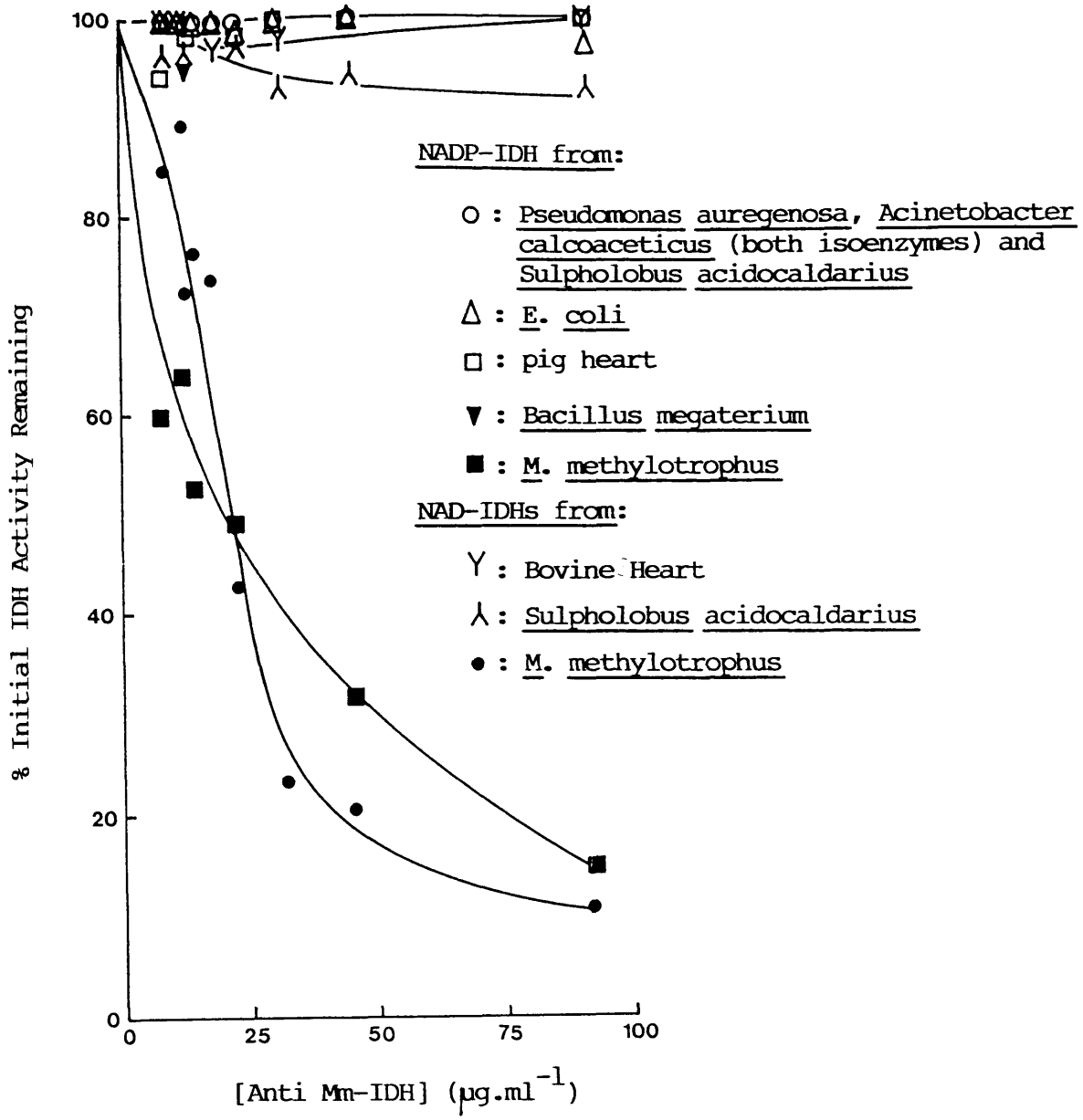
The NADP-IDH in extracts of M. methylotrophus was inhibited by anti-M.mIDH. Its response to anti-M.mIDH was similar to that of the NAD-linked activity (Figure 74). The pre-immune serum at 0.10 $mg.ml^{-1}$ did not inhibit NADP-IDH, so the effect was due to inhibition by anti-M.mIDH. This showed that, immunologically, a strong similarity existed between the NADP- and NAD-linked activities in M. methylotrophus. This and the ability of the pure IDH to use both NAD^+ and $NADP^+$ may suggest that a single IDH uses both coenzymes in crude extracts (**3.2.2.1.**).

5.1.6. Chemical Modification of M. methylotrophus IDH.

Although bacterial NADP-IDH is sensitive to thiol-directed reagents (**1.13.6.1.**), the bacterial NAD-IDHs have not been studied in this regard. Thus, the effect of thiol-directed reagents on M.

Figure 74

A Survey of the Sensitivities of IDHs of varying Coenzyme Preferences from Various Sources to Inhibition by Anti-M.m-IDH



M. methylotrophus NAD-linked IDH activity (purified enzyme) and bovine heart NAD-IDH (mitochondrial extract) were incubated with anti-M.m-IDH in 50 mM-Mops, 10 mM $MgCl_2$ (pH 7.0). Porcine heart NADP-IDH (ammonium sulphate suspension from Sigma) was incubated in MET-8.0 with anti-M.m-IDH. All other activities were in crude extracts and were incubated with anti-M.m-IDH in MET-8.0.

methylotrophus IDH was examined.

In all the experiments, unless otherwise specified, the purified IDH was desalted into MET-8.0 and was incubated at 0.24 $mg.ml^{-1}$ (2.6 μM with respect to dimer) with 0.2 mM-DTNB in 1 ml for 60 min at 25°C. 20 μl aliquots were withdrawn for assay of activity. The reaction between IDH thiol groups and DTNB was followed continuously at 412 nm.

5.1.6.1. The Effect of DTNB on IDH in MET-8.0 Alone.

5.1.6.1.1. The Effect on IDH activity. Incubation as above caused a loss of 86% of the activity. The activity without DTNB was completely stable over the same time period (Figure 75). A semi-logarithmic plot of the data suggested that inactivation was probably caused by more than one kinetically distinct process (Figure 76a). In the simplest case, there are two such processes, comprising a fast phase of inactivation (a) followed by a relatively slower phase (b).

5.1.6.1.2. Modification of Thiol Groups. After 1 h, 2.02 ± 0.12 (± S.E.M., n = 3) thiols per dimer reacted with DTNB (Figure 75) but the reaction had not reached completion. However, it is possible to estimate the final extent of the reaction at infinite time. According to Freedman and Radda (1968), if at times t_a, t_b and t_c where:

$$t_b - t_a = t_c - t_b$$

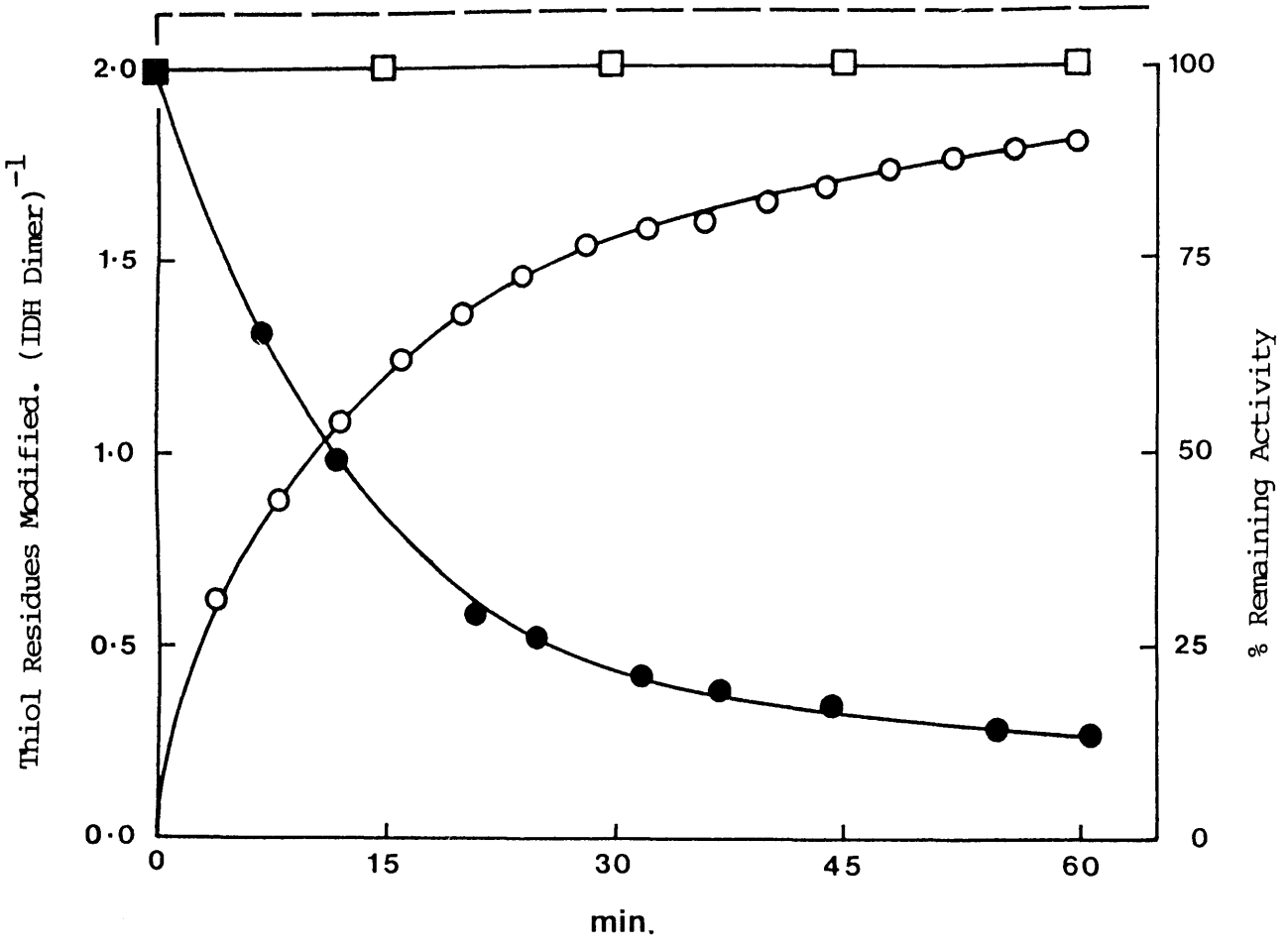
the increases in modification are A, B and C respectively, then the modification at infinite time (Z) is given by:

$$Z = \frac{B^2-(AC)}{2B-(A+C)}$$

Freedman and Radda (1968) found the increment of time (t_b-t_a) giving the most consistent value of Z was in the region of the half-life of the reaction. In this instance, the increases in thiol modification were estimated over 12 min every 4 min (i.e. t_b-t_a = 12 min where

Figure 75

Correlation Between Inactivation of IDH and Modification of Thiol Residues by DTNB



——— : Number of thiol groups modified at infinite time

○—○ : Thiol residues modified per dimer of IDH by DTNB

●—● : Activity remaining after incubation in the presence of DTNB

□—□ : Activity remaining after incubation in MET-8.0 alone

Concentrations of IDH dimer and DTNB were 2.6 μM and 0.2 mM respectively

Legend to Figure 76

Figure 76(a)

For graphs i and ii, points are experimental data and the lines are theoretically generated by substitution of time values into the equations:

Modification (line i)

$$\ln [\% \text{ Remaining Thiol}] = \ln [61e^{-0.108t} + 39.7e^{-0.014t}]$$

Inactivation (line ii)

$$\ln [\% \text{ Remaining Activity}] = \ln [68.7e^{-0.117t} + 35.1e^{-0.016t}]$$

Figure 76(b)

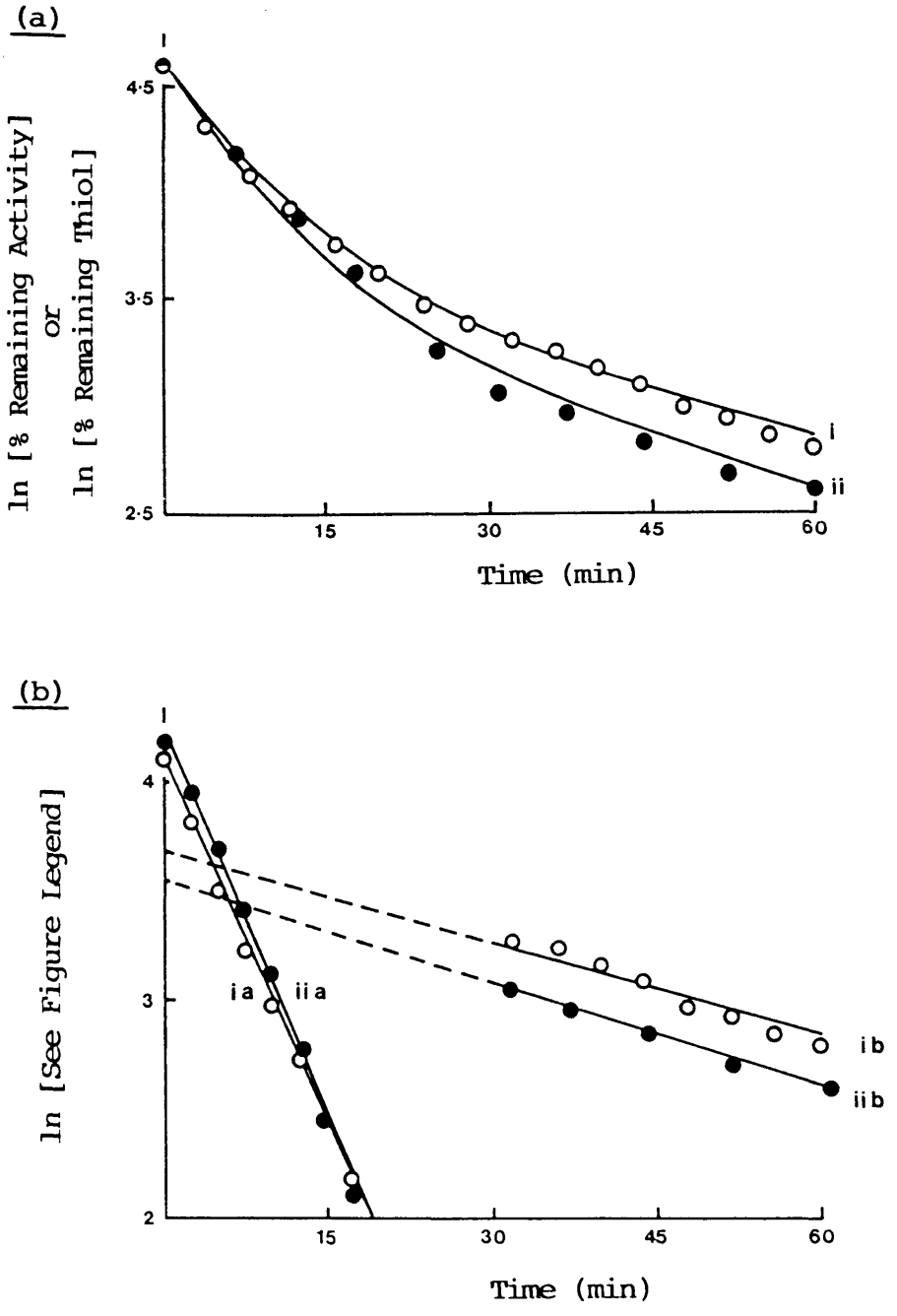
For ib, data corresponds to last 7 points of line i in Figure 76(a). For ii b, data corresponds to the last five points of line ii in Figure 76(a). For lines ib and iib, the ordinates are respectively; ln [% Remaining thiol] and In [% Remaining Activity]. The constants derived from these lines are B_o^m and $k_{(b)}^m$ from line i b and B_o^i and $k_{(b)}^i$ from line iib.

