STUDIES OF CARBON METABOLISM

BY THE

FACULTATIVE METHYLOTROPH *ARTHROBACTER* 2B2

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To Mom and Dad, for all their help.

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ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
DCPIP	Dichlorophenol-indophenol
DEAE-cellulose	Diethylaminoethyl-cellulose
DTT	Dithiothreitol
EDTA	Ethylene diaminetetraacetic acid
GSH	Reduced glutathione
HuMP	Hexulose 6-phosphate
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
RiMP	Ribose 5-phosphate
RuDP	Ribulose 1,5-diphosphate
RuMP	Ribulose 5-phosphate
SDS	Sodium dodecyl sulphate
TCA cycle	Tricarboxylic acid cycle
Tris	Tris (hydroxymethyl) methylamine
v/v	Volume per volume
w/v	Weight per volume

CONTENTS

ABSTRACT

The research has involved a study of the enzymology of carbon assimilation in the facultative methylotroph Arthrobacter 2B2, utilising methylamine and choline as a sole source of carbon. When growing on methylamine the organism uses the ribulose monophosphate cycle of formaldehyde fixation as the carbon assimilating pathway. The variant of the cycle used is that involving Embden-Meyerhof cleavage, coupled with transaldolase/transketolase re-arrangement. It is the most energetically favourable of the 4 possible variants but is rare amongst methylotrophs, the only other organism in which it has been reported to be of physiological significance is Arthrobacter P1 (Levering et al., 1982) Two key enzymes of the ribulose monophosphate cycle, hexulose phosphate synthase and phosphohexuloisomerase were purified and characterised. Many of the properties of the purified hexulose phosphate synthase from Arthrobacter 2B2 resembled those of similar enzymes purified from other methylotrophs. However, it differs from previously purified enzymes in having a molecular weight of 155000, consisting of two identical subunits. The specific activity of the purified enzyme was very low compared to those reported for similar enzymes from other organisms.

The phosphohexuloisomerase from Arthrobacter 2B2 was different to those previously studied in other organisms, in being a trimeric protein of total molecular weight 108000, constructed of three identical subunits. The final specific activity of the purified enzyme was very low compared to those reported for similar enzymes from other organisms.

When growing on methylamine as a sole source of carbon, the organism synthesises hydroxypyruvate reductase. This enzyme was purified and found to be strictly NADPH specific, and therefore, different from hydroxypyruvate reductases reported in other organisms.

When growing on choline as sole source of carbon the organism synthesises enzymes of the ribulose monophosphate cycle and the serine pathway indicating the possibility that choline metabolism proceeds in a similar manner to that described in Arthrobacter P1 (Levering et al.,1981b). However, crude extracts of choline-grown Arthrobacter 2B2 were shown to contain high specific activities of formaldehyde dehydrogenase, which were not detected in extracts of the organism grown on methylamine or glucose. This suggests that formaldehyde produced by the metabolism of choline, is oxidised by formaldehyde dehydrogenase. Crude extracts of choline grown Arthrobacter 2B2 contained high activities of NADPH-linked and NADH/NADPH-linked hydroxypyruvate reductase. Both enzymes were purified and their characteristics tested. Batch cultures of choline-grown cells produced the two enzymes at different stages of growth, the NADH/NADPH-linked enzyme being produced during early-to mid-exponential phases, and the NADPH-linked enzyme during late exponential and stationary phases. This effect was studied further in chemostat continuous culture. From these studies it has been proposed that the oxidation of choline is not completed in one uninterrupted sequence, but that an intermediate of the catabolic pathway is allowed to build up during the early stages of batch growth. If this intermediate repressed the production of the NADPH-linked enzyme, this could provide an explanation for its delayed appearance during the growth cycle. However, under conditions of strict carbon limitation, such as would be present at very low dilution rates in continuous culture, the intermediate would itself be degraded thus lifting the repression of the synthesis of the NADPH-linked enzyme.

A study was also made of the formaldehyde dehydrogenase of Methylophilus methylotrophus. This enzyme was purified, and its activity was demonstrated to be thiol-dependent. The thiol specificity was not limited to GSH, as cysteine was also active in this capacity.

CHAPTER 1

Introduction

In natural environments compounds containing a single carbon atom occur in abundance at all levels of oxidation from carbon dioxide to methane. It is therefore not surprising that a large number of microorganisms have taken advantage of these compounds and developed the ability to utilise them as their carbon and/or energy source.

The first isolation of an organism capable of using methane was reported in 1906 (Sohngen, 1906). In the sixty years after this, up to the year 1961, only sixteen species of bacteria had been characterised for their growth on C_1 compounds (Quayle, 1961). Since that time however, mainly due to the onset of industrial interest, many hundreds of species of organisms capable of utilising one carbon substrates have been isolated and described. Perhaps at this stage the question should be posed, why industrial interest has so recently increased? There are several reasons. Firstly, methane and its derivatives form an inexpensive substrate for organisms which could be used to produce single cell protein. This is currently being manufactured as an animal feedstock and has a possible future as a human foodsource. Secondly, there is a possibility of exploiting the capabilities of organisms able to metabolise reduced C_1 compounds, to remove prospective environmental contaminants such as carbon monoxide, cyanide and methyl sulphides. Finally, some of the organisms contain novel enzymes and enzyme systems which could be useful industrially, either in the production of new compounds or by lowering manufacturing costs of currently produced compounds which are at present being produced chemically.

Much recent work on C_1 utilising organisms has centred

on their ecology and their role in the carbon cycle. An idea of the importance of such organisms in the environment can be gained when it is known that the production of methane world-wide by ruminants has been estimated to be 4×10^5 tonnes per day (Large, 1983). The methane must be recycled into organic compounds by methanotrophs in order to make it available to other organisms.

This brief introduction shows why the study of these microorganisms is of both industrial and academic interest. The characterisation of C_1 fixation pathways in methylotrophs has been well documented for many organisms. In recent years Quayle (1972), Anthony (1975), Colby et al (1979), and Quayle (1980), have reviewed the area in great detail. It is intended that this initial chapter should bring the reader up to date with the current state of knowledge within the field of methylotrophy.

Methylotrophy and Autotrophy

Organisms which use compounds containing a single carbon atom as their source of carbon can be divided into two groups, the autotrophs, and the methylotrophs. The definition of these two terms has varied in the past but here methylotrophs are defined as organisms which are able to grow at the expense of reduced carbon compounds, containing one or more carbon atoms, but with no carbon-carbon bonds (Anthony, 1982). Autotrophs on the other hand, are those microorganisms which can synthesize all of their cell components from carbon dioxide, irrespective of the biochemical pathway involved (Quayle and Ferenci, 1978). The definition of autotrophy does not exclude an organism from being considered an autotroph

if it merely cannot synthesize an essential vitamin cofactor. It should also be noted that some organisms have the metabolic capabilities to be considered either a methylotroph or an autotroph. Paracoccus denitrificans for example can grow autotrophically on carbon dioxide. However, it can also grow on methanol, methylamine or formate as sole source of carbon and energy (Cox and Quayle, 1975; Pichinoty et al., 1977), and thus could be termed a methylotroph. However the reduced one carbon compound is assimilated via the ribulose bisphosphate cycle after being first oxidised to carbon dioxide. Thus Paracoccus denitrificans can assimilate carbon dioxide and reduced one carbon compounds via the same fixation pathway. On the other hand, Methylococcus capsulatus (Bath), has the capability to operate the ribulose monophosphate cycle to assimilate reduced one carbon compounds and, to a limited extent, the ribulose bisphosphate cycle to fix carbon dioxide. However it has been reported that the ribulose monophosphate cycle is the predominant assimilatory pathway in this organism (Taylor, 1977a), with only about 2.5 percent of cell carbon arising from carbon dioxide during growth on methane as carbon substrate, and no fully autotrophic growth on carbon dioxide plus an inorganic energy source yet being reported.

Methylotrophic Organisms

For the sake of convenience the methylotrophs have been split up into four groups. The groups are based on similar growth properties and sometimes structural features. They are not necessarily taxonomic groups with genetic relationships. The groups are:

1. Methanotrophs.

2. Obligate methylotrophs unable to utilise methane.

3. Facultative methylotrophs unable to utilise methane.

4. Methanogens.

1. Methanotrophs

A large part of our knowledge of methanotrophs and their classification comes from the description by Whittenbury *et al.*, (1970) of more than one hundred new isolates of such organisms. Prior to this only four methanotrophs had been described.

The obligate methanotrophic bacteria are a group of Gram negative, strictly aerobic organisms which are capable of growth using methane, methanol, methyl formate or dimethyl-carbonate as sole carbon and energy sources. There is a general consensus that a taxonomy of the methane utilisers should be based on the scheme of Whittenbury *et al* (1970). This divides the organisms into Type 1 and Type 2 methanotrophs, and is based on a number of biochemical and physiological characteristics.

Type I methanotrophs:

This group is characterised by the genera *Methylomonas*, *Methylococcus* and *Methylobacter*. Such genera have a complex internal membrane system consisting of bundles of disc-shaped vesicles which appear to have been formed by invagination of the cytoplasmic membrane. The bacteria within this group are also able to form a resting stage; this consists of a cyst, similar to those formed by *Azotobacter* species, and thus known as '*Azotobacter* type' cysts. Moving on to the

biochemistry of the Type I organisms, the mode of carbon assimilation employed is the ribulose monophosphate cycle of formaldehyde fixation, although as mentioned previously, low levels of ribulose bisphosphate cycle enzymes have been found in Methylococcus capsulatus (Bath). There appears to be an incomplete tricarboxylic acid (TCA) cycle in Type I organisms as they show a total lack of 2-oxoglutarate dehydrogenase and generally have lower levels of other TCA cycle enzymes than Type II organisms.

Type II methanotrophs:

This group is characterised by the genera Methylosinus, Methylocystis, and the facultative methanotroph Methylobacterium. As in Type I organisms, there is a complex internal membrane system present. However, in Type II methanotrophs this consists of a system of paired peripheral membranes. The resting stage formed by these organisms varies depending on the genus. Methylosinus sp. develop exospores which bear some resemblance to the endospores produced by some Bacillus species and the exospores produced by Rhodomicrobium vannielii (Dow and Whittenbury, 1980). A majority of Type II organisms are able to form rosettes of bacterial cells, held together and anchored at their non-flagellated poles by a polysaccharide material. Such aggregates can be visualised under the light microscope (Whittenbury et al., 1970). The mode of carbon assimilation in Type II organisms is the serine pathway. They also possess a complete TCA cycle.

The facultative methanotrophs have been assigned to the Type II organisms. The first facultative methanotroph was reported in 1974 (Patt et al., 1974), and was placed in a new genus Methylobacterium. Since then several more strains have been described, all being Gram negative rods with a

Type II membrane arrangement, and assimilating one carbon compounds via the serine pathway. It should be noted that their growth on methane is slow compared to growth on multicarbon compounds, and it has been suggested that the genetic information for growth on methane may be encoded on a plasmid, thus explaining the ease by which some facultative methanotrophs can lose this characteristic (O'Connor, 1981).

Methanotrophic yeasts:

The first reported isolation of a yeast able to oxidise methane was in 1979 (Wolf and Hanson, 1979). All the strains which are currently known belong to one of four species, Sporobolomyces roseus, Sporobolomyces gracilis, Rhodotorula glutinis, and Rhodotorula rubra (Wolf, 1981). These organisms are unable to grow on methanol or formate but some grow on methylamine and higher alkanes. Their growth rate is extremely slow, with a generation time on methane of more than two days. All are budding yeasts, forming bright pink colonies on yeast extract. At present little is known about the enzymes concerned with methane assimilation.

Moving away from the methanotrophs, a brief consideration will now be made of organisms unable to utilise methane as a source of carbon and energy.

2. Obligate methylotrophs unable to utilise methane

These organisms form a distinct group. They cannot grow on methane or formate, but all grow extremely well on methanol and a majority can grow on methylamine. All of the organisms contained within this group are strictly aerobic,

Gram negative, non-pigmented, non-sporulating rods, motile by a single polar flagellum. All utilise the ribulose monophosphate cycle as their method of carbon fixation, the variant of the cycle involved being that using Entner-Doudoroff enzymes in the cleavage phase, and transaldolase in the rarrangement phase. It has been suggested that all the organisms contained in this group should be placed in the genus Methylophilus, and it appears that many newly isolated strains are being classified in this way (Strand and Lidstrom, 1984). It has been noted that some obligate non-methane utilising methylotrophs, for example Methylophilus methylotrophus, Pseudomonas methanolica, Pseudomonas C (Rokum et al., 1978) and Pseudomonas W1 (Dahl et al., 1972), have a thick cell wall with an undulating outline. Sometimes the undulations may be so great that they form protrusions of the outer membrane. Analysis of the cell envelope of these organisms shows that the lipid content is up to five times greater than that present in the cell surface of facultative methylotrophs.

It is interesting to speculate as to the reason why certain organisms are obligate methylotrophs. In all the cases considered so far the reason appears to be that the organism lacks a complete TCA cycle. The TCA cycle normally has two functions: (1) to provide NAD(P)H and ATP for energy, and (2) to provide carbon precursor for biosynthesis. During methylotrophic growth only the second function needs to be carried out, and this does not require the enzyme 2-oxoglutarate dehydrogenase. Up to now all obligate methylotrophs that have been studied have been found to lack this enzyme. All facultative methylotrophs have a complete TCA cycle; however mutants of the facultative organism Pseudomonas AM1 which have lost the ability to

synthesise 2-oxoglutarate dehydrogenase, fail to grow on multicarbon compounds and effectively become obligate methylotrophs (Taylor and Anthony, 1976). It therefore appears that the biochemical basis of obligate methylotrophy is the lack of 2-oxoglutarate dehydrogenase. This makes the TCA cycle inoperable.

3. Facultative methylotrophs unable to utilise methane

This group contains a varied selection of differing methylotrophs and is best described by dividing the organisms up into sub-groups:

(i) Gram-negative facultative methylotrophs. This group contains the pink facultative methylotrophs which assimilate one carbon compounds via the serine pathway. They are motile, often produce red carotenoid pigments, and most belong to the genus Pseudomonas.

Also within this group are the non-pigmented Pseudomonads. These are rods, motile by a single polar flagellum, and most assimilate one carbon compounds via the serine pathway. The one exception to this is Pseudomonas oleovorans which has been reported to use the ribulose monophosphate cycle to assimilate its carbon (Loginova and Trotsenko, 1977; Sokolov and Trotsenko, 1978a).

Finally the group contains the Hyphomicrobia. These are restricted facultative methylotrophs. They are stalked, motile organisms, which reproduce by budding and have the ability to grow anaerobically on methanol using nitrate as a final electron acceptor.

(ii) Gram-negative or Gram-variable rods. All of the organisms in this group are Gram-negative at some stage of growth. However, Arthrobacter species can be Gram positive

and show rod/coccal pleomorphism. The assimilation of one carbon compounds is either via the ribulose monophosphate cycle or the serine pathway, Arthrobacter P1 (Levering et al., 1981a) being reported to have both pathways present during growth using choline as sole source of carbon (Levering et al., 1981b).

(iii) Gram-positive methylotrophs. Only a few of these organisms are known. Most are restricted facultative methylotrophs many belong to the genus Bacillus. The ribulose monophosphate cycle is used by these organisms to assimilate one carbon compounds. Such an organism (not belonging to the genus Bacillus) is Nocardia sp.239 (Streptomyces sp.239). This was originally reported to operate both the ribulose monophosphate cycle and the serine pathway (Kato et al., 1977a). However, more recent work indicates that the role of the serine pathway in the assimilation of one carbon compounds is a minor one compared to that of the ribulose monophosphate cycle (Hazeu et al., 1983).

(iv) Organisms using the ribulose bisphosphate cycle. Bacteria in this group assimilate reduced one carbon compounds by initially oxidising them to carbon dioxide, then using the ribulose bisphosphate cycle to fix the carbon. This group are often referred to as autotrophic methylotrophs or pseudomethylotrophs (Zatman, 1981). The Rhodopseudomonads account for a number of organisms within this group. These bacteria are red, motile, phototrophic and reproduce by budding. Rhodopseudomonas gelatinosa (Wertlieb and Vishniac, 1967) was the first species to be reported to photosynthetically incorporate low levels of ^{14}C methane into cell material under an atmosphere of methane and carbon dioxide. Rhodopseudomonas palustris (Yoch and Lindstrom, 1967; Stokes and Hoare, 1969)

was found to be capable of growth on formate anaerobically in the light, and Rhodopseudomonas acidophila will utilise methanol/carbon dioxide or formate anaerobically in the light (Quayle and Pfennig, 1975; Sahm et al., 1976a). More recently the latter organism has been found to grow at reduced oxygen tensions, anaerobically in the dark, using methanol/carbon dioxide or formate as sole source of carbon and energy (Seifert and Pfennig, 1979). During anaerobic growth on methanol, carbon dioxide is required in order to act as an electron acceptor.

A number of organisms in this group are chemoautotrophs, a good example being Paracoccus denitrificans which is able to grow anaerobically on methanol with nitrate as a terminal electron acceptor (Bamforth and Quayle, 1978).

Pseudomonas oxalaticus is another bacterium contained within this group, differing from those already mentioned in having no photosynthetic or chemoautotrophic capacities, but still using the ribulose bisphosphate cycle to fix its C_1 substrate, formate (Quayle and Keech, 1959a, 1959b). This organism has provided a good subject with which to study the regulation of the ribulose bisphosphate cycle using mixed substrate cultures (Dijkhuizen and Harder, 1979a, 1979b, 1984).

(v) Fungi. The methanotrophic yeasts have already been described. In this section a consideration will be made of the non-methane utilising fungi capable of using C_1 compounds as a carbon source. Three species of mycelial fungi able to utilise methanol have been isolated, these being Trichoderma lignorum (Tye and Willets, 1973), Gliocladium deliquescens (Sakaguchi et al., 1975), and Paecilomyces varioti (Sakaguchi et al., 1975). However a majority of fungal methylotrophs

are yeasts. The first reported isolation of a methylotrophic yeast was by Ogata et al., (1969), who isolated Kloeckera sp.2201 on methanol as carbon source. This organism is now believed to be Candida boidinii (van Dijken et al., 1981). Since then many other methylotrophic yeasts have been described, most being species of Hansenula, Candida, Pichia and Torulopsis. All of the yeasts divide by budding and share the dihydroxyacetone pathway as their mode of carbon assimilation. It has become clear that in contrast to methylotrophic bacteria, the yeasts form a homogeneous group with respect to their mode of methanol metabolism.

4. Methanogens

Methanogens, as the name indicates, are strictly anaerobic organisms which produce methane during the course of their growth. A few methanogens are able to grow at the expense of formate, methanol or methylated amines and thus can be considered as methylotrophs, whilst most methanogens assimilate their cell carbon at the level of carbon dioxide, obtaining the energy for this process from the anaerobic reduction of carbon dioxide to methane, using hydrogen as electron donor, and can thus be considered as autotrophs.

Methylotrophic methanogens are in most respects totally dissimilar to the aerobic methylotrophs. In fact the methanogens appear completely distinct from almost all other known bacteria with the exception of the extreme halophiles (Halobacterium sp.), and the thermoacidophiles (Thermoplasma sp. and Sulfolobus sp.). In the light of this it has been proposed (Woese et al., 1978) that a separate primary kingdom, the Archaebacteria, be recognised amongst the prokaryotes

to include these three groups of bacteria. All of the remaining bacteria would then be placed in the Eubacteria.

The methanogens are very stringent anaerobes. The best growth substrate for most of these organisms is hydrogen and carbon dioxide. The energy available from the coupled reduction of carbon dioxide by hydrogen being sufficient to support reasonable growth. Formate can be matabolised by most species of methanogens. However, it is probably first oxidised by a formate dehydrogenase to hydrogen and carbon dioxide. Perhaps the most metabolically diverse strains are species of Methanosarcina which can grow on the previously mentioned compounds and also on acetate, methanol, methylamine, dimethylamine, trimethylamine and ethyldimethylamine. In addition some methanogens can convert carbon monoxide to methane, and a few strains can grow on it.

Although as mentioned previously the organisms growing at the expense of carbon dioxide can be considered as autotrophs, the growth of these methanogens is totally different from that of other photoautotrophs and chemoautotrophs as it does not involve the ribulose bisphosphate pathway. None of the key enzymes of the ribulose monophosphate cycle, dihydroxyacetone pathway or serine pathway have been found in the methanogens. Recent work has indicated that the assimilation of carbon dioxide in methanogens could proceed via a carbon dioxide reduction pathway (Jansen et al., 1982).

Biochemistry of growth on C_1 compounds

The metabolic transformations of C_1 compounds can be divided into two areas. These are:

(1) The methods by which carbon is incorporated into cell

material. These are generally called assimilatory pathways.

(2) The methods by which the growth substrates are oxidised to yield energy. These are known as dissimilatory pathways.

Pathways of C_1 assimilation

In methylotrophic organisms, the incorporation of carbon into cellular material occurs mostly at the oxidation levels of formaldehyde and carbon dioxide. At present five assimilation sequences have been postulated for the incorporation of one carbon compounds by methylotrophs. These are: the ribulose bisphosphate cycle, the carbon dioxide reduction pathway of methanogens, the ribulose monophosphate cycle, the dihydroxyacetone pathway, and the serine pathway. Each of these assimilation sequences will now be considered in more detail.

1. The ribulose bisphosphate cycle.

The first C_1 assimilation sequence to be established was the ribulose bisphosphate cycle of carbon dioxide fixation (Bassham *et al.*, 1954). This was originally discovered in green plants, but also operates during non-photosynthetic growth in chemoautotrophs and methylotrophs. The cycle is shown in Figures 1 and 2. The overall reaction synthesises one molecule of glyceraldehyde 3-phosphate from three molecules of carbon dioxide:

$$3CO_2 + 6NAD(P)H_2 + 9ATP \longrightarrow \text{Glyceraldehyde 3-phosphate} + 6NAD(P)^+ + 9ADP + 8Pi$$

The equation clearly shows that carbon assimilation at the

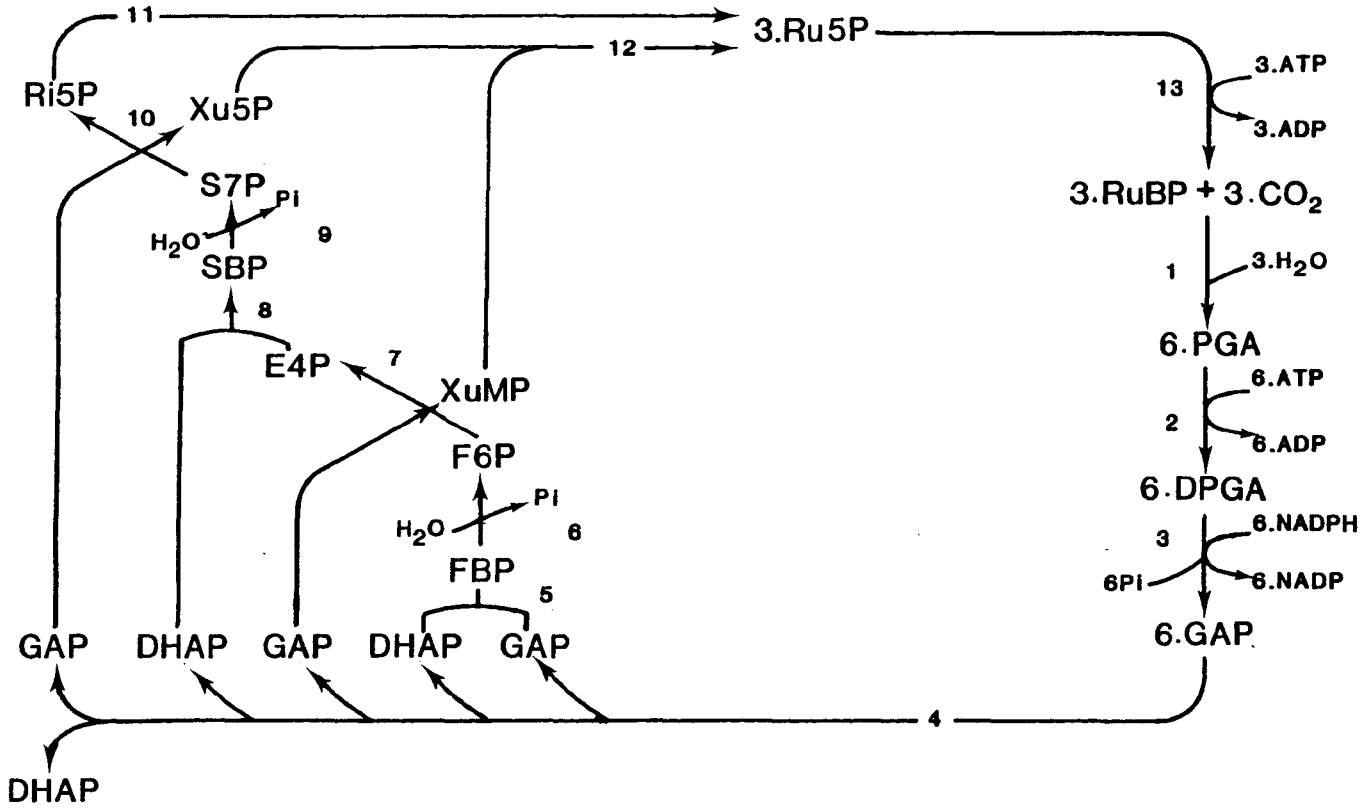
Figure 1 The ribulose bisphosphate cycle (sedoheptulose bisphosphatase variant) for the assimilation of CO_2 into cell constituents