For line ia, the ordinate is ln [% Total Modification - % Modification due to Slow Phase]. For line iia, the ordinate is ln [% Total Inactivation - % Inactivation in Slow Phase]. Thus, values were taken at certain times from the extrapolated portions of lines ib and iib (dashed line), antilogged, and subtracted from the antilogarithms of values obtained at equivalent times from the data in Figure 76(a). To do this, an initial fit was made to the data in Figure (76a) by eye. The ln [result] was then plotted vs. time.

$k_{(a)}^m$ and A_o^m were obtained from line ia and $k_{(a)}^i$ and A_o^i were obtained from line iia. With these values and $k_{(b)}^{i \& m}$ and $B_o^{i \& m}$, the equations, to which the lines in 76a were fitted, were generated.

Figure 76

Analysis of Inactivation and Modification of M. methylotrophus IDH by DTNB



(a) Loss of (i) free thiol residues with time (open symbols)
(ii) IDH activity with time (closed symbols)

(b) Determination of rate constants of modification (open symbols) and inactivation (closed symbols) of IDH by DTNB

For further details see adjacent legend.

$t_{a(i-n)}$ = 28 min, 32 min,....; $t_{b(i-n)}$ = 40 min, 44 min,.... and $t_{c(i-n)}$ = 52 min, 56 min, ... etc.). This calculation is only valid for curves composed of one exponential (Freedman and Radda, 1968). If the modification is described by 2 or more exponentials, then A, B and C should be estimated as near to the end of the measurement as was possible, as here the process was most likely to be described by a single exponential. By this method, 4 estimates of Z were obtained for each modification for which the average was calculated. From this, the number of thiol residues modified over three time-courses at infinite time was 2.14 $\pm$ 0.18 ($\pm$ S.E.M., n = 3) thiol residues per dimer. When this value was used as the 100% value, and the data plotted as ln[% remaining free thiol] vs. time (Figure 76a), it was found that, like the DTNB-inactivation of IDH, the modification of thiol residues also involved more than one kinetically distinct process.

5.1.6.2. Titration of Thiol Groups Under Denaturing Conditions. IDH was desalted into 6 M-urea in MET-8.0. The enzyme (1.2 μM with respect to [dimer]) was incubated for 15 h at 25°C in the dark with 0.2 mM-DTNB. 1.86 thiols per dimer (n = 2) were detected.

It thus appears that each IDH dimer has two free thiols under both native and denaturing conditions.

5.1.6.3. Treatment of Data. Thus far it has been assumed that the reaction of IDH with DTNB is a pseudo-first-order process. This is probably true as it can be shown from **5.1.6.1.2.** that at the end of modification only 3% of the DTNB would have been consumed. Thus, the DTNB concentration was effectively a constant.

The simplest explanation of the data in Figure 76a is that the reaction with DTNB comprised a fast phase (a) and a slow phase (b). As (a) becomes small relative to (b), there is a degree of curvature leading to (b). In the analysis below, it is assumed that the reaction has been followed until the decline in free thiol or activity is due to the slowest process. Certainly for the inactivation of IDH, the validity of this assumption is, admittedly, debatable. This is because the slow process only occurs at low activity (Figure 76a) which reduces the amount and accuracy of the data that can be collected.

Thus any further phases of inactivation are not easily monitored.

If the assumptions are correct, the relationship of % remaining activity (R.A) or remaining unmodified thiol (-SH) and time is:

(I) $$\ln[\%\ \text{R.A.}\ /\ \text{-SH}] = \ln[A_o e^{-k(a)t} + B_o e^{-k(b)t}]$$

where $k_{(a)}$ and $k_{(b)}$ would be pseudo-first-order rate constants for phases (a) and (b) of inactivation / modification (Frost and Pearson, 1961). A_o and B_o would be the proportions of IDH activity or thiol at zero time that were subject to loss during (a) and (b) respectively.

If, at the end of the time-courses in Figure 76a, phase (a) was over, the expression for ln[% R.A. / -SH] becomes:

(II) $$\ln[\%\ \text{R.A.}\ /\ \text{-SH}] = -k_{(b)}t + \ln[B_o]$$

and thus $k_{(b)}$ can be obtained from the gradient of the line at the end of the time course, whose intercept with the ordinate is B_o (Figure 76b). With this, the drop in activity or thiol residues due to (a) may be found by subtracting remaining activity or thiol content described by equation **(II)** (extrapolated line in Figure 76b) from remaining activity or thiol during (a) at various time intervals. Replotting as:

(III) $$\ln[\%\ \text{R.A.}\ /\ \text{-SH}] = -k_{(a)}t + \ln[A_o]$$

(Figure 76b) allows $k_{(a)}$ and A_o to be determined.

5.1.6.3.1. Modification of Thiol Residues of IDH by DTNB. Values of 0.108 min^{-1} and 0.014 min^{-1} were found for modification rate constants $k_{(a)}^m$ and $k_{(b)}^m$, and 61% and 39.7% for A_o^m and B_o^m respectively. $k_{(b)}^m$ was determined using a curve fitted by eye. The validity of this procedure could be tested by substitution of various values of t into the equation suggested by the above constants:

(IV) $$\ln[\%\ \text{-SH}] = \ln[61e^{-0.108t} + 39.7e^{-0.014t}]$$

This generated the theoretical relationship between ln[%-SH] and t. It

can be concluded that the experimental data are described by equation **(IV)** or one very like it, for the line (i) fitted to the modification data in Figure 76a is, in fact, the theoretically calculated line.

5.1.6.3.2. Inactivation of IDH by DTNB. The points representing loss of activity lie on a similar line to that described by the results of thiol modification (Figure 76a). If the data are analysed like the modification data, values of the inactivation rate constants $k_{(a)}{}^{i}$ and $k_{(b)}{}^{i}$ of 0.117 min^{-1} and 0.016 min^{-1} are found. $A_o{}^{i}$ and $B_o{}^{i}$ were 68.7 and 35.1% respectively. Again, these values were based on curves fitted by eye, so the line corresponding to the equation:

(V) $$\ln[\% \text{ R.A}] = \ln[68.7e^{-0.117t} + 35.1e^{-0.016t}]$$

was generated. Line (ii) in Figure 76a is, in fact, generated from this equation. Although not as precise a fit as that generated for the modification data, it does seem reasonable to propose that the data can be described by **(V)** or a very similar equation.

Thus as the IDH dimer has two thiols and as IDH inactivation parallels modification (Figure 76a), it may be the thiols are in or near the active site. Finally, the thiols are kinetically distinct.

5.1.6.4. Effects of Iodoacetamide on IDH Activity. Two issues arose that made the effects of iodoacetamide interesting to pursue:
A) DTNB-inactivation may have resulted either from modification of a catalytically crucial thiol residue and/or binding, or, because the introduction of TNB into vicinity of the active site sterically hindered binding of isocitrate or NAD^{+}. Thus, if modification with a reagent that introduced a smaller group did not inactivate IDH, then DTNB-inactivation may have been the result of steric hindrance of entrance and departure of substrates or products.
B) The biphasic kinetics of reaction of DTNB with IDH may not have resulted from the differential reactivity of two thiols. Thus, 1) modification of one thiol by DTNB may have slowed down modification of the other due to the presence of a bulky TNB group, or 2) modification of one thiol may have been followed by a slower attack by the other

thiol on the mixed disulphide formed with TNB. This would cause the formation of a disulphide bridge on IDH. Both 1) and 2) could be eliminated if it could be shown that inactivation and/or modification by iodoacetamide occurred in a manner similar to that shown by DTNB.

IDH was incubated under the conditions used above, except 2 mM-iodoacetamide replaced DTNB. No activity was lost in this incubation (Figure 77), so neither mechanism in B) could be eliminated. However, the results suggested that of the scenarios in A), steric hindrance may explain DTNB-inactivation. To confirm that IDH was modified by iodoacetamide, 0.2 mM-DTNB was added to iodoacetamide-treated IDH. Disappointingly, DTNB still inactivated IDH (Figure 77). Thus, the experiments conducted with iodoacetamide were inconclusive.

5.1.6.5. Effect of Substrates and Coenzymes on DTNB Inactivation. The effects of 1 mM-2-OG, 1 mM-NADH, 1 mM-NAD^+, 2 mM-D,L-isocitrate, 1mM-NAD^+ + 1 mM-2-OG (NAD^+ + 2-OG) and 1 mM-NADH + 2 mM-D,L-isocitrate on the inactivation of M. methylotrophus IDH by DTNB were examined:

The enzyme was incubated as previously, and the resulting data are plotted (Figures 78 and 79) as ln[% R.A] vs. time. The following features are immediately apparent:

1) IDH was protected from DTNB-inactivation by isocitrate (Figure 78).
2) NADH enhanced isocitrate protection and vice versa (Figure 78).
3) NAD^+ appeared to sensitise IDH to DTNB inactivation (Figure 79) and linearised the kinetics of inactivation.
4) Over the incubation, NADH with or without isocitrate and NAD^+ + 2-OG in combination linearised inactivation (Figure 78 and 79). Thus, NADH and NAD^+ + 2-OG increased the rate of phase (b) and NADH + isocitrate decreased the rate of phase (a).
5) 2-OG had little effect on its own, but slowed the inactivation of IDH in the presence of NAD^+ (Figure 79).
6) NAD^+ linearised inactivation in the presence of 2-OG (Figure 79).

The replots of data used to determine $k_{(a)}{}^i$ for the reactions of IDH with DTNB in the presence of 2-OG or isocitrate are shown in Figure 80. Values for $k(a)^i$ and $k_{(b)}{}^i$ are consistent with the above (1-6), e.g. NADH and NAD^+ + 2-OG linearised the inactivation of IDH by stimulation of phase (b) (2.8-fold in both cases), and by reducing the

Figure 77

The Effect of Sequential Addition of Iodoacetamide and DTNB on M. methylotrophus IDH Activity

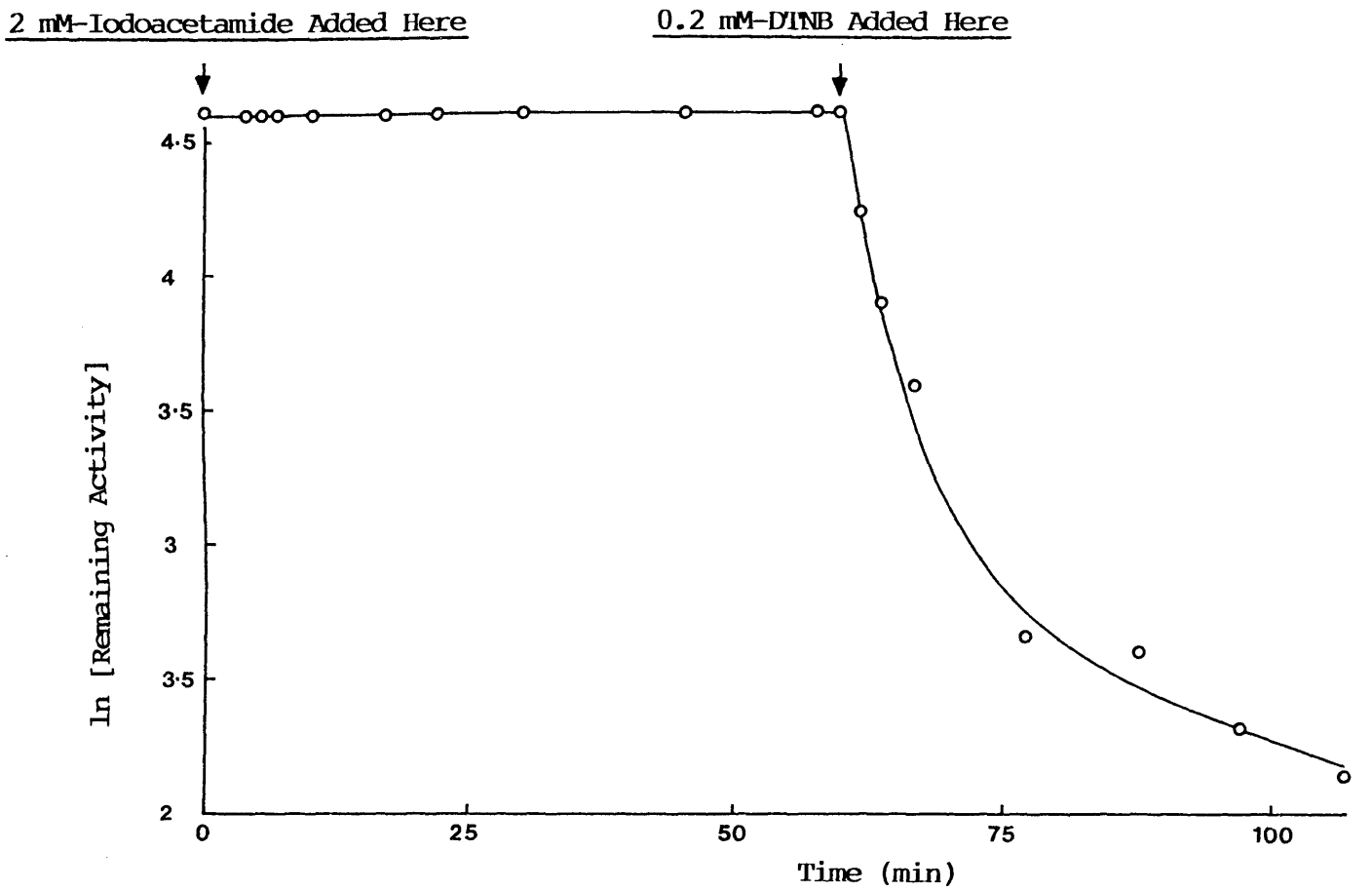


Figure 78

Effect of Isocitrate, NADH and a Mixture of NADH and Isocitrate on the DTNB-Inactivation of M. methylotrophus IDH