Enzymes:

1. Ribulose bisphosphate carboxylase
2. Phosphoglycerate kinase
3. Glycerol 3-phosphate dehydrogenase
4. Triose phosphate isomerase
5. Fructose bisphosphate aldolase
6. Fructose 1,6-bisphosphatase
7. Transketolase
8. Aldolase
9. Sedoheptulose 1,7-bisphosphatase
10. Transketolase
11. Phosphoriboisomerase
12. Ribulose phosphate 3-epimerase
13. Phosphoribulokinase

Abbreviations:

GAP	glyceraldehyde 3-phosphate
FBP	fructose 1,6-bisphosphate
F6P	fructose 6-phosphate
Xu5P	xylulose 5-phosphate
E4P	erythrose 4-phosphate
SBP	sedoheptulose 1,7-bisphosphate
S7P	sedoheptulose 7-phosphate
Ri5P	ribose 5-phosphate
Ru5P	ribulose 5-phosphate
RuBP	ribulose 1,5-bisphosphate
PGA	phosphoglycerate
DPGA	1,3-diphosphoglycerate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12
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Ri5P
10
Xu5P
13
3.ATP
3.ADP
S7P
Pi
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3.RuBP + 3.CO_2
1
3.H_2O
8
6.PGA
E4P
7
XuMP
6.ATP
2
6.ADP
F6P
6.DPGA
Pi
H_2O
6
3
6.NADPH
FBP
6Pi
6.NADP
5
6.GAP
GAP
DHAP
GAP
DHAP
GAP
4
DHAP

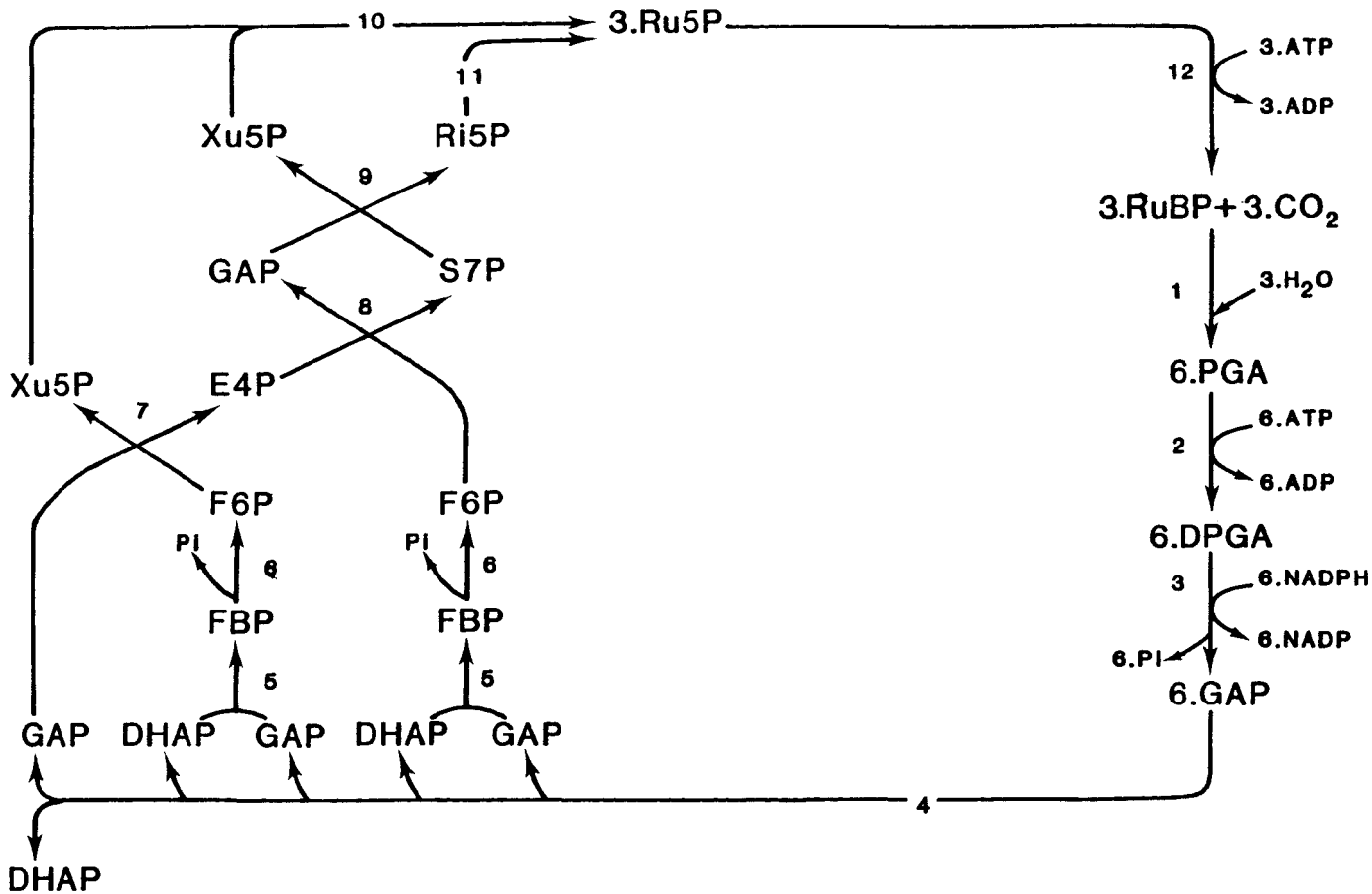
Figure 2 The ribulose bisphosphate cycle (transaldolase variant) for the assimilation of CO_2 into cell constituents

Enzymes:

1. Ribulose bisphosphate carboxylase
2. Phosphoglycerate kinase
3. Glycerol 3-phosphate dehydrogenase
4. Triose phosphate isomerase
5. Fructose bisphosphate aldolase
6. Fructose bisphosphatase
7. Transketolase
8. Transaldolase
9. Transketolase
10. Ribose phosphate 3-epimerase
11. Phosphoriboisomerase
12. Phosphoribulokinase

Abbreviations:

GAP	glyceraldehyde 3-phosphate
FBP	fructose 1,6-bisphosphate
F6P	fructose 6-phosphate
Xu5P	xylulose 5-phosphate
E4P	erythrose 4-phosphate
S7P	sedoheptulose 7-phosphate
Ri5P	ribose 5-phosphate
Ru5P	ribulose 5-phosphate
RuBP	ribulose 1,5-bisphosphate
PGA	phosphoglycerate
DPGA	1,3-diphosphoglycerate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11
12
3.ATP
3.ADP
Xu5P
Ri5P
9
3.RuBP+3.CO2
GAP
S7P
1
3.H2O
8
6.PGA
Xu5P
E4P
7
6.ATP
2
6.ADP
F6P
F6P
6.DPGA
PI
6
PI
6
3
6.NADPH
6.NADP
FBP
FBP
6.PI
6.GAP
5
5
GAP
DHAP
GAP
DHAP
GAP
4
DHAP

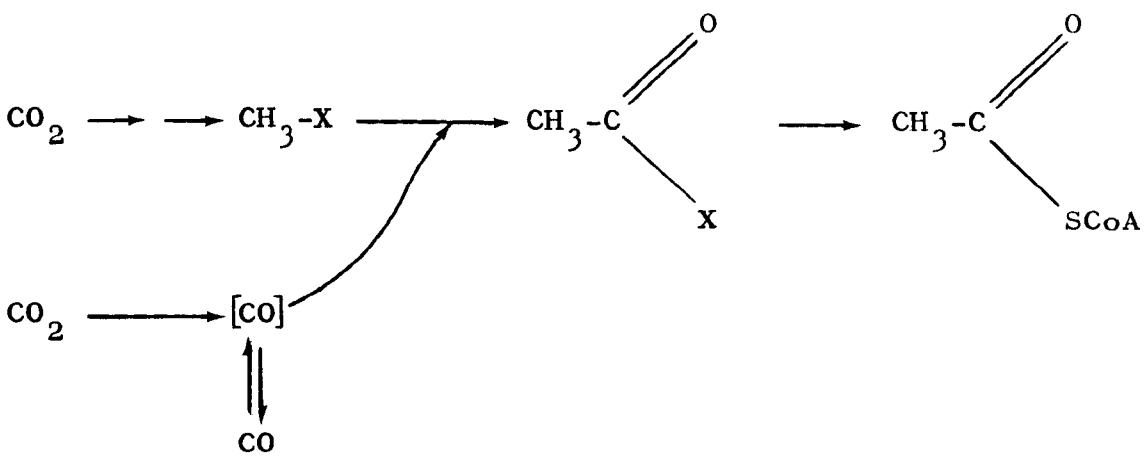
level of carbon dioxide is much less efficient than its assimilation at a higher reduction level, because of the heavy demands for ATP, and reducing power. The cycle itself can be divided into three parts. The first is known as the fixation phase in which three molecules of ribulose 1,5-bisphosphate are carboxylated to yield six molecules of 3-phosphoglycerate. The enzyme responsible for this step is ribulose 1,5-bisphosphate carboxylase, and is unique to the cycle. The second phase of the cycle is reduction. Here the six molecules of 3-phosphoglycerate are reduced at the expense of NADH, to yield six molecules of glyceraldehyde phosphate, one of which is the product of the cycle. Moving on to the third part of the cycle, this is called the rearrangement phase in which the five remaining molecules of glyceraldehyde phosphate are converted back into ribulose 1,5-bisphosphate. There are two possible methods of rearrangement depending on the enzymes used. One depends on sedoheptulose bisphosphatase (Figure 1), the other uses the enzyme transaldolase (Figure 2). Both rearrangement variants yield ribulose 5-phosphate which is converted back to ribulose 1,5-bisphosphate by the enzyme phosphoribulokinase which, like ribulose 1,5-bisphosphate carboxylase is unique to the cycle.

As previously mentioned, this pathway is an energetically very expensive method of assimilating reduced C_1 compounds. However, as most of the pseudomethylotrophs are also facultative autotrophs, the acquisition of the few enzymes required to oxidise reduced C_1 compounds to carbon dioxide confers the bonus of additional nutritional versatility on the organism.

2. The carbon dioxide reduction pathway.

Some methanogens, for example Methanobacterium

thermoautotrophicum, are able to grow autotrophically at the expense of carbon dioxide by a pathway other than the ribulose bisphosphate cycle. This metabolic sequence is known as the carbon dioxide reduction pathway or activated acetic acid pathway (Jansen et al., 1984), and proceeds via the formation of acetyl coenzyme A from two molecules of carbon dioxide. The formation of acetyl coenzyme A has been proposed to occur in the following way (Stupperich et al., 1983):



The enzyme responsible for the formation of carbon monoxide from carbon dioxide is carbon monoxide dehydrogenase. It is thought that a bound form of carbon monoxide takes part in the subsequent reaction; however, there appears to be some exchange of free with bound carbon monoxide within the cell.

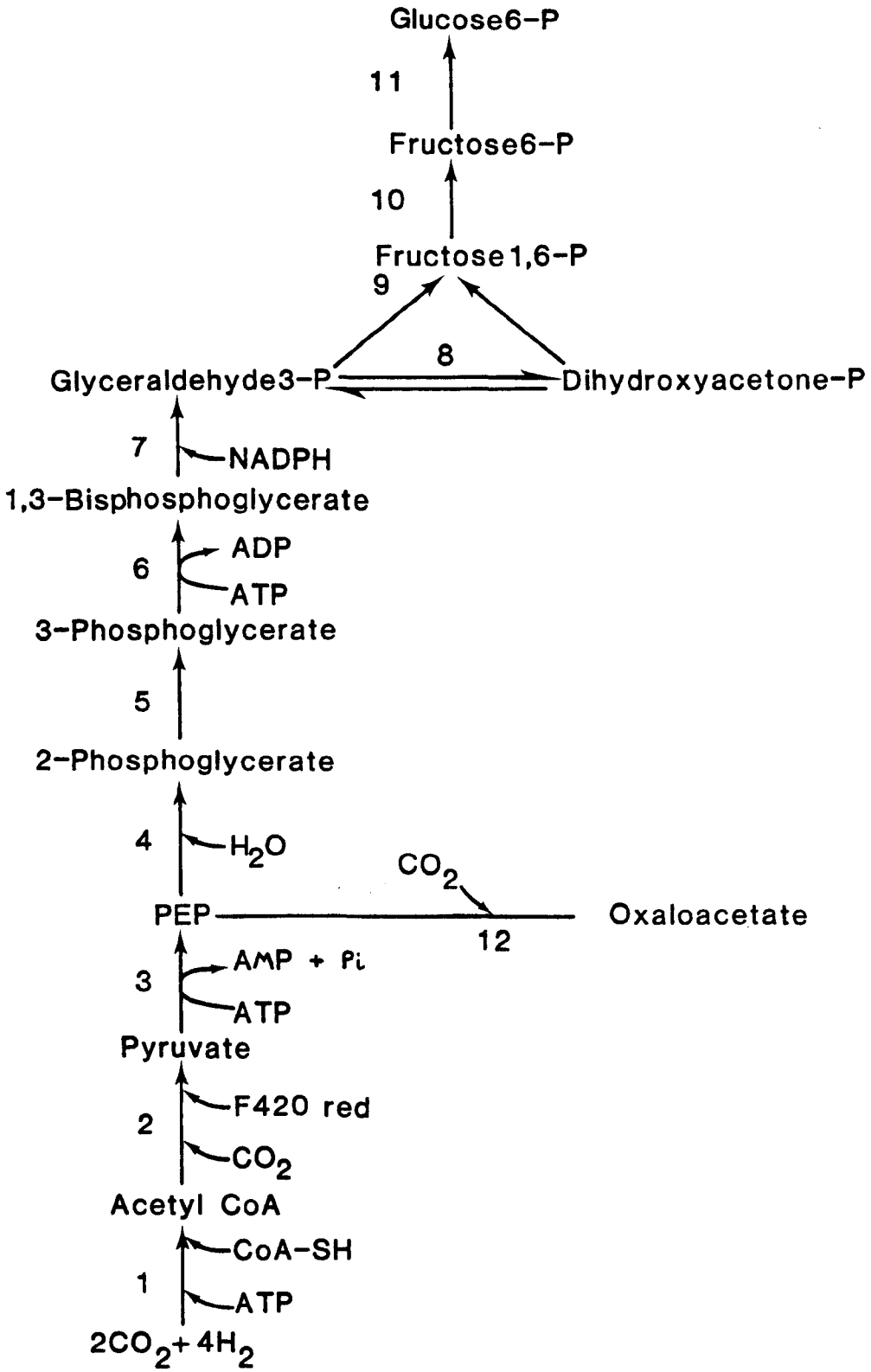
The metabolism of the acetyl coenzyme A produced by this reaction proceeds via reductive carboxylation, catalysed by a Factor 420 dependent pyruvate synthetase, to yield pyruvate. The pyruvate thus formed can then be metabolised via the pathway shown in Figure 3 (Jansen et al., 1982).

The pathway is reported to be exergonic under standard conditions, but not under physiological conditions, requiring four to five ATP equivalents to produce a molecule of triose

Figure 3 CO_2 reduction pathway

Enzymes:

1. Synthesis of acetyl CoA
2. Pyruvate synthase
3. PEP synthetase
4. Enolase
5. Phosphoglycerate mutase
6. Phosphoglycerate kinase
7. Glyceraldehyde P dehydrogenase
8. Triase phosphate isomerase
9. FBP aldolase
10. FBP phosphatase
11. Hexose phosphate isomerase
12. PEP carboxylase

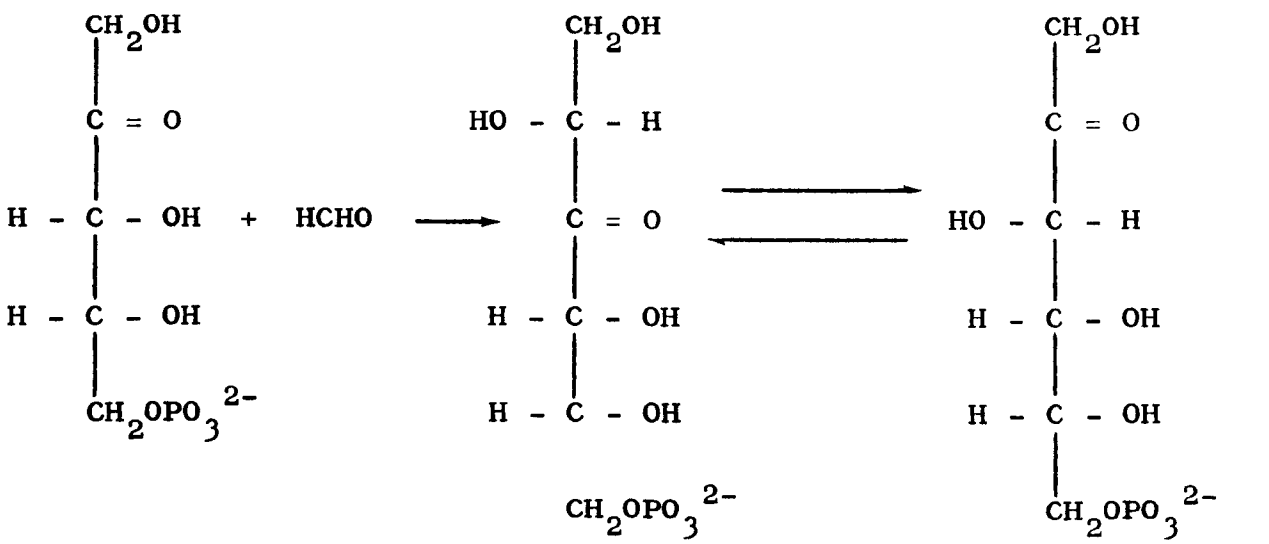

Glucose6-P
11
Fructose6-P
10
Fructose 1,6-P
9
8
Glyceraldehyde3-P
Dihydroxyacetone-P
7
NADPH
1,3-Bisphosphoglycerate
6
ADP
ATP
3-Phosphoglycerate
5
2-Phosphoglycerate
4
H_2O
CO_2
PEP
Oxaloacetate
12
3
AMP + Pi
ATP
Pyruvate
2
F420 red
CO_2
Acetyl CoA
1
CoA-SH
ATP
$2CO_2 + 4H_2$

phosphate. The ribulose bisphosphate cycle requires twice as much energy (nine ATP per triose phosphate), making the carbon dioxide reduction pathway a much more economical mode of autotrophic growth.

Recent work has demonstrated that this pathway is not confined to autotrophic methanogens, but it has also been found to be present in the autotrophic homoacetogenic bacterium Acetobacterium woodii (Eden and Fuchs, 1982; Diekert et al., 1984), and in the sulphate reducing bacterium Desulfovibrio barsii (Jansen et al., 1984).

3. The ribulose monophosphate cycle.

The initial work on this cycle was carried out using Pseudomonas methanica (Johnson and Quayle, 1965; Kemp and Quayle, 1966). The biosynthetic sequence that emerged from subsequent work (Kemp, 1974), showed that the key reactions were catalysed by 3-hexulose phosphate synthase and phospho 3-hexuloisomerase.



Ribulose 5-phosphate

3-Hexulose 6-phosphate

Fructose 6-phosphate

The presence of these enzymes enabled a net assimilation sequence to be constructed (Figures 4 and 5) (Strøm et al., 1974).

The pathway has been divided into three stages, these being the fixation, cleavage and rearrangement phases. The entry of C_1 compounds into the cycle is at the oxidation level of formaldehyde which is combined with ribulose 5-phosphate by a condensation reaction catalysed by hexulose phosphate synthase to yield 3-hexulose 6-phosphate. This is then isomerised to fructose 6-phosphate by phosphohexuloisomerase. These initial reactions are known as the fixation phase. The second phase of the pathway involves the cleavage of fructose 6-phosphate by one of two alternative routes. In one route cleavage is using enzymes of the Embden-Meyerhof pathway, fructose 6-phosphate being converted into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, by fructose bisphosphate aldolase. The alternative to this is the utilisation of enzymes of the Entner-Doudoroff pathway with fructose 6-phosphate being converted sequentially into glucose 6-phosphate, then 6-phosphogluconate. The latter compound is then cleaved via 2-keto 3-deoxy 6-phosphogluconate to give glyceraldehyde 3-phosphate and pyruvate. The last phase of the cycle involves the rearrangement of triose and hexose phosphates in order to regenerate the formaldehyde acceptor molecule ribulose 5-phosphate. However, there are two possible alternative routes for the rearrangement reactions, one involving transaldolase, the other sedoheptulose bisphosphatase.

As there are two methods of cleavage, and two of rearrangement, there are four possible variants of the ribulose monophosphate cycle. However, at present only three of the

Figure 4 The ribulose monophosphate cycle showing both methods of cleavage and sedoheptulose phosphatase rearrangement

Enzymes:

hps	hexulose phosphate synthase
phi	phosphohexuloisomerase
pgi	phosphoglucoisomerase
pfk	phosphofructokinase
gpd	glucose 6-phosphate dehydrogenase
fda	fructose phosphate aldolase
tk	transketolase
tda	aldolase
sda	sedoheptulose phosphatase
rpe	ribulose phosphate 3-epimerase
pri	phosphoriboisomerase