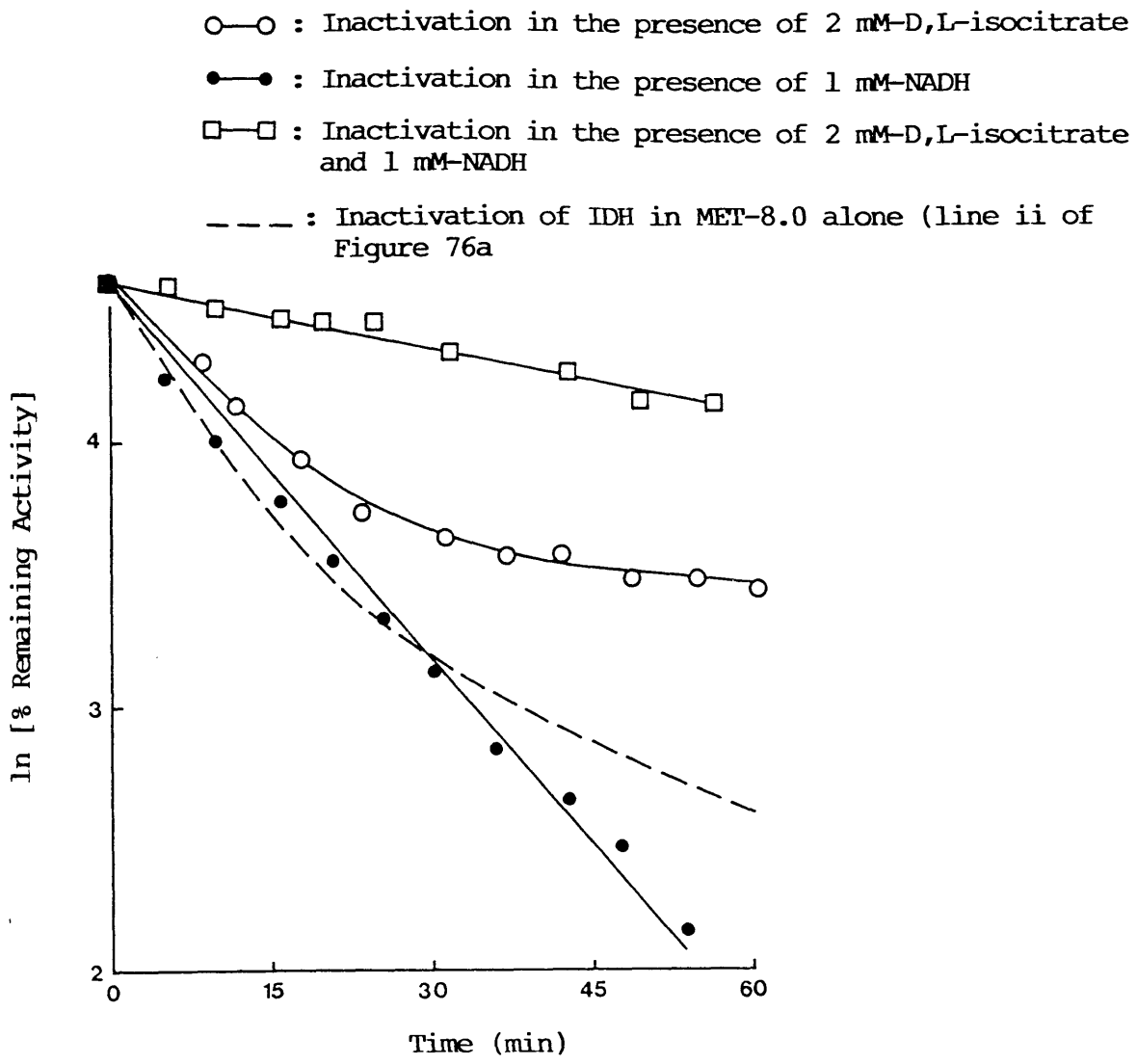


Figure 79

Effect of NAD^+, 2-OG and a Mixture of NAD^+ and 2-OG on DTNB-Inactivation of M. methylotrophus IDH

– – – – : Inactivation of IDH in MET-8.0 alone (line ii Figure 76a

o—o : Inactivation in the presence of 1 mM-2-OG

△—△ : Inactivation in the presence of 1 mM-NAD^+

□—□ : Inactivation in the presence of 1 mM-NAD^+ and 1 mM-2-OG

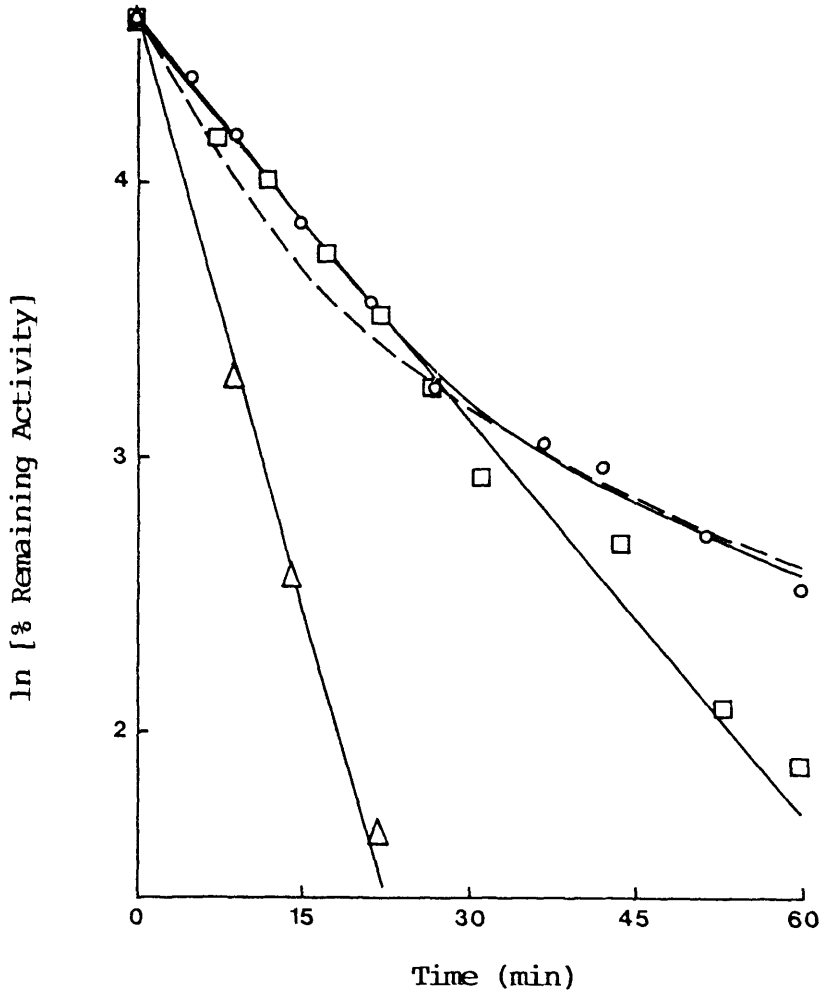
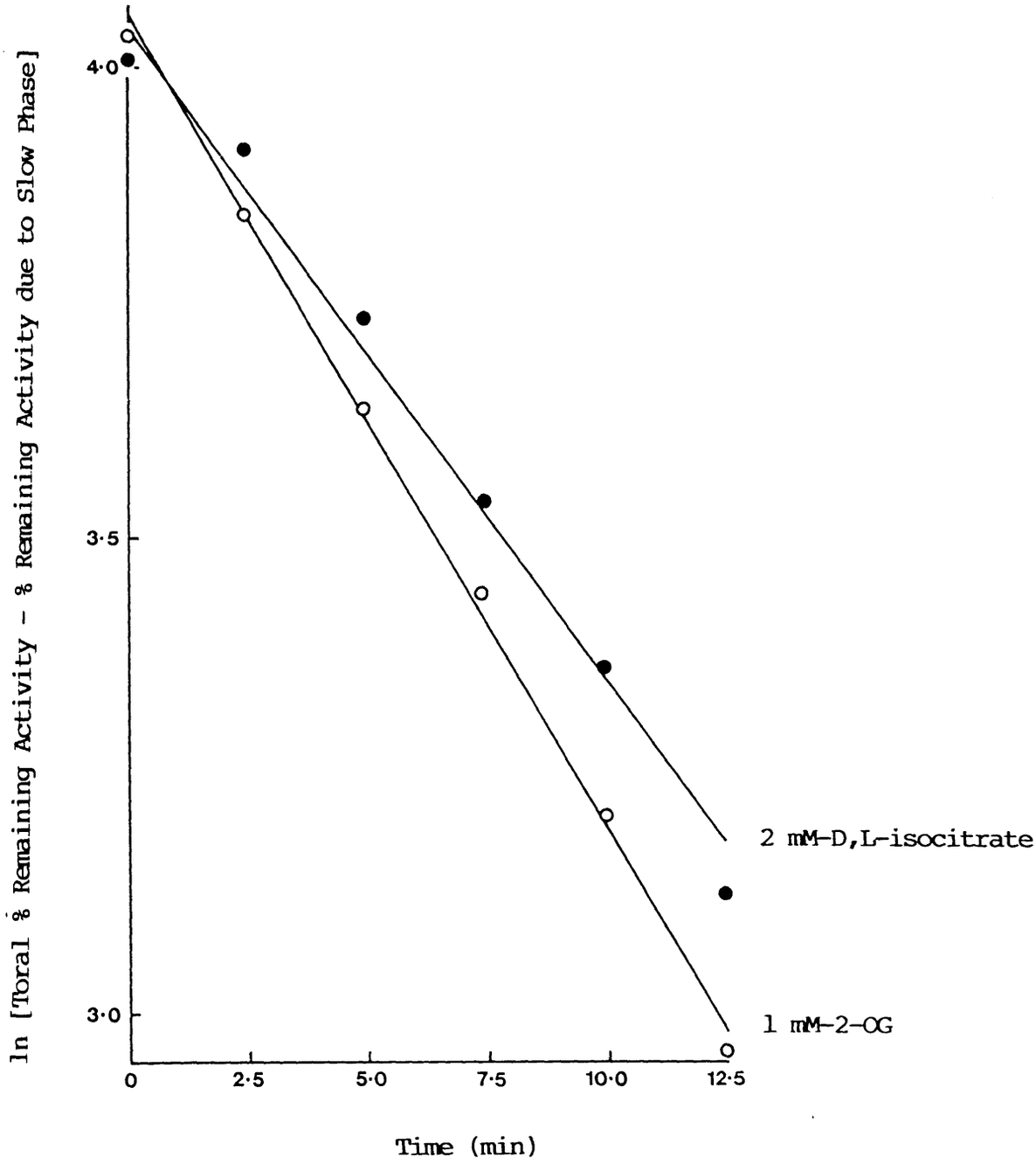


Figure 80

Inactivation of M. methylotrophus IDH by DTNB in the Presence of Isocitrate and 2-OG: Fast Phase (Phase a) of Inactivation



$k_{(a)}{}^{i}$ was found in both cases from the gradients of the above lines.

rate of phase (a) (2.7-fold in both cases, see Table 33).

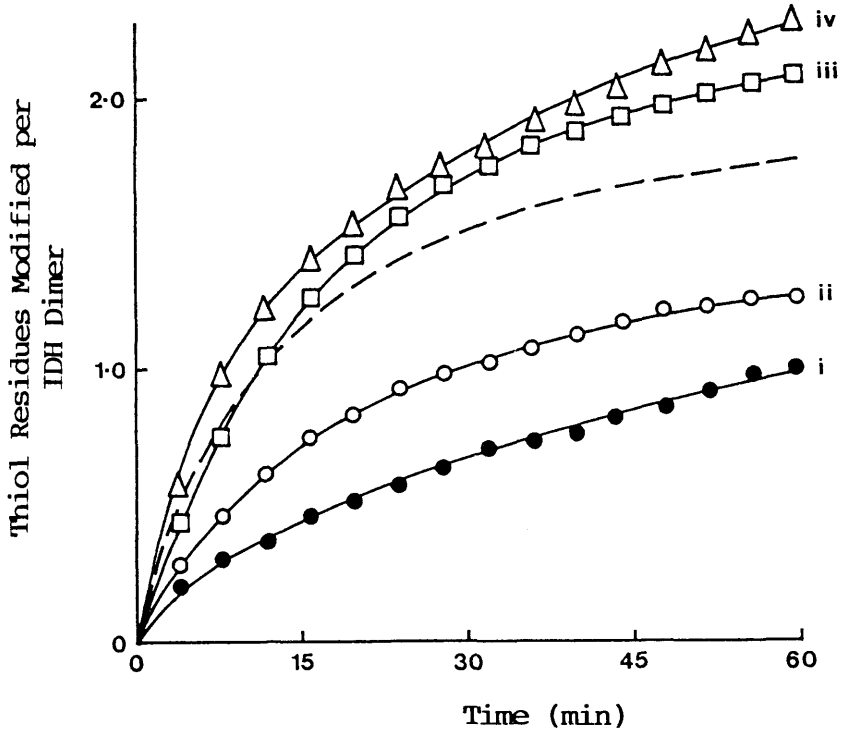
5.1.6.6. Effect of Substrates and Coenzymes on DTNB-Modification.
The effects of 2 mM-D,L-isocitrate, 1 mM-NAD^+ and mixtures of 1 mM-NAD^+ + 1 mM-2-OG and 2 mM-D,L-isocitrate + 1 mM-NADH on modification were studied. Isocitrate and its combination with NADH attenuated modification (Figure 81). This mirrors the ability of these compounds to protect activity. Titration of IDH thiols in the presence of NAD^+ with or without 2-OG led to an accumulation of more TNB than would be predicted from the titrations carried out in urea (Figure 81; **5.1.6.2.**). The cause of this is unknown. It does, however, mean that the data obtained with these ligands must be interpreted with care.