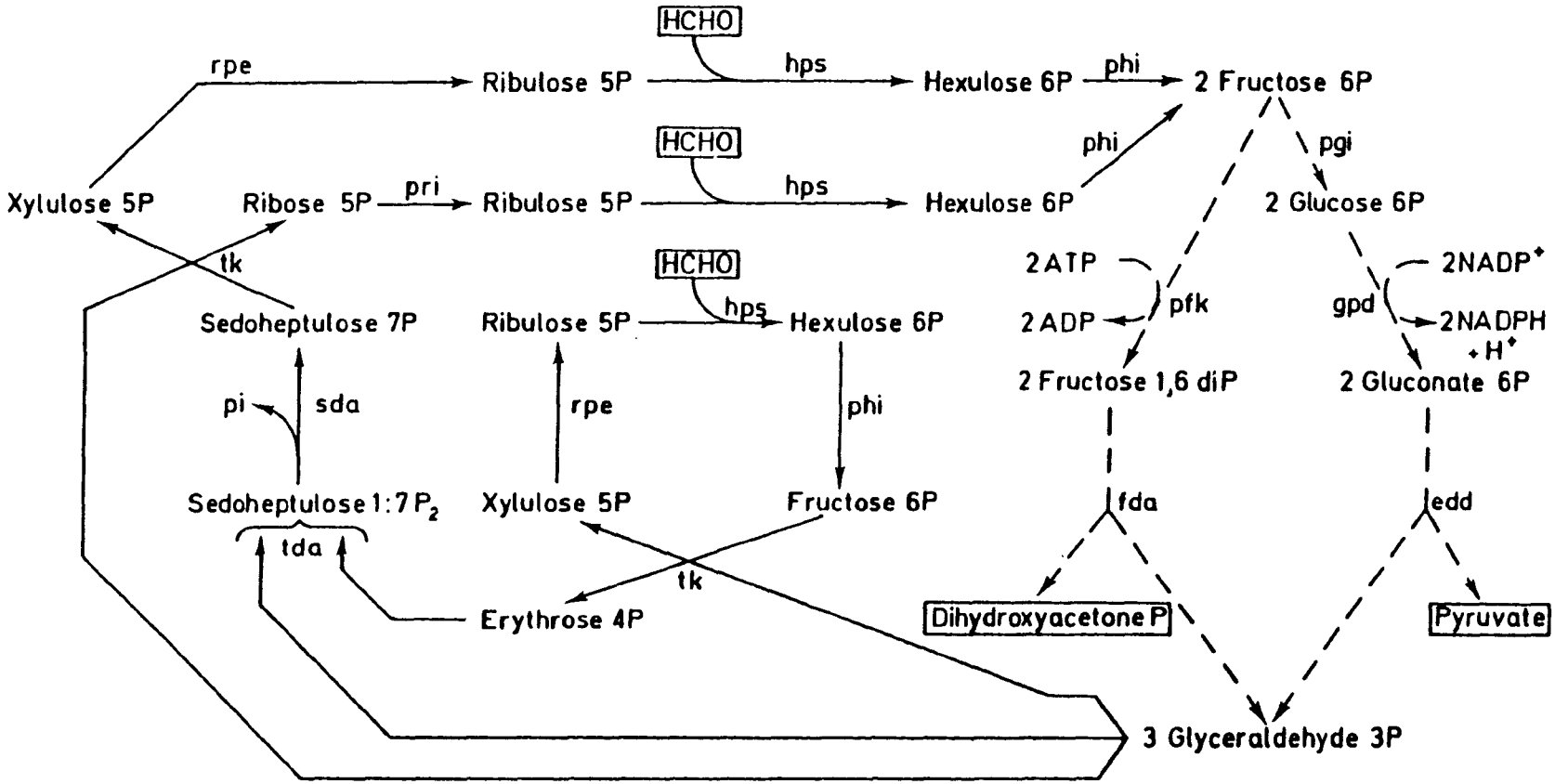
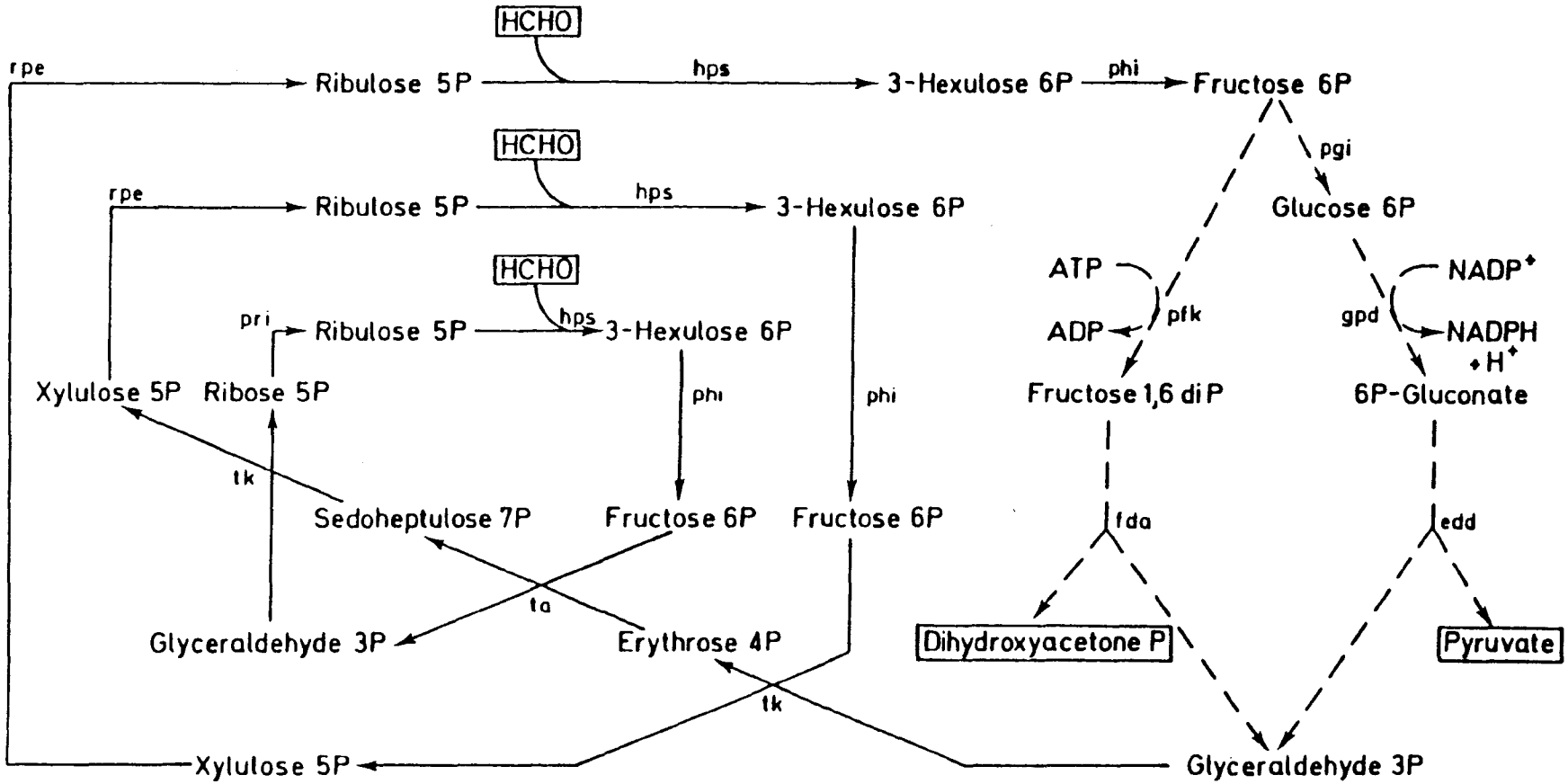

HCHO
rpe
hps
phi
Ribulose 5P
Hexulose 6P
2 Fructose 6P
HCHO
phi
pgi
Xylulose 5P
Ribose 5P
pri
Ribulose 5P
hps
Hexulose 6P
2 Glucose 6P
2ATP
2NADP+
tk
HCHO
pfk
gpd
2ADP
2NADPH
+H+
hps
Sedoheptulose 7P
Ribulose 5P
Hexulose 6P
2 Fructose 1,6 diP
2 Gluconate 6P
pi
sda
rpe
phi
fda
edd
Sedoheptulose 1:7 P2
Xylulose 5P
Fructose 6P
tda
tk
Erythrose 4P
Dihydroxyacetone P
Pyruvate
3 Glyceraldehyde 3P

Figure 5 The ribulose monophosphate cycle showing both methods of cleavage and the transaldolase rearrangement

Enzymes:

hps	hexulose phosphate synthase
phi	phosphohexuloisomerase
pgi	phosphoglucoisomerase
gpd	glucose 6-phosphate dehydrogenase
pfk	phosphofructokinase
fda	fructose bisphosphate aldolase
tk	transketolase
ta	transaldolase
pri	phosphoriboisomerase
rpe	ribulose phosphate 3-epimerase


HCHO
rpe
Ribulose 5P
hps
3-Hexulose 6P
phi
Fructose 6P
pgi
Glucose 6P
HCHO
rpe
Ribulose 5P
hps
3-Hexulose 6P
HCHO
pri
Ribulose 5P
hps
3-Hexulose 6P
ATP
ADP
pfk
Fructose 1,6 diP
NADP+
gpd
NADPH
+H+
6P-Gluconate
Xylulose 5P
Ribose 5P
phi
phi
tk
Sedoheptulose 7P
Fructose 6P
Fructose 6P
fda
edd
ta
Glyceraldehyde 3P
Erythrose 4P
Dihydroxyacetone P
Pyruvate
tk
Xylulose 5P
Glyceraldehyde 3P

variants have been found in methylotrophic bacteria. The 2-keto 3-deoxy 6-phosphogluconate/transaldolase variant appears to be found mainly in obligate methanotrophs such as Methylococcus capsulatus and Methylomonas methanica (Strøm et al., 1974), and the obligate non-methane utilising methylotrophs like Methylophilus methylotrophus (Beardsmore et al., 1982). The fructose bisphosphate aldolase/ sedoheptulose bisphosphatase variant occurs mainly in facultative bacteria such as Organisms PM6 and S2A1 (Colby and Zatman, 1975). At present the only organism that has been shown to use the fructose bisphosphate aldolase/transaldolase variant has been the facultative methylotroph Arthrobacter P1 (Levering et al., 1982). As yet the 2-keto 3-deoxy 6-phosphogluconate/ sedoheptulose bisphosphatase variant of the cycle has not been reported in any organism.

4. The serine pathway.

This pathway is widespread amongst methylotrophic bacteria, examples being Pseudomonas MA, Hyphomicrobium species, Pseudomonas aminovorans, Pseudomonas AM1 and Bacterium 5H2. The establishment of the serine pathway has come from the work of a number of research groups over many years (Quayle, 1972; Anthony, 1975; Hersh and Bellion, 1972; Salem et al., 1972, 1973a, b; Harder et al., 1973; Attwood and Harder, 1978). The net reaction of the pathway is:

$$2HCHO + CO_2 + 2ATP + 2NADH + FMN \longrightarrow \text{Phosphoglycerate} + 2NAD + 2ADP + FMNH_2$$

The equation shows that two thirds of the assimilated carbon enters the pathway at the oxidation level of formaldehyde, the remaining one third enters as carbon dioxide.

The complete serine pathway is shown in Figure 6. Until 1972 a basic problem remained as to how the glycine skeleton necessary for each passage through the serine pathway was synthesised. This was solved by Bellion and Hersh (1972) for certain organisms such as Pseudomonas MA. They found that during growth on methylamine, the organism produced high levels of isocitrate lyase and an ATP and coenzyme A-dependent, malate cleavage system. The malate cleavage system was subsequently discovered to be separable into two reactions, the first being malate thiokinase, the second being malyl CoA lyase. The latter cleaves malyl CoA into glyoxylate and acetyl CoA. However, it is known that certain serine pathway methylotrophs such as Pseudomonas AM1 possess neither isocitrate lyase nor malate thiokinase, and therefore there must be a second variant of the serine pathway. A possible solution to the problem of organisms lacking isocitrate lyase (ICL^- organisms), was put forward by Kortstee (1980, 1981), who proposed the oxidation of acetyl CoA to glyoxylate by a homoisocitrate cycle (Figure 7). This route involves the use of homocitrate synthase which condenses acetyl CoA with 2-oxoglutarate, to give homocitrate. A key enzyme of this cycle is homoisocitrate lyase which cleaves homoisocitrate into glyoxylate and glutarate. Such a cycle in ICL^- organisms, although appearing attractive, requires much more work to be done in order to confirm its presence, especially in the light of the difficulty experienced by some workers in repeating the experiments of Kortstee (Bellion et al., 1981).

Before moving on to the dihydroxyacetone pathway, which is confined to methylotrophic yeasts, a brief consideration will be made of the energetics of the three different

Figure 6 The serine pathway of C_1 fixation involving isocitrate lyase

Enzymes:

1. serine transhydroxymethylase
2. serine-glyoxylate aminotransferase
3. hydroxypyruvate reductase
4. glycerate kinase
5. enolase
6. PEP carboxylase
7. malate dehydrogenase
8. malate thiokinase
9. malyl CoA lyase
10. citrate synthase
11. aconitase
12. isocitrate lyase
13. succinate dehydrogenase
14. fumarase

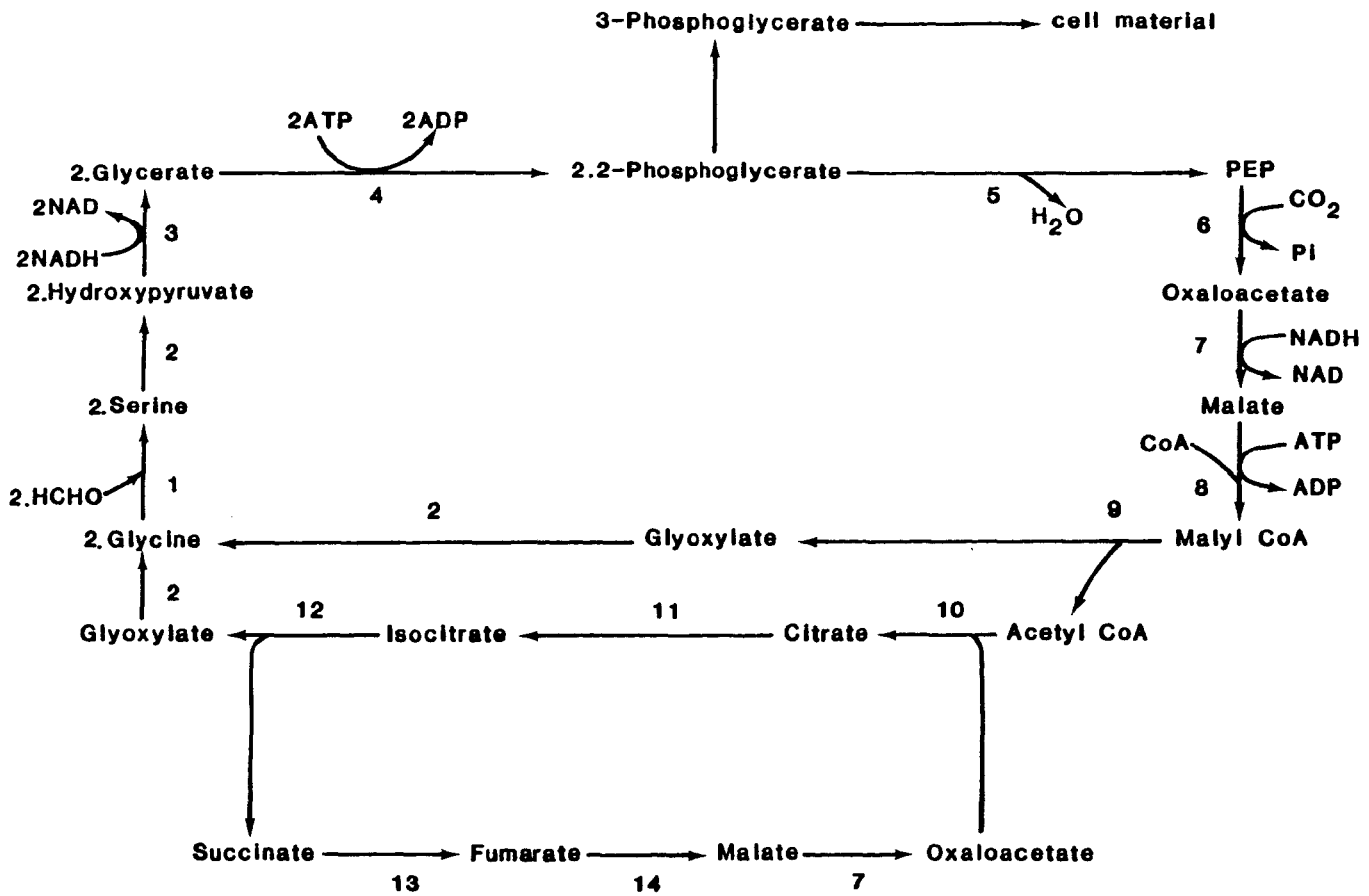

3-Phosphoglycerate
cell material
2ATP
2ADP
2.Glycerate
4
2.2-Phosphoglycerate
5
H_2O
PEP
2NAD
2NADH
3
2.Hydroxypyruvate
6
CO_2
Pi
Oxaloacetate
2
7
NADH
NAD
2.Serine
Malate
CoA
ATP
2.HCHO
1
8
ADP
2
9
2.Glycine
Glyoxylate
Malyl CoA
2
12
11
10
Glyoxylate
Isocitrate
Citrate
Acetyl CoA
Succinate
Fumarate
Malate
Oxaloacetate
13
14
7

Figure 7 The homoisocitrate cycle for oxidation of acetyl-CoA to glyoxylate in ICL^- serine pathway organisms

Enzymes:

1. homocitrate synthase
2. homoisocitrate lyase
3. glutarate dehydrogenase
4. 2,3-dihydroglutarate hydratase
5. 2-hydroxyglutarate dehydrogenase

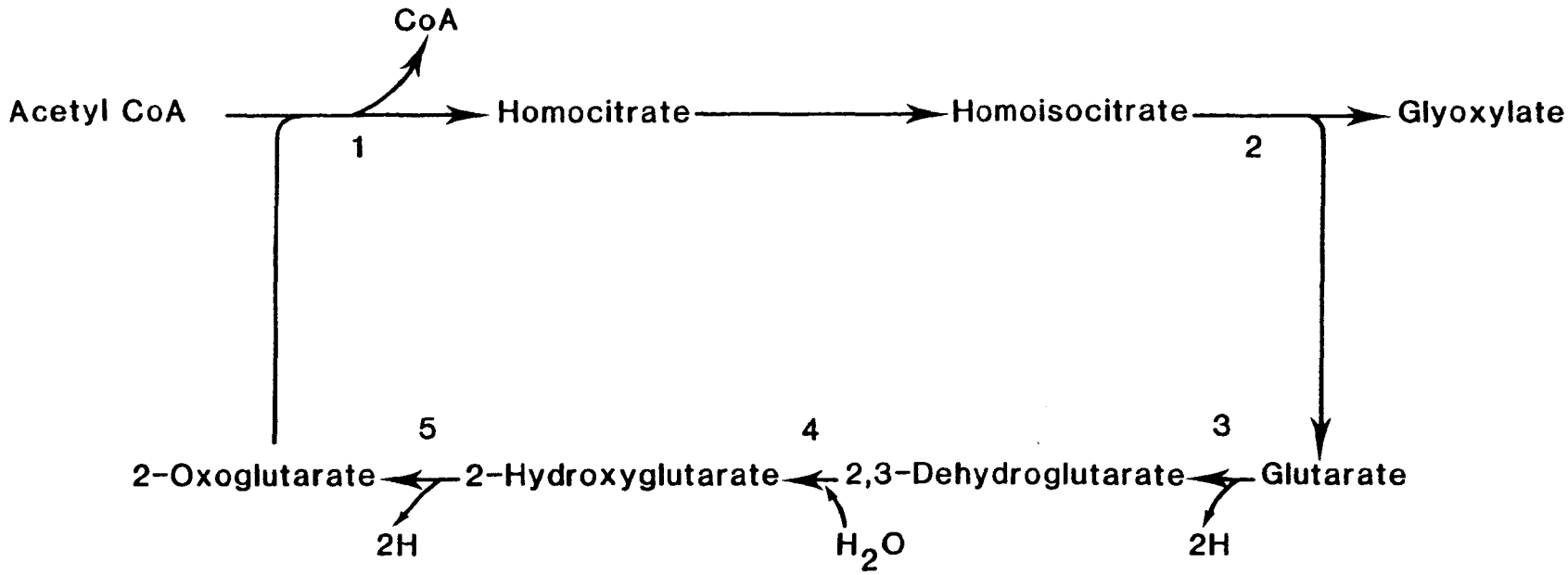

CoA
Acetyl CoA
Homocitrate
Homoisocitrate
Glyoxylate
1
2
5
4
3
2-Oxoglutarate
2-Hydroxyglutarate
2,3-Dehydroglutarate
Glutarate
2H
H_2O
2H

TABLE 1 Energy budgets for C_1 assimilation sequences normalised to pyruvate production

Cycle	Cleavage phase: FDP aldolase	Cleavage phase: KDPG aldolase	Rearrangement phase: Transaldolase	Rearrangement phase: SDP-ase	Reactants	Product	Energy change: NAD(P)H	Energy change: FPH	Energy change: ATP
RuMP	-	+	-	+	3HCHO		+ 1	0	- 3
	-	+	+	-	3HCHO		+ 1	0	0
	+	-	-	+	3HCHO		+ 1	0	0
	+	-	+	-	3HCHO		+ 1	0	+ 1
						Pyruvate			
ICL$^+$ / Serine					2HCHO + $1CO_2$		- 2	+ 1	- 2
RuDP			-	+	$3CO_2$		- 5	0	- 7
			+	-	$3CO_2$		- 5	0	- 7

bacterial C_1 assimilation pathways (Ferenci and Quayle, 1978; Anthony, 1982). In this analysis, where necessary, dihydroxyacetone phosphate is converted to pyruvate by established glycolytic steps, and account is taken of the resulting changes in energy budget. Table 1 shows the results of this examination. Comparison of the energetics of the ribulose monophosphate cycle, with those of the ICL^+ serine pathway and the ribulose bisphosphate cycle shows the great energetic cost to an organism of using carbon dioxide rather than formaldehyde as a C_1 unit in the assimilation process. However, it should be borne in mind that methylotrophs using the ribulose bisphosphate cycle oxidise reduced C_1 compounds to carbon dioxide before they can enter the cycle, and this process does generate reducing power. When the ribulose monophosphate cycle is examined, the most energetically favourable of the four variants is the fructose bisphosphate aldolase/transaldolase cycle with a net yield of 1 ATP and 1 NAD(P)H. The most unfavourable is the 2-keto 3-deoxy 6-phosphogluconate aldolase/sedoheptulose bisphosphatase variant which has a net production of 1 NAD(P)H, but has a requirement for 3 ATP. It is therefore not surprising that no organisms have yet been reported which use this variant of the pathway. The two energetically intermediate variants, 2-keto 3-deoxy 6-phosphogluconate aldolase/transaldolase and fructose bisphosphate aldolase/sedoheptulose bisphosphatase, have net yields of 1 NAD(P)H only. The ICL^+ serine pathway requires the use of 2 NAD(P)H and 2 ATP for every 2 formaldehyde and 1 carbon dioxide assimilated. However, one reduced flavoprotein is generated. This suggests that the pathway is less favourable than the ribulose monophosphate cycle in energetic terms.

5. The dihydroxyacetone pathway.

Yeasts like bacteria oxidise methanol to formaldehyde which is then used as the precursor of cell material. Early work on the elucidation of the formaldehyde fixing pathway showed that ^{14}C methanol was rapidly incorporated into hexose phosphates (Fujii and Tonomura, 1973; Fujii et al., 1974). The labelling patterns were similar to those obtained when bacteria containing the ribulose monophosphate cycle were given labelled methanol. Thus it was originally believed that yeasts had the same ribulose monophosphate cycle of C_1 assimilation as bacteria. However, when the activities of the characteristic enzymes of the ribulose monophosphate cycle were measured, "hexulose phosphate synthase" was found to be very low and the pentose phosphate fixation of formaldehyde, catalysed by cell-free extracts, often proved to be dependent on the present of ATP; phosphohexuloisomerase was undetectable. Later work (van Dijken et al., 1978), proposed that formaldehyde fixation may be mediated by a transketolase using xylulose 5-phosphate as ketol donor and formaldehyde as acceptor, thus yielding dihydroxyacetone and glyceraldehyde phosphate. Such a pathway was first considered a possible route for formaldehyde assimilation during the growth of Arthrobacter 2B2 on trimethylamine (Cox and Zatman, 1974). However, subsequently this organism was shown to use the ribulose monophosphate cycle.

Further work confirmed the operation of such a cycle (Waites and Quayle, 1983). The complete dihydroxyacetone cycle (or xylulose monophosphate cycle) is shown in Figure 8. The formaldehyde is fixed by a transketolase (now known as dihydroxyacetone synthase in order to differentiate it from

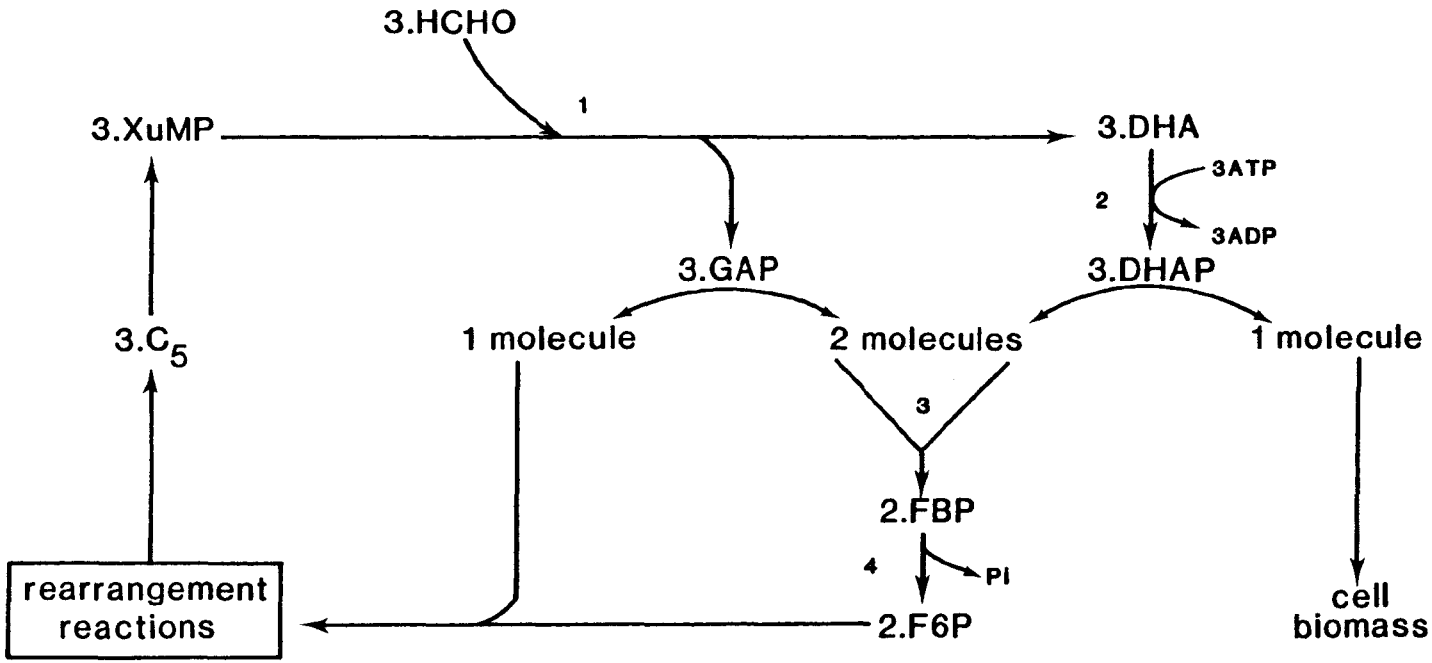
Figure 8 The dihydroxyacetone pathway of formaldehyde fixation