The modification data were plotted semi-logarithmically (Figure 82). Values of % remaining thiol for data obtained with isocitrate and its combination with NADH were calculated using the number of thiols modified at infinite time in MET-8.0 alone (**5.1.6.1.2.**) as the 100% value. This assumes that if IDH is protected against reaction with DTNB by a ligand, the number of thiols that react is not altered by that ligand. It is also assumed that an equilibrium between the enzyme (E), its ligand (lig) and the Elig complex exists and DTNB only reacts with E as the thiol residues are otherwise inaccessible in Elig. The reaction with DTNB is irreversible, so the equilibrium E + lig / Elig is continually displaced to E. Thus all thiols on E will eventually react with DTNB. This situation may account for protection of IDH where lig = isocitrate or lig = (NADH + isocitrate). For data obtained with NAD^+ or its combination with 2-OG, infinite time values were obtained from Figure 81. These values were 2.47 and 2.21 thiols per subunit respectively. Again it should be stressed that here, the discrepancy between the number of thiols detected in the presence of NAD^+ $\pm$ 2-OG and those present in IDH in MET-8.0 alone (c.f. **5.1.6.1-2.**) makes interpretation difficult.

Probably all the plots in Figure 82 are curved. This contrasts with inactivation in the presence of NAD^+ + 2-OG or NADH + isocitrate. However, the curvature is probably small enough in these instances (Figure 82) that if it occurred in inactivation, it would have been obscured by the scatter of the data (Figures 78, 79). Thus, the IDH

Figure 81

Time Courses of Modification of M. methylotrophus IDH by DTNB in the Presence of Substrates and Coenzymes



Curve i: Modification of IDH in the presence of NADH and D,L-isocitrate

Curve ii: Modification of IDH in the presence of D,L-isocitrate

Curve iii: Modification of IDH in the presence of 2-OG and NAD^+

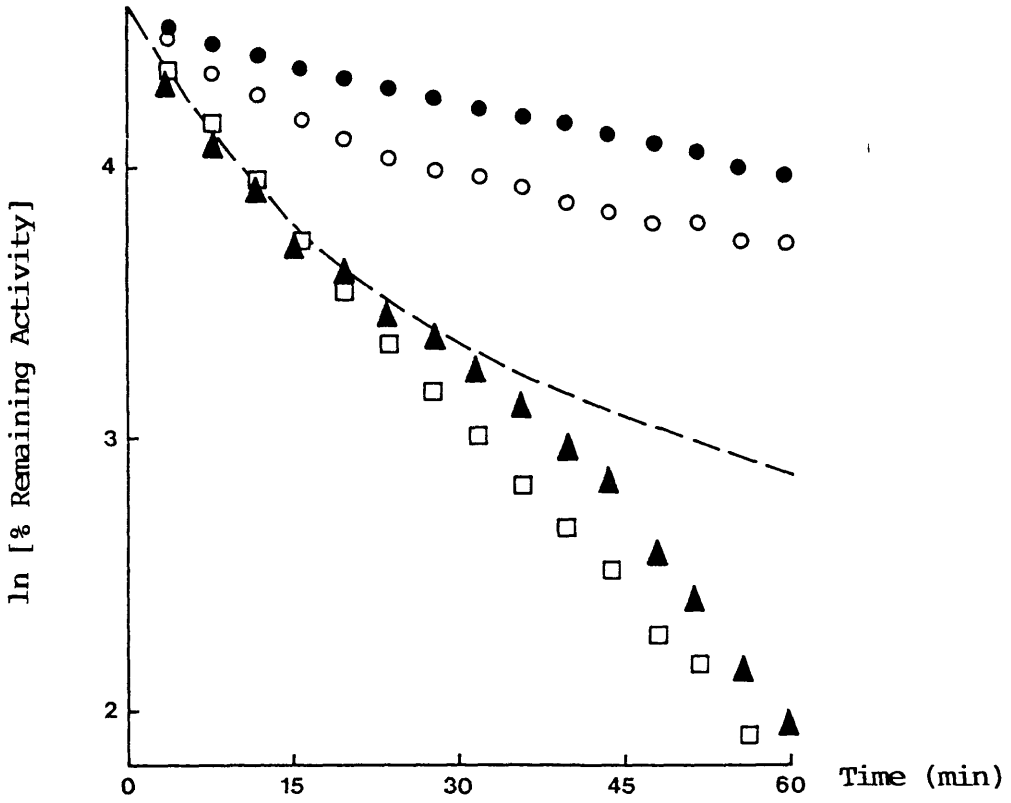
Curve iv: Modification of IDH in the presence of NAD^+

The dashed line is the time course of modification in the absence of coenzyme or substrate (i.e. from Figure 75).

NAD^+, NADH and 2-OG were present at 1 mM. D,L-isocitrate was present at 2 mM.

Figure 82

Semi-Logarithmic Plots of DTNB Modification of M. methylotrophus IDH Thiol Residues in the Presence and Absence of Substrates and Coenzymes



Modification of IDH thiol residues in the presence of

● : NADH and isocitrate

o : isocitrate

▲ : NAD^+

□ : NAD^+ and 2-OG

Dashed line (- - - -) represents the modification of the enzyme in the absence of coenzymes or substrates and is taken from Figure 76a.

NAD^+, NADH and 2-OG were present at 1 mM. D,L-isocitrate was present at 2 mM.

Table 33

Pseudo-First-Order Rate Constants of Inactivation of M. methylotrophus IDH by DTNB in the Presence of Substrates and Coenzymes

Addition	Monophasic Inactivation	Biphasic Inactivation	
	k (min^{-1})	$k_{(a)}^{i}$ (min^{-1})	$k_{(b)}^{i}$ (min^{-1})
None	-	0.117	0.016
2 mM-D,L-isocitrate	-	0.077	0.005
1 mM-2-OG	-	0.094	0.021
1 mM-NAD^+	0.142	-	-
1 mM-NADH	0.044	-	-
1 mM-NAD^+ + 1 mM-2-OG	0.044	-	-
2 mM-D,L-isocitrate + 1 mM-NADH	0.008	-	-

modification in the presence of NAD^+ + 2-OG, NADH + isocitrate or NAD^+ may be described approximately by one constant. Thus, constants for modification in the presence of NAD^+ + 2-OG, NAD^+ and NADH + isocitrate were found from the last 30 min of reaction and were assumed to be estimates of $k_{(b)}{}^m$ and $k_{(a)}{}^m$. For the data obtained with isocitrate, $k_{(a)}{}^m$ and $k_{(b)}{}^m$ were obtained as in **5.1.6.3.** (Figure 83).

With the exception of NAD^+, generally, values of $k_{(a)}{}^m$ and $k_{(b)}{}^m$ corresponded to those of $k_{(a)}{}^i$ and $k_{(b)}{}^i$ (Table 34, Table 33). Despite the scatter of the data obtained in the presence of NAD^+ (Figure 82), it seems that here, modification occurred at a similar rate to that in the presence of NAD^+ + 2-OG. Figure 79 shows that this was not the case with inactivation, where the inactivation rate in the presence of NAD^+ far exceeded that in the presence of NAD^+ + 2-OG. This may show that the enhanced inactivation rate caused by NAD^+ was not all due to increased rate of reaction of thiols, but was due to a secondary effect caused by the modification. Whatever this was, it could be eliminated by 2-OG. However, if the semi log plots were constructed using the the number of thiols modified at infinite time in MET-8.0 alone as the 100% value for the modification data obtained with NAD^+ $\pm$ 2-OG, the apparant rate constants for modification would more closley resemble those for inactivation. Thus, because of these difficulties in interpretation, detailed comment on the nature and effects of NAD^+ on the reaction of DTNB with IDH is, perhaps, premature.

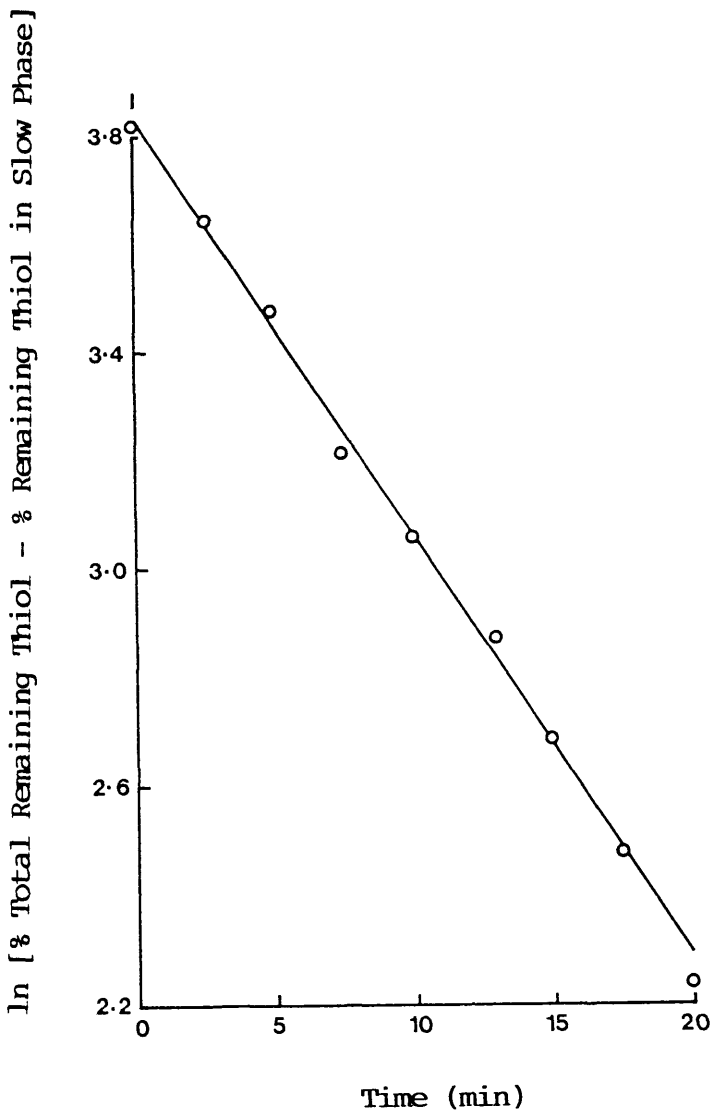
5.2. Discussion

5.2.1. Thermal Stability of *M. methylotrophus* IDH

At physiological temperatures, the native state of a protein is far from static; there are, for example, concerted motions within an enzyme that are connected with its catalytic activity. Nevertheless, there are limits to the conformations that can be adopted that are set by a complex balance of intramolecular non-covalent interactions such as hydrophobic, ionic and van der Waals interactions and hydrogen bonds. At increased temperatures that vary with the enzyme, the native

Figure 83

Modification of M. methylotrophus IDH by DTNB in the Presence of Isocitrate During the Fast Phase (Phase a) of Modification



$k_{(a)}^{m}$ was determined from the gradient of the above plot. The D,L-isocitrate concentration was 2 mM.

Table 34

Pseudo-First-Order Rate Constants of Modification of M. methylotrophus IDH by DTNB in the Presence of Substrates and Coenzymes

Addition	Rate Constant of Fast Phase $k_{(a)}^{m}$ (min^{-1})	Rate Constant of Slow Phase $k_{(b)}^{m}$ (min^{-1})
No Addition (MET-8.0)	0.108	0.014
+ 2 mM-D,L-Isocitrate	0.078	0.007
+ 2 mM-D,L-Isocitrate and 1 mM-NADH	0.008 (Approx.)*	0.008
+ 1 mM-NAD^+	0.039 (Approx.)	0.039
+ 1 mM-(NAD^+ + 2-OG)	0.042 (Approx.)	0.042

*: Although the modification plots of In[% -SH] with time were slightly curved, the degree of curvature was not great enough to obtain accurate estimates of both rate constants. However, because the degree of curvature was so slight it is likely that both thiol residues were behaving more or less identically. Therefore estimation of $k_{(b)}^{m}$ from the gradient of the plots during the last 30 min of reaction are given as approximate estimates of $k_{(a)}^{m}$.

structure of the protein cannot prevail against the drive towards increased entropy of the unfolded state expressed as rapid random motion, and the protein loses most of its ordered tertiary and secondary structure. This denaturation causes loss of activity by dispersal of those amino acids that make up the active site.

In addition to the above, Ahern and Klibanov (1988) have found chemical mechanisms that cause **irreversible** inactivation above 80°C. These involve loss of peptide chain integrity, loss of disulphide bonds and degradation of asparaginyl and cysteinyl residues.

Thermal inactivation of IDH in MET-8.0 alone occurs at too low a temperature and too quickly for inactivation by chemical degradation (Figures 53, 54; Ahern and Klibanov, 1988). This suggests inactivation of M. methylotrophus IDH is due to denaturation of secondary, tertiary or quaternary structure. The work of Hibino et al. (1974) on Bacillus stearothermophilus NADP-IDH supports this conclusion: Bacillus IDH at 60°C-70°C undergoes a sharp decline in alpha-helical structure. The decline in secondary structure closely parallels the loss of activity, suggesting that denaturation caused inactivation.

Isocitrate prevented thermal inactivation of M. methylotrophus IDH. In this context it is notable that the isocitrate concentration required for half-maximal protection was fifty-fold greater than the isocitrate K_m of the IDH. This disparity may have a number of causes: a) the affinity of IDH for isocitrate falls with rising temperature; b) the affinity of IDH for isocitrate is far higher in the presence of the coenzyme; c) increasing temperature causes dissociation of the Mg:isocitrate chelate and reduces the concentration of isocitrate that can bind to the enzyme (if the true substrate is the Mg^{2+}:isocitrate chelate); d) isocitrate binds in more than one way to IDH; e) isocitrate is degraded to a certain extent in extracts at 70°C.

The above suggests that stabilisation of M. methylotrophus IDH by isocitrate was due to the prevention of randomisation of the enzyme structure. With Bacillus stearothermophilus NADP-IDH this is probably true. At temperatures that would otherwise destroy any alpha-helical structure, the conformation of the IDH remained stable if isocitrate was present. This does suggest that isocitrate prevents the loss of activity by stabilising the structure of IDH at elevated temperatures.

The ability of isocitrate to prevent thermal inactivation is shared by M. methylotrophus IDH with the Bacillus stearothermophilus and Mycobacterium phlei NADP-IDHs (Dharwinal and Venkitasubramanian, 1987; Howard and Becker, 1970; Nagaoka et al., 1977) and the eukaryotic NADP-IDH from pea leaves and roots (Chen et al., 1988). It is of interest that the Bacillus NADP-IDH is protected by isocitrate only in the presence of Mg^{2+}, and this suggests that protection may be exerted by the metal chelate of isocitrate. Unfortunately the results obtained with the M. methylotrophus IDH do not bear on this point.

Thus it is likely that isocitrate, perhaps as the Mg:isocitrate chelate, binds residues at the active site of M. methylotrophus IDH which are dispersed in its primary structure, holding it in an active conformation. This suggests isocitrate binding to M. methylotrophus IDH is energetically more favourable than denaturation at temperatures that inactivate the enzyme in the absence of isocitrate. A corollary of this is that, relative to the enzyme in MET-8.0 alone, the rise in temperature required to bring about inactivation of M. methylotrophus IDH in the presence of isocitrate may be to a large extent that required to offset the binding energy of isocitrate to IDH.

5.2.2. The Interaction of M. methylotrophus IDH with Triazine Dyes

In the purification of the M. methylotrophus IDH fifteen immobilised dyes were screened. The enzyme bound to only two of these - Blue HE-RD and Green HE-4BD. Neither has been used for purification of IDH before, but at the same time as binding of M. methylotrophus IDH to Blue HE-RD was shown, it was found in Professor Weitzman's laboratory that Blue HE-RD also bound both NADP-IDH isoenzymes from A. calcoaceticus (O'Neil and Weitzman, 1988). Thus, Blue HE-RD may be useful for purification of IDHs in general. Otherwise, both dyes have been shown to bind Bacillus stearothermophilus aminoacyl-tRNA synthetases (Bruton and Atkinson, 1979) and Blue HE-RD has also been shown to bind to lactate dehydrogenase (Clonis and Lowe, 1980).

5.2.2.1. Interaction of M. methylotrophus IDH with Blue HE-RD. There is one instance of use of a triazine dye (Cibacron Blue F3GA) to

isolate (but not to homogeneity) a bacterial NAD-IDH. This is the IDH from the methylotroph organism W6 (Hofmann and Babel, 1980). However, more is known about the interaction of NADP-IDH with triazine dyes. Of these, Procion Red HE-3B and Cibacron Blue F3GA have been particularly useful. These dyes have been used to purify NADP-IDHs from Vibrio ABE-1 (isoenzyme I), pea, E. coli, Bacillus KSM-1050, Bacillus stearothermophilus and human heart (Nagaoka et al., 1977; Seelig and Colman, 1977; Vasquez and Reeves et al., 1979; Nimmo and Holms, 1980; Ishii et al., 1987; Shikata et al., 1988; Chen et al., 1988).

Of these, there is evidence for human NADP-IDH eluted from Cibacron Blue F3GA and the E. coli enzyme eluted from Red HE-3B that the dye interacts with the $NADP^+$ binding site and that binding and elution are biospecific. E. coli NADP-IDH is reversibly phosphorylated at its $NADP^+$ binding site (**1.13.8.5.**). The phospho-IDH cannot bind $NADP^+$ or Red HE-3B (Borthwick et al., 1984). For the human enzyme, Cibacron Blue is a competitive inhibitor with respect to $NADP^+$ and further, the coenzyme fragments nicotinamide mononucleotide and ATP act at the same site on the IDH as the Cibacron Blue F3GA (Seelig and Colman, 1977). Thus, this dye may bind to the complete $NADP^+$ binding site. Seelig and Colman (1977) also found hydrophobic interactions to be involved in the binding of human NADP-IDH to Cibacron Blue F3GA.

Other interactions with triazine dyes are notable. Bacillus stearothermophilus NADP-IDH was eluted from Cibacron Blue F3GA by EDTA. This showed that the metal ion was part of the dye-IDH interaction (Nagaoka et al., 1977). Isocitrate also eluted human NADP-IDH from Cibacron Blue F3GA and a 0-40 mM-citrate gradient eluted Vibrio ABE-1 isoenzyme 1 from Red HE-3B (Ishii et al., 1987; Seelig and Colman, 1977). For human NADP-IDH, large amounts of isocitrate were required (Seelig and Colman, 1977); thus it may be that ionic strength eluted the enzyme. A similar conclusion may apply to elution of Vibrio ABE-1 NADP-IDH from Red HE-3B (Ishii et al., 1987); however, as the IDH was loaded with Mg^{2+}, elution may have resulted in a similar way to elution of Bacillus stearothermophilus NADP-IDH.

Turning to binding and elution of M. methylotrophus IDH from Blue HE-RD, apart from KCl, the only effective eluant was isocitrate (Table 28). Free Blue HE-RD in solution inhibited IDH (Figure 61). This

inhibition was found to be non-linear (Figure 62). It seemed either Blue HE-RD interacted with IDH co-operatively or in more than one way. The non-linearity of Blue HE-RD-inhibition is reminiscent of ATP-inhibition of NADP-IDH when isocitrate was the variable substrate (**1.13.8.1.**). It is thus tempting to speculate that:
a) isocitrate eluted M. methylotrophus IDH by decreasing the Mg^{2+} concentration (c.f. elution of B. stearothermophilus and Vibrio ABE-1 NADP-IDHs from Cibacron Blue F3GA and Red HE-3B above).
b) If the dye acted like ATP, it may also have bound to IDH in a similar manner to that of the binding of the ATP pyrophosphate moiety to NADP-IDH. It should be pointed out that the similarity of the kinetics of Blue HE-RD-inhibition of this IDH to ATP-inhibition of NADP-IDH from other organisms ends when the inhibition data obtained at variable NAD^+ for IDH is examined; for here, the kinetics of inhibition are still non-linear (Figure 62b).

A puzzling aspect of the interaction of immobilised Blue HE-RD with IDH was the large volume of isocitrate needed to elute the enzyme (Table 29). It has been postulated that the larger the number of interactions between an immobilised ligand and a protein, the broader the width of peak of elution by that ligand (Fulton, 1980). For the interaction of Blue HE-RD with IDH this may be the case, i.e., there are several interactions with Blue HE-RD that are not connected directly with binding of isocitrate to IDH. This is consistent with the nature of the Blue HE-RD-inhibition of IDH (Figure 62).

5.2.3. Characteristics of Purified IDH from M. methylotrophus

To my knowledge, the purification of M. methylotrophus IDH is the first reported purification of a non-eukaryotic IDH that is primarily NAD-linked and the first purification of an IDH from a methylotroph.

5.2.3.1. Quaternary Structure. M. methylotrophus IDH has a quaternary structure that is markedly similar to that of the group A1 NADP-IDHs and the dual-specific enzyme from Thermus thermophilus, all of which are dimers of subunit M_r 42,000 to 57,000 (**1.13.4.**; Table 4).

5.2.3.2. Substrate and Coenzyme Dependences. M. methylotrophus IDH has hyperbolic dependences on NAD^+ and isocitrate; however, the form of isocitrate used (e.g. di- and tri-basic forms and their Mg^{2+} chelates) was not established. The data here do not allow conclusions to be drawn about the protonation state of isocitrate that is used by M. methylotrophus IDH. However, as the pH optimum for IDH activity is about 8 (Figure 72) and as the highest pKa of isocitric acid is 5.75 (Grzybowski et al., 1970), it seems likely that optimal activity is expressed with the tribasic $(isocitrate)^{3-}$ anion, although IDH might be active with other ionisation states of isocitrate.

The isocitrate and coenzyme dependences and affinities of M. methylotrophus IDH resemble those of Group A1 bacterial NADP-IDHs. These enzymes have hyperbolic dependences and affinities for isocitrate and coenzyme; K_ms usually range from 3.6 to 25 μM-D,L-isocitrate and 7 to 55 μM-$NADP^+$ (Marr and Weber, 1968; Ozaki and Shiio, 1968; Chung and Franzen, 1969, Howard and Becker, 1970; Chung and Braginski, 1972; Reeves et al., 1972; Self and Weitzman, 1972; Ramaley and Hudock, 1973; Bennett and Holms, 1975; Chandrasekharan-Nambiar and Shethna, 1976; Ochiai et al., 1979; Walsh and Koshland, 1984; Dharwinal and Venkitasubramanian, 1987; Thorness and Koshland, 1987; Shikata et al., 1988). Further, like M. methylotrophus IDH, the Rhodopseudomonas spheroides NADP-IDH has no co-operative interactions towards either $NADP^+$ or isocitrate (Chung and Braginski, 1972).

Curiously, the resemblance of isocitrate and coenzyme dependences of M. methylotrophus IDH to those of the prokaryotic NADP-IDHs is not shared by the bacterial NAD-IDHs. Although these IDHs have hyperbolic dependences on NAD^+ and isocitrate, apart from Thiobacillus thio-oxidans and Organism W6 NAD-IDH, the D,L-isocitrate K_m of Rhizobium CC829, Acetobacter peroxydans and Alcaligenes eutropha NAD-IDHs are 100-fold greater than that of M. methylotrophus IDH (Hathaway and Atkinson, 1963; Glaeser and Schlegel, 1972a; Moustafa and Leong, 1975). Similarly, the M. methylotrophus IDH NAD^+ K_m is low compared with values for W6, Thiobacillus, Rhizobium and Alcaligenes NAD-IDHs.

M. methylotrophus IDH could also use $NADP^+$ as well as NAD^+, and therefore difered from the methylotrophic Organism W6 which had separate NAD- and NADP-linked IDHs (Hofmann and Babel, 1980).

M. methylotrophus IDH is more specific about coenzyme preference than the Sulpholobus acidocaldarius or Rhodomicrobium vanielli dual-specific IDHs and less specific than the dual-specific Thermus thermophilus IDH. The coenzyme specificity of M. methylotrophus IDH is also unique as it is the only dual-specific IDH that is preferentially NAD-linked (Danson and Wood, 1984; Leyland et al., 1989; Eguchi et al., 1989). As is the case with the predominantly NADP-linked dual-specific IDHs, it is unlikely that the least preferred coenzyme of the M. methylotrophus IDH (i.e. $NADP^+$) will be used in vivo. This is because it is unlikely that $NADP^+$ could accumulate to such an extent that it could provide an alternative to NAD^+.

It is also unlikely that the 'substrate' inhibition shown by $NADP^+$ is relevant in vivo because of the levels of $NADP^+$ required to bring it about. Substrate inhibition by $NADP^+$ is also shown by the Thiobacillus novellus NADP-IDH (Charles, 1970). Where enzymes catalyse reactions involving multiple interactions between the active site and a ligand like $NADP^+$, inhibition may result from the ability of two (or more) sites on the protein each to bind a molecule of $NADP^+$ via the normal points of attachment of the coenzyme. This may result in an inactive ES_2 complex. If substrate inhibition had also been a feature of NAD-dependence of the IDH, it may not have been noticed due to the lower NAD^+ concentrations used to study NAD-dependence. However, substrate inhibition may explain the disparity between the V_{max} values found when varying isocitrate and NAD^+ (Wharton and Eisenthal, 1981).

5.2.3.3. Metal Ion Requirement. M. methylotrophus IDH used Mg^{2+} and Mn^{2+} with about equal efficiency but Co^{2+} was a poor cofactor. As the ionic radii of these ions (Å) are 0.65, 0.80 and 0.72 respectively (Cotton and Wilkinson, 1966), there may be no relationship between ionic radius and activity. IDH showed no activity with (ionic radii (Å) in square brackets) Zn^{2+} [0.74], Cd^{2+} [0.97], Ca^{2+} [0.99] or Fe^{2+} [0.76] (Cotton and Wilkinson, 1966). Again, the ineffectiveness of these ions was not due to their size. Thus, although Ca^{2+} and Cd^{2+} are larger than ions supporting activity, Zn^{2+} and Fe^{2+} are smaller than Mn^{2+} and larger than Mg^{2+}, but although the latter two ions support activity, Zn^{2+} and Fe^{2+} do not. Similarly, Colman (1972) found no

correlation between cation size and preference of porcine heart NADP-IDH. If M. methylotrophus IDH binds metal ion as an isocitrate chelate, metal ion preference may reflect stability of the chelate.