Enzymes:

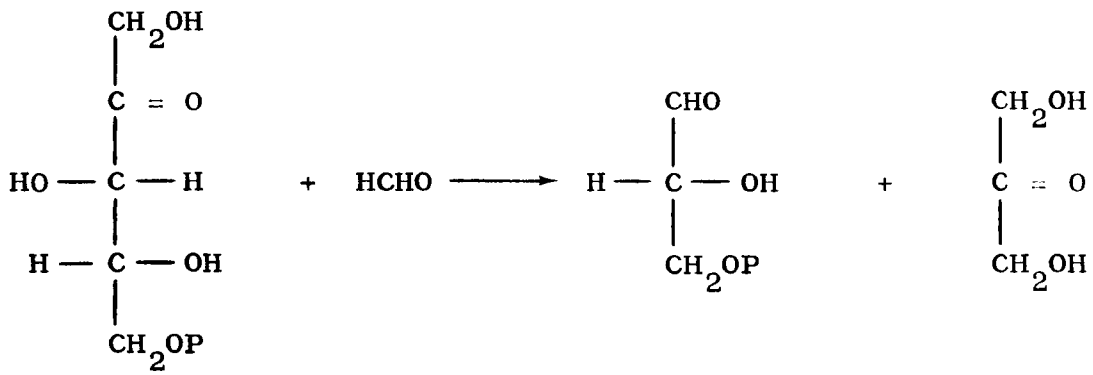
1. dihydroxyacetone synthase
2. triokinase
3. fructose bisphosphate aldolase
4. fructose bisphosphatase

Abbreviations:

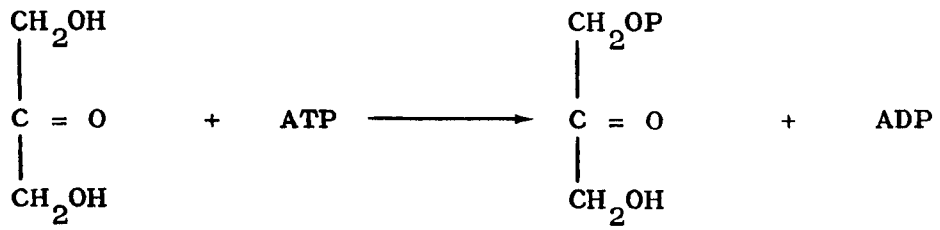
Xu5P	xylulose 5-phosphate
GAP	glyceraldehyde phosphate
DHAP	dihydroxyacetone phosphate
DHA	dihydroxyacetone
FBP	fructose 1,6-bisphosphate
F6P	fructose 6-phosphate


3.HCHO
1
3.XuMP
3.DHA
3ATP
2
3ADP
3.GAP
3.DHAP
3.C5
1 molecule
2 molecules
1 molecule
3
2.FBP
4
PI
rearrangement reactions
2.F6P
cell biomass

the classical transketolase contained within the organisms), and reacted with xylulose 5-phosphate to give glyceraldehyde 3-phosphate and dihydroxyacetone.



The dihydroxyacetone is then phosphorylated by triokinase to yield dihydroxyacetone phosphate.



Dihydroxyacetone phosphate is then condensed with glyceraldehyde phosphate by fructose bisphosphate aldolase to give fructose bisphosphate which is subsequently hydrolysed to fructose 6-phosphate by fructose bisphosphatase. Two molecules of fructose 6-phosphate together with one molecule of glyceraldehyde phosphate then enter a series of rearrangement reactions which could involve either transaldolase or sedoheptulose bisphosphatase in a similar way to the rearrangements of the ribulose monophosphate cycle, to reform three molecules of xylulose 5-phosphate.

Pathways of C_1 dissimilation

The dissimilatory pathways make both energy and reducing

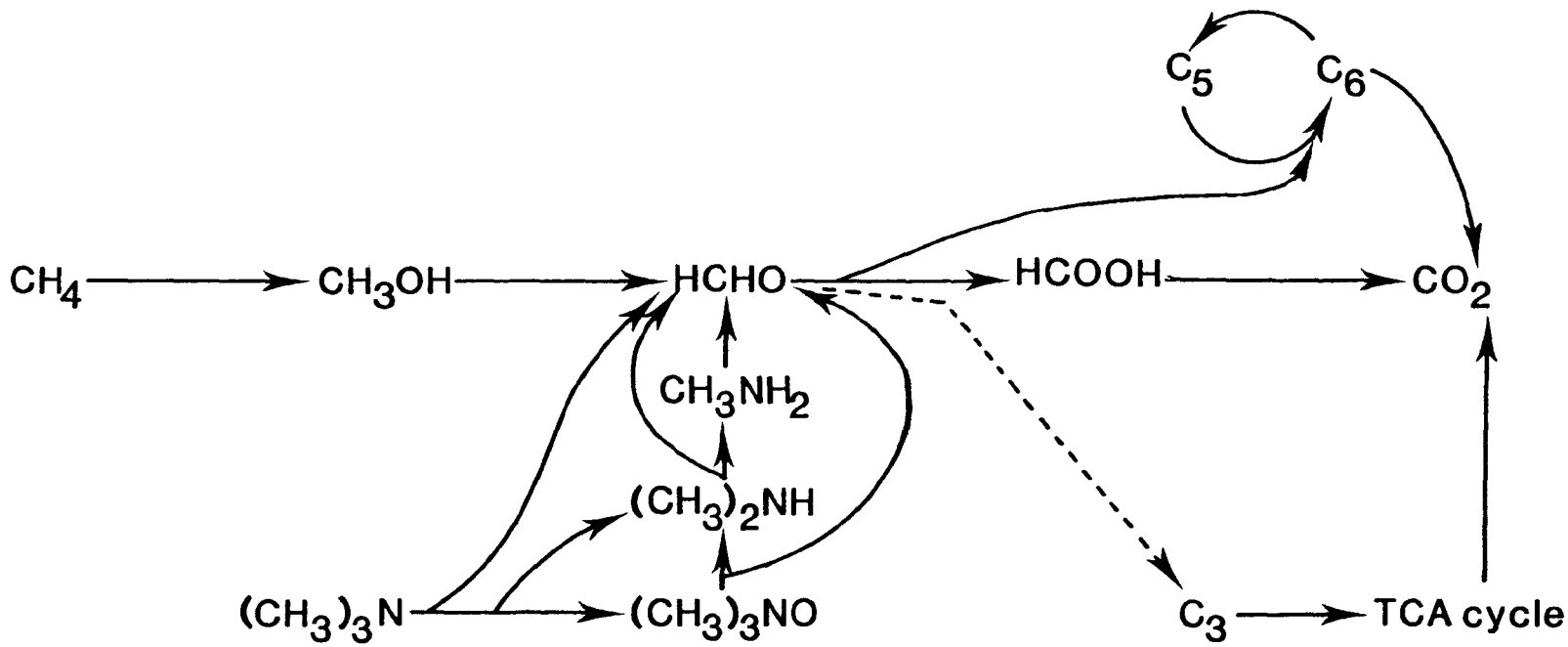
power available to the cell. They also take part in the assimilatory pathways which use either formaldehyde or carbon dioxide as their fixation substrate. Thus more reduced C_1 compounds must initially be oxidised before assimilation can occur.

A general summary of the C_1 oxidation pathways is shown in Figure 9. The oxidation of the following compounds will be considered in more detail: (1) methane, (2) methanol, (3) formaldehyde, (4) formate and (5) methylated amines.

(1) Methane oxidation.

Methane can only be attacked by a substitution mechanism. It was shown in 1970 (Higgins and Quayle, 1970), that growth on methane is accompanied by the incorporation of an atom of oxygen derived from gaseous oxygen, into the methane molecule, giving methanol. The enzyme responsible for this oxidation step is methane monooxygenase, and currently some controversy surrounds this enzyme. At present methane monooxygenases derived from Methylococcus capsulatus (Bath), and Methylosinus trichosporium have been fully described. The enzyme from Methylococcus capsulatus (Bath) appears to be soluble and quite non-specific in its substrate specificity. It has been reported to oxidise a variety of alkanes, alkenes, ethers and cyclic compounds (Colby et al., 1977), as well as ammonia (Dalton, 1977), and use NADH as an electron donor. The enzyme has been shown to be composed of three components called A, B and C (Colby and Dalton, 1978, 1979; Colby et al, 1979; Dalton, 1981). Component A consists of two subunits and has an iron-sulphur core. It is thought to be responsible for the binding of the hydrocarbon substrate. Component C contains an iron-sulphur centre and a molecule of FAD. It

Figure 9 General oxidation pathways of C_1 compounds


C5
C6
CH4
CH3OH
HCHO
HCOOH
CO2
CH3NH2
(CH3)2NH
(CH3)3N
(CH3)3NO
C3
TCA cycle

can be directly reduced by NADH and can pass its electrons to a variety of acceptors including oxygen and oxidised protein A. The role of component B is unknown. It has no measurable catalytic activity but is necessary in the monooxygenase reduction. The site of binding and activation of oxygen is unknown but the non-haem iron species of components A and C are prime candidates. The methane monooxygenase from Methylosinus trichosporium OB36 has been purified and studied (Tonge et al., 1977). The enzyme was found to be particulate and to consist of two protein components plus soluble cytochrome c. Equal amounts of each component were required for maximum activity. The two most important characters of the enzymes which differ between organisms are the distribution of activity between the soluble and membrane bound fractions and the nature of the electron donor, Methylococcus capsulatus (Bath) using NADH, and Methylosinus trichosporium OB36 using cytochrome c. Further studies by Stirling and Dalton (1979) using the enzyme from Methylosinus trichosporium OB36 have indicated fundamental differences from characteristics reported previously. The enzyme was found to be soluble and only NAD(P)H would serve as an electron donor. The enzyme has been resolved into two inactive components (further purification being impossible due to extreme instability). However, activity could be restored by the addition of purified components from Methylococcus capsulatus (Bath), showing the two enzymes to be very similar.

There has been an indication that the two reported methane monooxygenases in Methylosinus trichosporium OB36 are produced in response to different culture conditions (Higgins et al., 1981). In continuous culture the organism

contains a soluble NADH-linked enzyme, similar to that found in Methylococcus capsulatus. In batch culture under conditions of oxygen limitation, between 30% and 60% of the total enzyme activity was found to be particulate. It has been concluded that the soluble and particulate activities are different and related to culture conditions. It has also been proposed that the particulate activity is associated with the extensive intracellular membrane systems of methanotrophs (Higgins et al., 1981)

(2) Methanol oxidation

Bacteria oxidise methanol via a dehydrogenase which was intially described in Pseudomonas M27 (Anthony and Zatman,1964a,b,1965,1967a). Very similar enzymes have subsequently been described in a wide range of organisms (Yamanaka,1981). Methanol dehydrogenase is NAD(P) independent, and responsible for the oxidation of methanol for both the serine pathway, and the ribulose monophosphate cycle in obligate and facultative methylotrophs. All methanol dehydrogenases so far examined show the following characteristics in vitro: formaldehyde dehydrogenase activity, an inability to use NAD(P) as an electron acceptor, a broad substrate specificity, an affinity for primary aliphatic alcohols, and a high optimum pH. The ability to oxidise formaldehyde was thought to be due to a structural similarity between the hydrated form of formaldehyde and methanol. However it has more recently been suggested that alcohol and aldehyde oxidation occur at different catalytic sites on the enzyme (Duine and Frank,1981). The oxidation of formaldehyde by this enzyme is unlikely to be of significance in vivo as:1, it is rare for one enzyme to catalyse 2 successive reactions, and 2.certain mutants of Hyphomicrobium X and Pseudomonas AM1, oxidise formaldehyde at similar rates to wild type organisms, even though lacking methanol dehydrogenase.