Comparison with other NADP-IDHs shows M. methylotrophus IDH to be unusual as Mg^{2+} is used as efficiently as Mn^{2+} (**1.13.5.**). However, Acetobacter peroxydans NAD-IDH and the Azotobacter vinelandii NADP-IDH use both metal ions equally well (Chung and Franzen, 1969; Hathaway and Atkinson, 1963). Interestingly, the Acetobacter peroxydans enzyme, in contrast to M. methylotrophus IDH, can also use Zn^{2+}. This and the observation that for bacterial NADP-IDHs (apart from Mn^{2+} and Mg^{2+}) it is difficult to find any trend in metal ion usage (**1.13.5.**) suggests that, in addition to the degree of stability of isocitrate: metal chelates (if indeed these are the true substrates), the individual characteristics of active sites of IDH from different organisms may also cause the diverse metal ion preferences.

5.2.3.4. Putative Regulatory Features of M. methylotrophus IDH. Only ADP and ATP significantly affected M. methylotrophus IDH (Table 32), although it should be noted that assays were carried out at saturating isocitrate. This may have reduced the effect of some of metabolites tested and it is appreciated that a more detailed investigation into their effects may reveal other phenomena of regulatory significance.

ADP was the most effective adenine nucleotide, exerting up to 60% inhibition. ATP was less potent. This may be due to the greater resemblance of ADP to NAD^+ than ATP to NAD^+. However, inhibition was not reduced by NAD^+ as may have been expected from comparison with other bacterial IDHs (**1.13.8.1.**; **1.13.9.2.**). Alternatively, it may be that the pyrophosphate of ADP is important in inhibition of the M. methylotrophus enzyme, which would explain the ineffectiveness of AMP. Pyrophosphate is a competitive inhibitor with respect to isocitrate of the Salmonella typhimurium NADP-IDH (Marr and Weber, 1968) and it may be that the pyrophosphate of ADP behaves like this here, explaining the uncompetitive effect with respect to NAD^+ (Table 32).

ATP, ADP, and AMP concentrations in M. methylotrophus are 2.38, 0.63, and 0.16 mM respectively. Under low-energy conditions, ATP falls to 0.13 mM while ADP and AMP rise to 1.05 mM and 3.34 mM respectively

(Jones et al., 1984). Thus, the effects of 2 mM-ADP and 2 mM-ATP may be worthy of further study, as IDH inhibition by ADP in low energy conditions would be consistent with the biosynthetic role of the CAC in M. methylotrophus. However, it should be noted that although, teleologically, ATP-inhibition differs greatly in significance from ADP-inhibition, the quantitative difference between the effect of ATP and ADP on M. methylotrophus IDH may have been small enough to question the specificity and relevance of regulation of IDH by ADP.

5.2.4. Immunological Relationships of M. methylotrophus IDH

It appears that eukaryotic NADP-IDH, the bacterial dual-coenzyme-specific IDH and all bacterial Group A and B NADP-IDHs tested were not immunologically related to M. methylotrophus IDH (**5.1.5.**). Few studies have been conducted concerning the degree of relatedness of bacterial NADP-IDHs. For mammalian IDHs, Seelig and Colman (1978) have shown anti-porcine heart NADP-IDH antibody reacts with the corresponding human enzyme, but not porcine heart NAD-IDH. Seelig and Colman (1978) have noted that amino acid compositions of the Azotobacter vinelandii, Bacillus stearothermophilus, Rhodopseudomonas spheroides and E. coli NADP-IDHs suggest that they share little sequence homology. Thus it is hardly surprising that M. methylotrophus IDH (differing from all NADP-IDHs in its coenzyme preference) should be immunologically unrelated to any NADP-IDH.

That NADP-IDH activity in M. methylotrophus crude extracts was inactivated by anti.Mm-IDH was evidence that suggested that NADP-IDH activity from M. methylotrophus was due to the same or very similar protein as that responsible for NAD-IDH activity. Indeed, taken with the observation that pure IDH can also reduce $NADP^+$, this argument is very persuasive. This situation differs from that of the obligate methylotroph organism W6 and a variety of facultative autotrophs (**1.13.9.**) where NAD- and NADP-linked IDHs are separate enzymes.

5.2.5. Chemical Modification of M. methylotrophus IDH

Until now, no bacterial NAD-IDH has been examined for sensitivity

to thiol-directed reagents. However, **5.1.6.** shows that IDH from M. methylotrophus is like other NADP-IDHs in that it is inactivated by DTNB. As inactivation closely follows modification (Figures 75, 76), it seems that loss of activity is the result of thiol modification.

M. methylotrophus IDH has two thiol residues per dimer in native and denaturing conditions and so resembles the Thermus thermophilus dual-specific IDH and the Bacillus stearothermophilus NADP-IDH (Nagaoka et al., 1977; Eguchi et al., 1989). As it is not known if the subunits of M. methylotrophus IDH are identical, it is impossible to discuss the distribution of the thiols between the subunits.

Both inactivation and modification by DTNB were biphasic. There are three (or more) possible models for this:

1) The environment of each thiol was different.

2) The environment of each thiol was identical, but both were near enough to each other for modification of one thiol to sterically or otherwise to hinder the modification of the second thiol.

3) DTNB reacted with one thiol; then, the second thiol displaced TNB from the modified thiol to form a disulphide bridge on the enzyme.

Model 1) may result if IDH is a heterodimer. It could also result from an asymmetrical arrangement of two identical subunits where for each subunit the accessibility of the thiol is different.

Models 2) and 3) are both consequences of using DTNB and so they can be tested by employing a smaller reagent that does not react by disulphide bond exchange. This was attempted here (**5.1.6.4.**) using iodoacetamide. However, the results of this experiment were inconclusive. This was surprising; however, such a disparity has been found with the Bacillus KSM-1050 and Thiobacillus novellus NADP-IDHs (Shikata et al., 1988; Charles, 1970).

Another aspect of Model 2) is a possibility that close proximity of two thiols causes the dissociation of one thiol to increase the pKa of the second thiol (Lolkema et al., 1986). Such a change in pKa may have affected the rate of reaction with DTNB and may explain the biphasic kinetics of inactivation and modification seen here. Model 2) is testable with reagents that are specific for vicinal thiols (**5.3.**).

Model 3) has a precedent in the chemical modification of IDH by DTNB. The partially modified porcine heart and Azotobacter vinelandii

NADP-IDHs expel TNB as a result of thiol-disulphide exchange (Colman, 1969; Braginski et al., 1970).

The kinetics of reaction of bacterial NADP-IDHs with DTNB have not been studied in detail. The Bacillus stearothermophilus NADP-IDH, however, differs from M. methylotrophus IDH in that inactivation of the former by DTNB does not correlate closely with modification. The modification of porcine heart NADP-IDH has been studied in detail. In contrast to M. methylotrophus IDH, inactivation is monophasic, and five thiol residues become modified (Colman, 1968).

5.2.5.1. Effect of DTNB in the Presence of Substrates and Coenzymes. It is impossible to rationalise all of the data in **5.1.6.5.** This is especially so for data obtained with NAD^+, not only because of the higher than expected thiol content, but also because the rate of inactivation is far faster than the rate of modification. Why this latter effect occurs is unclear, and there must be a post-modification effect that further inactivates IDH. This may be peculiar to M. methylotrophus IDH as inactivation of Bacillus stearothermophilus NADP-IDH by DTNB is not affected by $NADP^+$ (Nagaoka et al., 1977). What follows is an attempt to explain some of the data.

The kinetics of inactivation and modification of IDH in the presence of isocitrate were similar (Table 33, 34). The results may suggest isocitrate exerts greater protection on the slower reacting thiol than on the faster reacting thiol. Similarly, isocitrate reduces $k_{(b)}{}^i$ far more than $K_{(a)}i$ (Tables 33 and 34). This may suggest the slower reacting thiol is at the isocitrate binding site. When the experiment was repeated with isocitrate and NADH, the faster reacting thiol residues were subject to far greater protection than the slower reacting group (Table 34) although the latter was protected to the same extent as by isocitrate alone. Thus, it may be that the faster reacting thiol is located in the coenzyme binding site of this IDH. This result also suggests IDH can form the NADH:E:isocitrate ternary dead-end complex. Kinetic analysis of Azotobacter vinelandii NADP-IDH has shown that such a complex can also be formed (Wicken et al., 1972). Further, if one thiol is located in the isocitrate binding site and one thiol is located in the coenzyme binding site, M.

methylotrophus IDH may only have one active site per dimer. If so, IDH from M. methylotrophus may resemble the dimeric NADP-IDH from Rhodopseudomonas spheroides but differ from the dimeric E. coli NADP-IDH in this respect (**1.13.6.1.**).

It is difficult to envisage how isocitrate, NAD^+ and NADH all affect the reaction between M. methylotrophus IDH and DTNB without binding to the enzyme. If they can all bind to IDH independently, this has important consequences for any proposed mechanism for this IDH. It would for example be consistent with a random addition of NAD^+ and isocitrate to the enzyme **if** the binding of coenzyme and substrate to the enzyme in the presence of DTNB is identical to that involved in the formation of active ternary complex.

Inactivation of other bacterial IDHs by thiol modification is also attenuated by isocitrate and in some cases coenzyme (**1.13.6.1.**), indicating that certainly for the substrate, cysteine plays a crucial role in the interaction of the bacterial IDH with isocitrate.

5.2.6. Comparison of M. methylotrophus and Eukaryotic NAD-IDHs

There are several differences between M. methylotrophus IDH and eukaryotic NAD-IDH.

The eukaryotic NAD-IDH structure is more complex than that of the M. methylotrophus IDH: The pig heart NAD-IDH is probably a tetramer of three types of subunit (native M_r = 160,000). The Saccharomyces cerevisiae NAD-IDH is an octamer with an M_r of 300,000 (Barnes et al., 1971; Colman, 1983). In contrast, the M. methylotrophus IDH dimer is far simpler and smaller (2 X 45,600). The marked difference in structure between eukaryotic and methylotrophic IDHs may explain the insensitivity of bovine NAD-IDH to anti Mm-IDH (Figure 74; **5.1.5.**).

The eukaryotic NAD-IDHs are allosteric, showing positive co-operativity towards isocitrate. Although a Hill plot of saturation of M. methylotrophus IDH with isocitrate was inconclusive, it appears that this IDH is hyperbolically dependent on isocitrate. Like the eukaryotic enzymes, M. methylotrophus IDH is hyperbolically dependent on NAD^+ (Sanwal and Cook 1966; Barnes et al., 1971; Plaut et al., 1974; Denton et al., 1978; Willson and Tipton, 1980).

The activation of mammalian NAD-IDHs (Plaut, 1970) by ADP and Neurospora and Saccharomyces NAD-IDHs by AMP (Hathaway and Atkinson, 1963; Sanwal and Cook, 1966) contrasts with the ADP-inhibition of M. methylotrophus IDH. The basis of this dichotomy may possibly be the role of the CAC in a eukaryote and an obligate methylotroph. In the latter, this may only be biosynthetic (**1.10.1.**). The function of the eukaryotic CAC includes ATP production. Thus, as the biosynthetic potential of a cell or organism may be inversely proportional to ADP, high levels of ADP may inhibit the biosynthetically orientated bacterial IDH and stimulate the catabolic mammalian enzyme.

Mammalian NAD-IDHs are like M. methylotrophus IDH in that they are inactivated by DTNB (Fan and Plaut, 1974; Colman, 1983). Mn^{2+} and isocitrate protect against inactivation. This resembles the effects of isocitrate on M. methylotrophus IDH and other NADP-IDHs (**1.13.6.1.**). Thus, it may be vital to every IDH, irrespective of coenzyme preference, that a free thiol residue is located in the vicinity of the active site. It is of interest that modification of the bovine NAD-IDH thiol residues is biphasic; however, heterogeneity of subunit composition of this enzyme prevents detailed interpretation of the data (Fan and Plaut, 1974; Colman, 1983).

5.3. Further Work

Future investigations may require cloning and amplified expression of the IDH gene, because of the low cellular abundance of the IDH in M. methylotrophus (0.07% of extractable cell protein). This goal may not be too far away as a cosmid library of the genome of M. methylotrophus is available (Lyon et al., 1988). Thus, it may be worthwhile to use a DNA probe synthesised on the basis of results obtained from the IDH protein with automated Edman degradation.

In the short term, there are a number of issues arising from the results that may be dealt with without recourse to molecular biology.

With regard to the purification, conditions need to be found that reduce the elution volume of IDH from Blue HE-RD, for reasons of economy and dilution of the IDH. This may involve finding conditions that reduce the number of interactions between IDH and the immobilised

dye. Raising the ionic strength of the isocitrate wash or the previous low salt (0.1 M-KCl) wash may achieve this (Fulton, 1980). Otherwise, immuno-affinity chromatography may be of use to purify IDH if it can be eluted with preservation of activity. The feasibility of this idea is increased by the stability of IDH in the presence of isocitrate.

Although comparison with other bacterial IDHs may suggest M. methylotrophus IDH is composed of identical subunits, eukaryotic NAD-IDHs are composed of more than one subunit type and so it is of interest to determine subunit composition of M. methylotrophus IDH. This may be achieved by N-terminal amino acid analysis or amino acid composition and proteolysis studies. Amino acid analysis may also facilitate more detailed comparisons with other IDHs.

The identity of the form of isocitrate used by M. methylotrophus in relation to the metal ion remains to be determined. This may be revealed by study of the effects of metal ion on the isocitrate K_m and vice versa, and whether the K_m value for the metal ion-isocitrate chelate remains constant in these experiments (Wicken et al., 1972; Marr and Weber, 1973). In connection with this, ADP-inhibition of IDH is curious as the uncompetitive nature of inhibition with respect to NAD^+ may have resulted from sequestration of Mg^{2+} and/or competitive inhibition with respect to isocitrate. Thus it may be of interest to examine the kinetics of ADP-inhibition with respect to isocitrate and Mg^{2+}. It should be noted that if kinetic work is undertaken, the very low isocitrate K_m of M. methylotrophus IDH will make the use of a more sensitive assay necessary. A possible candidate for this would be to monitor NADH production fluorimetrically.

A criticism that can be levelled at the immunological comparison of IDHs that was made is that IDH inhibition would not have detected the interaction of antibody at sites non-essential for activity. Thus, the immunological relationships between M. methylotrophus IDH and other IDHs may be closer than was suggested by the results. To test this, immobilised protein A (e.g as intact Staphylococcus aureus cells) could be added at the end of the incubation to bind all the IgG added. This would remove all of the enzyme that interacted with the antibody including that bound at non-essential sites.

The chemical modification of M. methylotrophus IDH with DTNB left

a number of questions. The first of these concerns the essentiality of the thiol residues to activity. When the DTNB-modified NADP-IDHs from Azotobacter vinelandii and pig heart are treated with cyanide, this cleaves the disulphide bond to form TNB and enzyme-thiocyanate (Johanson and Colman, 1981; Chung et al., 1971). That this occurs in preference to formation of enzyme-SH and TNB-thiocyanate is due to the resonance stabilisation of TNB (Vanaman and Stark, 1970). Thus treatment of IDH with DTNB and cyanide introduces a very small group into the enzyme. This method has shown that a thiol is essential for the activity of the porcine NADP-IDH because cyanolysis of the TNB derivative of IDH releases TNB without restoration of activity. On the other hand, similar treatment restores activity to the modified Azotobacter vinelandii NADP-IDH indicating here that inactivation results from steric effects caused by the size of the TNB group. This approach may prove useful in determining if M. methylotrophus IDH thiols are essential. In this context, the reagent 2-nitro-5-thiocyanobenzoate has been introduced by Degani and Patchornik (1974) as a specific cyanylating reagent for thiol residues and achieves exactly the same result as sequential modification with DTNB and cyanide.

The biphasic inactivation and modification of M. methylotrophus IDH also requires study. If biphasic kinetics result from close proximity of two thiol groups, then IDH should react with reagents that are specific for vicinal dithiols such as the arsenite: 2,3-dimercaptopropanol conjugate or phenylarsine oxide (Carlson et al., 1978; Russo and Bump, 1988; Alvear et al., 1989).

A further hypothesis put forward was that the slow phase of inactivation or modification was due to disulphide exchange resulting from the attack of the second thiol on the IDH-S-S-TNB disulphide to form an intramolecular disulphide. This idea may be tested in two ways. If an equimolar mixture of IDH and DTNB is allowed to react, at completion of reaction there will be (for two thiol residues per IDH dimer) one molecule of TNB per molecule DTNB originally reacted. If however, thiol disulphide-exchange has occurred, this ratio will be doubled. Otherwise, partially modified IDH may be desalted to remove excess DTNB. Thiol disulphide exchange can be monitored as a release of TNB from the enzyme without DTNB.

With regard to the effects of substrates and coenzymes on the reaction of IDH with DTNB, there is a need to establish the number of substrate and coenzyme binding sites and types of subunit before further interpretations can be made. The tentative identification of the location of the fast-reacting and slow reacting-thiol residues as the coenzyme and substrate binding sites respectively might be testable by: a) studying the effect of isocitrate on $k_{(b)}{}^{i}$ and $k_{(a)}{}^{i}$; b) studying the effect of NAD^{+} on the rate constant of $k_{(a)}{}^{m}$, if conditions can be found where the latter has an explicable effect on DTNB modification. From such data, dissociation constants of coenzyme and substrate may be found. If these values are comparable with the K_m values for coenzyme and substrate, this may confirm the location of the two thiol groups. Alternatively, affinity labelling with reactive substrate and coenzyme analogues may prove useful in the location of these thiol residues.

CHAPTER 6

General Discussion

General Discussion

"Hindsight is always twenty-twenty" - Billy Wilder.

The findings that have been presented and individually discussed in Chapters 3 to 5 generally assemble themselves into two groups. The first group bears on the route *via* the CAC by which pyruvate is converted to glutamate in *M. methylotrophus*. The second group concerns itself with some of the enzymes that are components of the *M. methylotrophus* CAC, i.e CS and IDH. These groups are the subject of the first two sections of the Discussion. The last section concerns itself with the regulation of the CAC in *M. methylotrophus*.

6.1. Aspects of the State and Origins of the CAC and Related Reactions in M. methylotrophus

Let us consider the CAC as a whole. The results show that *M. methylotrophus* has the enzymic potential to convert 2 pyruvate molecules from the C_1 source to acetyl-CoA and OAA, which are then used to synthesise citrate, isocitrate and 2-OG. This latter metabolite and NADH, from IDH, are probably used in the GS/GOGAT system of ammonia assimilation. The end product of this is glutamate (Figure 84). As *M. methylotrophus* is impermeable to glutamate (Windass *et al.*, 1980), the eventual conversion of 2 pyruvate plus ammonia to glutamate and CO_2 is crucial to the survival of the organism. The CAC can produce NADH stoichiometrically with respect to 2-OG. However, in the absence of CAC dehydrogenases that oxidise 2-OG, the CAC cannot *catalytically* make reducing equivalents and so cannot generate them in amounts approaching those extracted by the complete CAC. Thus, if the CAC were to be used in energy generation in *M. methylotrophus*, the process would be extremely wasteful as 2-OG would have to be accumulated in large amounts as a waste product (c.f. Baldwin and Krebs, 1981). Thus, this (incomplete) CAC is probably primarily biosynthetic.

Figure 84 shows the centrality of pyruvate to the intermediary metabolism of *M. methylotrophus*. Aperghis (1981) has noted that

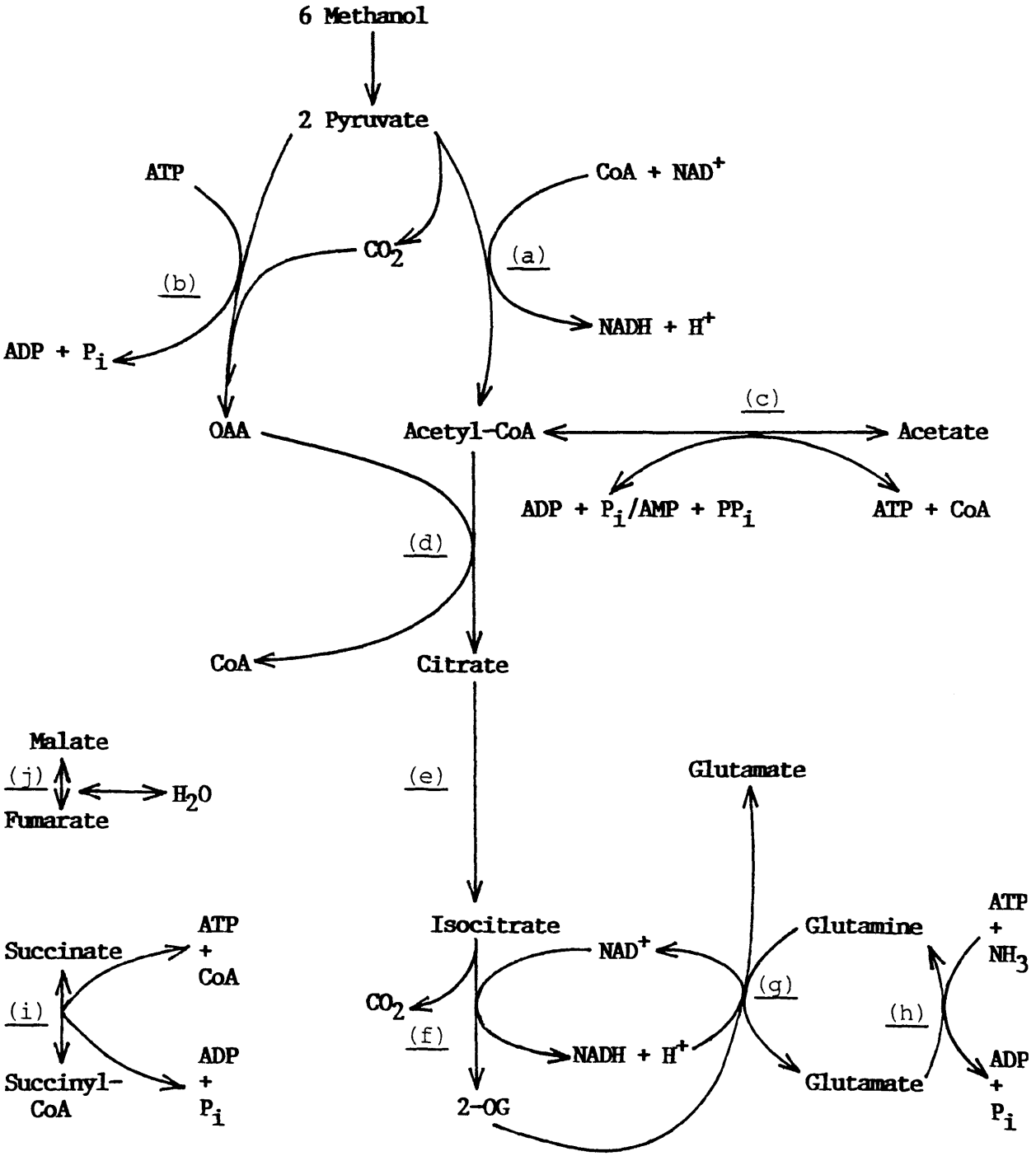
Legend to Figure 84

The enzymes labelled (a) to (j) are:

(a): Pyruvate Dehydrogenase
(b): Pyruvate Carboxylase
(c): Acetyl-CoA Synthetase
(d): CS
(e): Aconitase
(f): IDH
(g): GOGAT
(h): GS
(i): STK
(j): Fumarase

Figure 84

The CAC of M. methylotrophus and Some Related Reactions:
A Summary of Some of the Results in Chapter 3



pyruvate can be regarded as the end product of the RuMP cycle in M. methylotrophus. From here, it may be used for the synthesis of amino acids such as alanine, valine and leucine. Otherwise, pyruvate is at a branch-point between, on the one hand, acetyl-CoA for the production of citrate and fatty acids, and on the other, OAA for the production of citrate, triose phosphates and aspartate-related amino acids. It is possible that formation and use of pyruvate may form a locus for CAC regulation and this is considered later (**6.3.**).

6.1.1. Evolutionary Consideration of the CAC of M. methylotrophus

The observation of acetyl-CoA synthetase in M. methylotrophus during growth on methanol alone (**3.2.1.**) is hard to explain. As it was not established whether this activity led to production of AMP and PPi or whether it produced ADP from ATP, the reversibility of acetyl-CoA synthesis was not clear. This point may be of interest from an evolutionary point of view.

Quayle and Ferenci (1978) considered the antiquity of the RuMP cycle of formaldehyde fixation. They considered that as formaldehyde formation may have featured in prebiotic chemical evolution, it may, by its ability to condense with itself, have led to the formation of a variety of sugars. As the consensus of opinion suggests oxygen did not reach significant atmospheric concentrations until the rise of the oxygenic photosynthetic prokaryotes, and as a life-style such as theirs is highly developed, oxygenic photosynthesis must have been preceded by a long period of evolution from simpler organisms. Thus the earliest organisms were probably anaerobic, living by fermentation of substrates such as those produced from formaldehyde itself (Quayle and Ferenci, 1978; Gest, 1987). As the supply of such preformed biomolecules decayed, it follows that selection pressure for pathways that could exploit molecules of ever decreasing complexity would have developed. This is the basis of "retro-evolution" as proposed by Horowitz (1945). This selection may have led to evolution of pathways which exploited formaldehyde itself as a carbon source, or as Quayle and Ferenci (1978) put it: "We suggest....that the earliest sugar shortage was overcome by evolution of a pathway of net sugar synthesis

from formaldehyde similar to the present day RuMP cycle". As the early methylotrophs that used these carbon sources were anaerobic, they would have had to conserve energy by substrate level phosphorylation. As Quayle and Ferenci (1978) noted, one RuMP cycle variant (fructose 1,6 bis-phosphate aldolase/transaldolase) can actually synthesise ATP from the exergonic formation of pyruvate from formaldehyde:

$$3\ HCHO + ADP + Pi + NAD(P)^{+} \longrightarrow Pyruvate + NAD(P)H + H^{+} + ATP$$

from which it might be possible to construct the fermentation

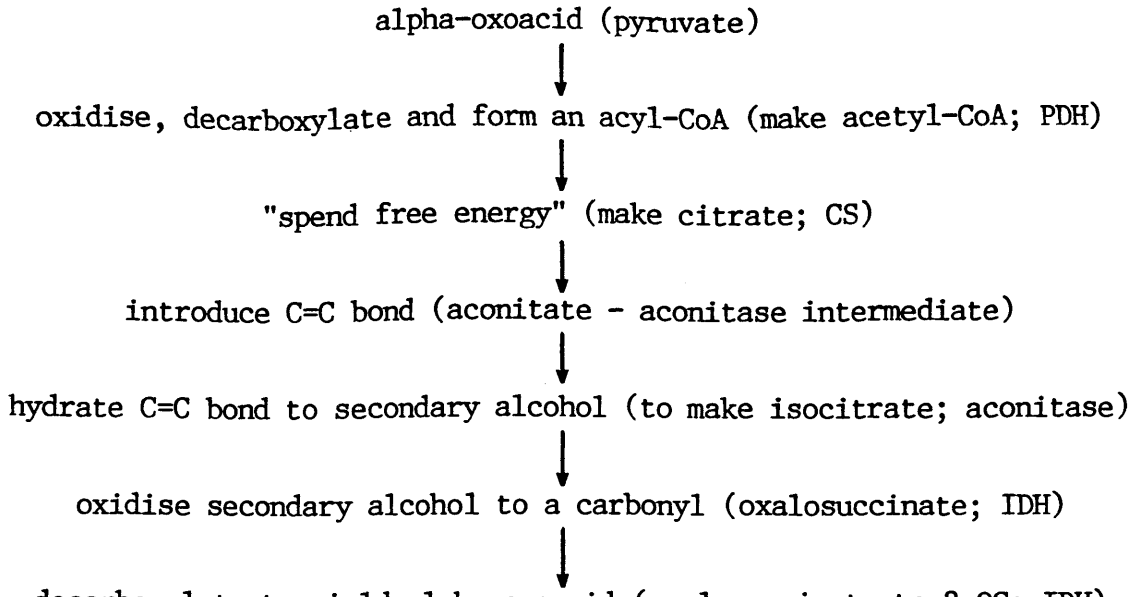
$$3HCHO + ADP + Pi \longrightarrow Lactate + ATP.$$

However, M. methylotrophus possesses the KDPG aldolase/transaldolase variant of the RuMP cycle (Beardsmore et al., 1982) which does not allow formation of ATP during pyruvate synthesis:

$$3\ HCHO + NAD(P)^{+} \longrightarrow Pyruvate + NAD(P)H + H^{+}$$

Another potential substrate level phosphorylation may be facilitated by decarboxylation of pyruvate to acetyl-CoA, followed by conservation of the free energy of hydrolysis of acetyl-CoA as ATP. This has been proposed for the archaebacterium Thermoplasma acidophilum which has an ADP-forming acetyl-CoA synthetase (Danson, 1988). Thus, if the acetyl-CoA synthetase in M. methylotrophus is found to be an ADP-forming enzyme (as opposed to the more common AMP-forming type), might its presence in this obligate aerobe be a vestige of its anaerobic past ? Further, might the retention of the synthetase to the present day by M. methylotrophus, augment the supply of ATP at low O_2 tension ? Such suggestions may provide another contribution to 3.4., i.e. a study of the ability of M. methylotrophus to excrete acetate during O_2-limited growth on methanol.

Turning to the CAC proper, Weitzman (1981, 1985) has noted a striking duplication within the CAC that may be evolutionarily significant. This may also be particularly applicable to the CAC of M. methylotrophus. From pyruvate to 2-OG, the general reaction scheme is:



If this sequence of general reactions is repeated with 2-OG as the initial oxoacid, it will be oxidatively decarboxylated to an acyl-CoA (succinyl-CoA), whose free energy will be "spent" to produce a phosphodiester bond. A double bond is then introduced into succinate to make fumarate, which is hydrated to malate - a secondary alcohol. This can be oxidised to OAA, and decarboxylated to pyruvate which yields the first alpha-oxoacid - pyruvate. If the last decarboxylation is abandoned and OAA is recirculated around the two contiguous sequences, the CAC results where pyruvate is the C_2 source. Weitzman (1985) has postulated that the two halves of the CAC suggested by this type of scheme are evolutionarily related. Thus, the reactions of the CAC may have evolved by gene duplication and diversification or by recruitment of enzymes of broad specificity. Although this hypothesis is stretched because it pairs reactions like STK with CS and succinate dehydrogenase with aconitase, it provides an attractive framework for the evolution of most, if not all of the complete CAC.

Returning to the methylotrophs, M. methylotrophus and similar RuMP cycle utilisers have a CAC that has the 'outgoing section', that is PDH to IDH. However, these organisms have an incomplete set of enzymes that catalyse the second 'return section' (2-OG to OAA), with deficiencies in OGDH and often other CAC enzymes (**3.2.2.**; Figure 84; Dahl et al., 1972; Davey et al., 1972; Colby and Zatman, 1972, 1975a).

From the foregoing hypothesis, the patchy distribution of these enzymes may be of evolutionary significance. This is because the theory of Weitzman (1981, 1985) suggests these C_1-utilisers had reached different stages of duplication, diversification or recruitment of enzymes during the development of a fully oxidative CAC. Such a view would be consistent with that of Ferenci and Quayle (1978) who proposed that the RuMP cycle may be a "primitive pathway". This may suggest RuMP utilisers like M. methylotrophus may mark early stages in the evolution of an oxidative CAC from an anaerobic past. This would also be evolutionarily consistent with the OGDH lesion in M. methylotrophus and similar organisms which, it is thought, would have evolved later, when there was a selection pressure to exploit oxygen as a terminal electron acceptor (Weitzman, 1985; Gest, 1987).

Interestingly, as the last two sentences imply, methylotrophs like M. methylotrophus may have never responded to the presence of O_2 by evolution of an oxidative CAC. If the RuMP cycle was functional by the time O_2 was present, its modification to oxidise formaldehyde (**1.7.2.2.**) cyclically by the acquisition of 6-phospho gluconate dehydrogenase and lactonase would have been far less daunting than the duplicative reorganisation of the CAC to perform the same task. Thus, is it possible that the gaps in the oxidative sequence between 2-OG and OAA in, e.g. M. methylotrophus, show the stage of CAC evolution that had been reached before the dissimilatory variant of the RuMP cycle evolved ? If so, did this relieve the selection pressure on evolution of an oxidative CAC in M. methylotrophus and similar organisms ?

6.2. CS and IDH from M. methylotrophus

The novelty of the large NADH-insensitive M. methylotrophus CS has already been noted (**1.12.12.2.1.**). It seems from the amino acid analysis of CSs from M. methylotrophus and A. calcoaceticus, that there may be a close similarity between the two, but unfortunately, these results have to be treated with a degree of scepticism (**4.3.1.**). However, if it is assumed the two proteins are related in evolutionary terms (an assumption given credence by the similarity of substrate

dependences, molecular weights, and gross quaternary structure between CSs from M. methylotrophus and A. calcoaceticus [**4.3.1.**; **4.3.2.**]), a few comments on evolution of NADH sensitivity of CS may be considered.

If the CAC in obligate RuMP cycle utilisers has never evolved so that it has a catabolic role (**6.1.1.**), this may suggest that the M. methylotrophus CS was never under pressure to develop a sensitive allosteric inhibitory response to NADH. Consequently, this CS may be a protein template in the evolution of NADH-sensitivity, as opposed to a CS that has lost it.

The novelty of the primarily NAD-linked M. methylotrophus IDH has also been noted (**1.13.9.1.**). Thus, the fact that this and the novel large NADH-insensitive CS occur in the same organism prompts consideration of the literature regarding their co-occurrence.

6.2.1. Co-occurrence of NAD-Linked IDH with NADH-insensitive CS

If reference to the Gram-negative organisms in Table 8 with NAD-linked IDHs is made, and comparisons of the NADH sensitivities of their CSs (where known) are drawn, the following pattern emerges. The autotrophic Thiobacillus neapolitanus, Thiobacillus novellus, the obligate RuMP cycle-utilising methylotrophs and type M RFMs C2A1, 4B6, Methylococcus albus BG8, and M. methylotrophus all have NADH-insensitive CSs, as well as NAD-IDH activities. However, only in the case of M. methylotrophus is it explicitly known that the reason for the NADH-insensitivity of the CS does not lie with the size of the enzyme (Otto, 1986; **4.2.2.2.**).

Although, there may be an association between NAD-IDH and NADH-insensitive CSs in Gram-negative organisms, there remains a lot of work to be done before such a trend becomes established. Thus, the NADH sensitivity of CSs from Gram-negative organisms in Table 8 not yet mentioned here needs to be characterised. Further, the coenzyme specificity of IDHs from Acetobacter xylinum, Branhamella, Kingella, Moraxella, Neisseria, and Thiobacillus denitrificans, all of which have NADH-insensitive CSs, needs to be established (Taylor, 1970; Swissa and Benziman, 1976; Weitzman, 1981).

If it transpires that occurrence of NAD-IDH in Gram-negative

organisms depends on the presence of an NADH-insensitive CS, the opposite trend, i.e. the dependence of occurrence of NADH-insensitive CS on the presence of NAD-IDH does not hold true. Thus, for example, the cyanobacteria have NADH-insensitive CSs but only have NADP-IDH, not NAD-IDH, and a similar situation exists in Thermus aquaticus (Pearce and Carr, 1967; Pearce et al., 1969; Ramaley and Hudock, 1973; Taylor, 1973; Lucas and Weitzman, 1975; Weitzman, 1978).

6.2.1.1. Apparent Exceptions to the Trend. One such exception is the photoheterotroph Rhodomicrobium vanielli, which has an NADH-sensitive CS and both NADP- and NAD-linked IDH activities (Morgan et al., 1986); however, the IDH in this organism is a dual-specific enzyme, whose coenzyme specificity suggests NAD-IDH activity in vivo would be insignificant (**1.13.3.1.**; Leyland et al., 1989). Another partial exception to this trend is the methylotroph Pseudomonas oleovorans which has two CSs, only one of which is NADH-sensitive. The organism also has an NAD-IDH, but its use of NADP-IDH has not been commented on. However, during C_1 growth, only NADH-insensitive CS is expressed, which is consistent with the above trend (Babel and Müller-Kraft, 1979; Anthony, 1982).

6.2.1.2. Teleological Interpretation of the Trend. Previously, in this thesis, separate hypotheses have been put forward to explain the presence of NADH-insensitive CS and NAD-linked IDH in Gram-negative M. methylotrophus and other organisms. These concern either the solely biosynthetic nature of an OGDH-deficient CAC, the inability of the latter to produce sufficient NADH, and/or a particular need for NADH by the organism (**1.12.6.2.2.**; **1.12.7.3.**; **1.12.12.2.1.**; **1.13.9.1.**; **1.13.10.**; **3.3.2.2.**; **3.3.3.**; **4.3.3.5.**; **5.2.3.4.**; **5.2.6.**). It is a matter of conjecture as to whether NAD-linkage of isocitrate oxidation and NADH-insensitivity of CS developed independently as a response to one or more of these situations, or developed in response to each other. However, under any circumstances, it may make little sense to place an NAD-linked IDH downstream of an NADH-sensitive CS. This may be because the production of NADH by IDH would prematurely curtail CAC activity through NADH-inhibition of CS before the cycle had fulfilled

the demands of energy conservation and/or biosynthesis that may be placed upon it. Thus, it may be for this reason that, in Gram-negative organisms with NADH-sensitive CSs, the corresponding IDH is practically always solely NADP-linked (c.f. Ragland et al., 1966; Weitzman and Jones, 1968). If this is the case, then it makes little difference what coenzyme preference IDH has if the CS is NADH-insensitive. This may account for the fact that NAD-IDH seems only to occur in Gram-negative bacteria if CS is NADH-insensitive, but NADH-insensitivity of CS is not inextricably linked with NAD-IDH.

6.3. Regulation of CAC and Related Enzymes

The effects of 2-OG and succinyl-CoA on CS activity, and the effects of ADP on IDH activity can be rationalised in terms of the biosynthetic function of the CAC as it exists in M. methylotrophus. However, relative to the magnitude of their effects in other organisms (inhibitory or activatory), these effectors are not as potent with M. methylotrophus (c.f. Wright et al., 1967; Hampton and Hanson, 1969; Lucas and Weitzman, 1977; Denton et al., 1978; Willson and Tipton, 1980). One potential effector that was not tested on CS here was glutamate. This has been found to inhibit the Salmonella typhimurium CS (Flechtner and Hanson, 1970). However, Aperghis (1981) and Colby and Zatman (1975c) found M. methylotrophus and other methylotrophs to be less sensitive to glutamate than they are to 2-OG. Teleologically this makes sense because 2-OG is not only used for glutamate synthesis. If glutamate were to inhibit CS, this may cause shortages of other amino acids derived from 2-OG.

Thus it appears that control of C_5 skeleton and possibly succinyl-CoA synthesis by energy indicators and end products acting on IDH and CS might only be effective if these metabolites accumulated to very high levels.

This leads to consideration of what other features could be relevant to the control of the CAC in M. methylotrophus. First, let us consider the supply of OAA and acetyl-CoA to the CAC. Aperghis (1981) has shown that the enzyme that produces OAA from pyruvate - pyruvate carboxylase - is inhibited by ADP. Aperghis (1981) considered that

this effect may control the replenishment of the RuMP cycle with triose phosphates which would gear the assimilatory and thus biosynthetic metabolism of M. methylotrophus to the energy status of the cell. Inhibition by ADP may also prevent entry of acetyl-CoA into the CAC which would spare wasting energy-rich thioester bonds, when there was insufficient ATP to conduct biosynthesis from CAC products. Such a hypothesis is also consistent with the lack of sensitivity of M. methylotrophus pyruvate carboxylase to activation by acetyl-CoA which contrasts with the behaviour of this enzyme when it services a catabolically functioning CAC (Aperghis, 1981).

Let us now turn to the supply of acetyl-CoA, in other bacteria, PDH is regulated by product inhibition and by AMP activation (Weitzman, 1981). The latter effect and the NADH-product inhibition are only of relevance where PDH supplies acetyl-CoA for catabolic purposes and it seems that these effects would be of little relevance in M. methylotrophus. However, acetyl-CoA-product inhibition of PDH may provide a precaution against unnecessary loss of carbon if the requirements for the CAC or fatty acid synthesis for acetyl-CoA had fallen. It should be noted that a disadvantage of this idea is that it may not discriminate between the demands of fatty acid synthesis and those of the CAC. In this context, PDH regulation by C_2 units may occur at the level of the gene, for in the presence of acetate, M. methylotrophus PDH is repressed (Tables 14, 17). Further work is required to ascertain whether it occurs at the level of the protein.

Thus far, all concern with regulatory behaviour has been directed towards the effects of relevant metabolites on individual enzymes. In the present thesis, this approach may have provided data that were more equivocal than was desirable. A main cause of this is a lack of knowledge regarding intracellular metabolite concentrations. However, even if this were not the case, the following would still apply.

It is now apparent that the study of enzyme regulation also has to consider control of higher levels of organisation, particularly the effects of enzyme-enzyme association. Thus, it is now believed that the enzymes of major pathways such as glycolysis (Ovádi, 1988), the RubP cycle in plants (Gontero et al., 1988), the CAC in bacteria and mitochondria (Barnes and Weitzman, 1986; Robinson and Srere, 1985),

mammalian glycogen degradation and synthesis (Cohen, 1988) and others (Srere, 1987) are associated in multi-enzyme complexes or clusters and, in the case of glycogen metabolism, with the somewhat sizable substrate. It is thought that arrangements such as these may offer the advantages of protection of unstable intermediates, substrate channelling and raised local concentrations of substrates in the generation of pathway end products (Srere, 1987). It is also known that such enzyme-enzyme associations may themselves be regulated (as opposed to direct regulation of enzyme activity) by signals relevant to the role of the pathway which could, in turn, modify the flux through that pathway (Beeckmans and Kanarek, 1987; Ovádi, 1988; Cohen, 1988).

Future approaches designed to examine organisation and control of organisation of the enzymes of the CAC in M. methylotrophus may shed more light on how the CAC in this organism is controlled.

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