All methanol dehydrogenases from methylotrophic bacteria contain a prosthetic group which was originally classified as a pteridine moiety (Anthony and Zatman 1967b). However, subsequently the enzyme was found to have the properties of a quinone derivative (Duine et al.,1978). After a purification procedure for

the compound had been developed (Duine and Frank, 1980), the structure of the prosthetic group was determined by nuclear magnetic resonance and mass spectroscopy (Duine et al., 1980). The prosthetic group was called pyrrolo quinoline quinone (PQQ), and its presence has been established not only in several methanol dehydrogenases (Duine and Frank, 1980), but also in alcohol dehydrogenases from non-methylotrophs (Duine and Frank, 1981), glucose dehydrogenase (Duine et al., 1979), and methylamine dehydrogenase (de Beer et al., 1980). Quinoprotein is a general name which has been proposed for enzymes containing PQQ.

In methylotrophic yeasts the first enzyme which takes part in the oxidation of methanol is not a dehydrogenase as in bacteria, but an alcohol oxidase. The enzyme has been purified and characterised from the following organisms: Kloeckera sp2201 (Tani et al., 1972a, b); Candida N-16 (Fujii and Tonomura, 1972); Candida boidinii (Sahm and Wagner, 1973); Hansenula polymorpha (van Dijken et al., 1976a) and Pichia pastoris (Cauderc and Baratti, 1980). All the enzymes appear very similar having the same absorption spectra, a flavine prosthetic group, and a molecular weight of between 600000 and 670000 composed of eight identical subunits each containing a molecule of FAD. All the enzymes have a wide substrate specificity, oxidising primary aliphatic alcohols up to C_5, and formaldehyde, although oxygen is the only electron acceptor.

Methanol oxidase, along with catalase, is induced by the growth of the yeast on methanol, the catalase being required to remove hydrogen peroxide from the cell. Both enzymes are present in microbodies or peroxisomes.

$$CH_3OH + O_2 \longrightarrow HCHO + H_2O_2$$

(3) Formaldehyde oxidation.

Several different enzymes catalysing the direct oxidation of formaldehyde occur in methylotrophic bacteria, the best characterised being: (a) methanol dehydrogenase, which as mentioned previously can *in vitro* oxidise formaldehyde, as well as primary aliphatic alcohols. However, as previously mentioned on page 25, the oxidation of formaldehyde by this enzyme is thought to be of minor significance *in vivo*; (b) NAD(P) linked formaldehyde dehydrogenase, which is sometimes GSH dependent; and (c) a dye linked, non-specific aldehyde dehydrogenase, and example being that from *Hyphomicrobium X* (Marison and Attwood, 1980).

The dye linked aldehyde dehydrogenases have been purified and characterised from *Pseudomonas AM1* (Johnson and Quayle, 1964), *Methylomonas methylovora* (Patel *et al.*, 1979), and *Hyphomicrobium X* (Marison and Attwood, 1980). They all oxidise a wide range of aldehydes, and formaldehyde is not usually the best substrate, either in terms of maximum velocity or binding. The dye linked enzymes, with one exception, are not induced during methylotrophic growth, and have specific activities which are too low for the growth rates observed, making such enzymes unlikely to be solely responsible for formaldehyde oxidation. The one exception is the dye linked enzyme from *Pseudomonas* RJ1 (Mehta, 1975), which is formaldehyde specific, has a high Km for formaldehyde and is induced during growth on methylamine and oxalate, but is absent during growth on methanol.

A proposal has been put forward that the oxidation of formaldehyde to carbon dioxide in *Pseudomonas MA* could be mediated by way of the serine pathway (Newaz and Hersh, 1975). The conversion of formaldehyde to acetyl coenzyme A could be

carried out via the serine pathway, and the oxidation of the latter compound to yield carbon dioxide, could be completed by way of the TCA cycle. At present, however, there is little evidence to support such a pathway.

A route of formaldehyde oxidation was described by Strøm et al (1974), and Colby and Zatman (1975) which involved the use of enzymes of the ribulose monophosphate cycle. This dissimilatory ribulose monophosphate cycle is shown in Figure 10. Formaldehyde is fixed via hexulose phosphate synthase, and the hexulose 6-phosphate produced is converted to glucose 6-phosphate by the sequential action of phospho-hexuloisomerase and phosphoglucoisomerase. Glucose 6-phosphate is then oxidised to 6-phosphogluconate by glucose 6-phosphate dehydrogenase, and this compound is then decarboxylated and oxidised by 6-phosphogluconate dehydrogenase, to yield the formaldehyde acceptor molecule ribulose 5-phosphate. The operation of this cycle has been confirmed in Pseudomonas C (Ben Bassat and Goldberg, 1977; Ben Bassat et al., 1980), Pseudomonas oleovorans (Sokolov an Trotsenko, 1977, 1978b), and Methylophilus methylotrophus (Beardsmore et al., 1982). It has been suggested that all non-methane utilising methylotrophic organisms utilising the ribulose monophosphate cycle, oxidise formaldehyde via the dissimilatory ribulose monophosphate cycle (Zatman, 1981), as all such organisms so far studied have low levels of formaldehyde and formate dehydrogenase and high levels of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. On the other hand obligate methane utilisers have low activities of latter two enzymes and high levels of formaldehyde and formate dehydrogenases, suggesting that these organisms use a linear pathway of formaldehyde oxidation.

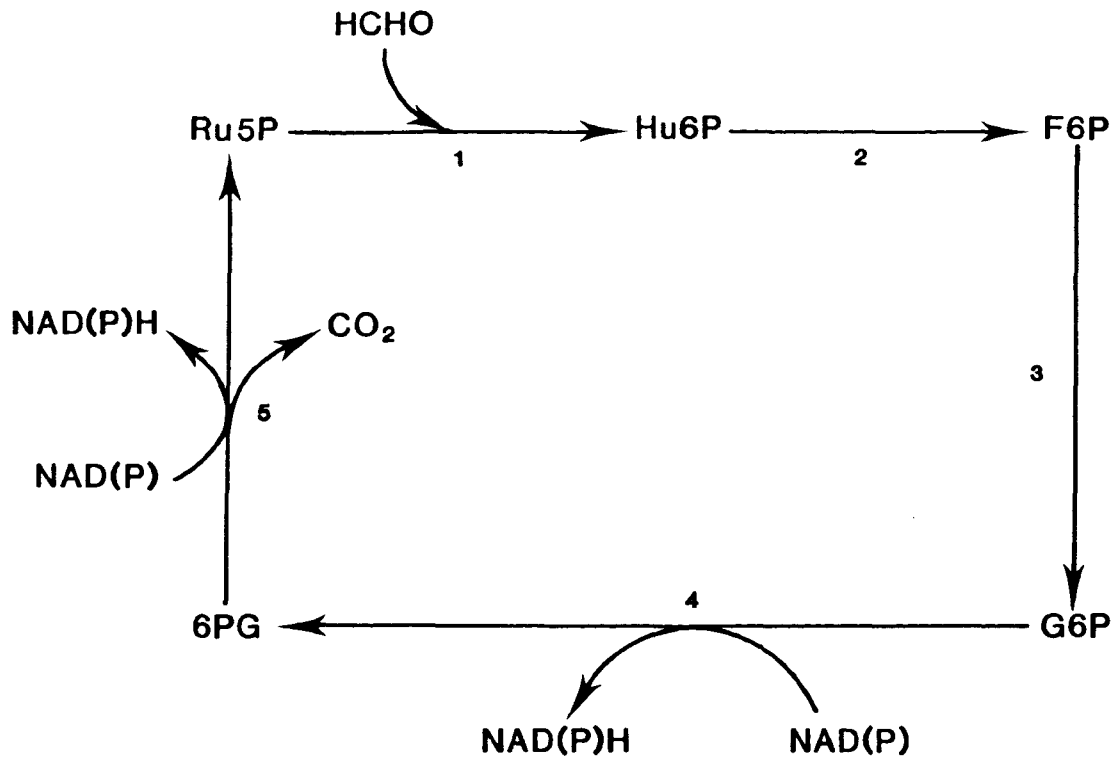
Figure 10 The dissimilatory ribulose monophosphate cycle of formaldehyde oxidation

Enzymes:

1. hexulose phosphate synthase
2. phosphohexuloisomerase
3. phosphoglucoisomerase
4. glucose 6-phosphate dehydrogenase
5. 6-phosphogluconate dehydrogenase

Abbreviations:

Ru5P	ribulose 5-phosphate
Hu6P	hexulose 6-phosphate
F6P	fructose 6-phosphate
G6P	glucose 6-phosphate
6PG	6-phosphogluconate


HCHO
Ru5P
1
Hu6P
2
F6P
3
NAD(P)H
CO_2
5
NAD(P)
6PG
4
G6P
NAD(P)H
NAD(P)

The dissimilatory ribulose monophosphate cycle provides a method of control between the assimilation of formaldehyde and its oxidation to carbon dioxide with a branch point at the level of 6-phosphogluconate dehydrogenase. An enzyme possessing such a regulatory role would be expected to be inhibited by its end products NAD(P)H and ATP. Indeed this has been found to be true in the case of the enzyme from Methylophilus methylotrophus (Beardsmore et al.,1982), and Pseudomonas C (Ben Bassat and Goldberg, 1980).

In methylotrophic yeasts formaldehyde oxidation is mediated via an inducible NAD and GSH specific formaldehyde dehydrogenase (van Dijken et al., 1981), the substrate for the enzyme being S-hydroxymethylglutathione, the product being S-formylglutathione. As well as this direct oxidation pathway in yeasts, a non-linear dissimilatory pathway of formaldehyde oxidation has been proposed (Egli et al., 1983). This pathway involves the use of the dihydroxyacetone pathway to form triose phosphate. Hexose phosphate can then be produced from two triose phosphate molecules. The hexose phosphate can then be oxidised via the pentose phosphate pathway. An alternative method of oxidation would be to convert the triose phosphate produced by the dihydroxyacetone pathway into pyruvate and then to oxidise this via the TCA cycle.

(4) Formate oxidation.

This reaction is carried out by formate dehydrogenase. Two types of this enzyme have been described, one being membrane bound, the other soluble. Most methylotrophs appear to have the soluble enzyme, which is specific for formate and NAD (Anthony, 1982). The enzyme has not been

studied in great detail, the only one to be purified to homogeneity being that from Pseudomonas oxalaticus (Muller et al., 1978).

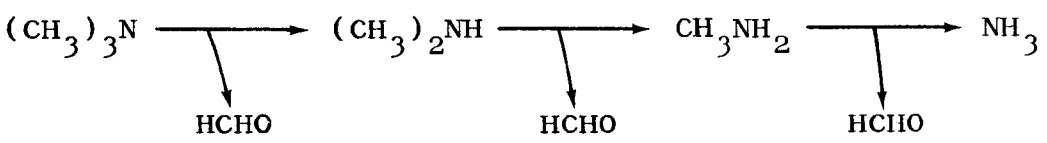
In methylotrophic yeasts the product of the reaction involving formaldehyde dehydrogenase is S-formylglutathione. The oxidation of this compound can proceed in one of two ways (van Dijken et al., 1981), either by hydrolysis to yield GSH and free formate, which is subsequently oxidised by formate dehydrogenase, or via direct oxidation. In the first case a formylglutathione hydrolase is required. In the second the oxidation is mediated by formate dehydrogenase. The partially purified NAD linked formate dehydrogenase from Hansenula polymorpha has been reported to have a forty-fold higher affinity for S-formylglutathione than for formate (van Dikjen et al., 1976b), suggesting that in this organism the former is the physiological substrate. However, an S-formylglutathione hydrolase has been reported to be present in Kloeckera sp.2201. The purified enzyme could hydrolyse S-formylglutathione to yield formate. The formate dehydrogenase produced by the organism showed no reaction with S-formylglutathione (Kato et al., 1980).

(5) The oxidation of N-methyl compounds.

This is a complex subject with a large array of different enzymes involved. The description of the oxidative pathways given here will be necessarily brief. Fuller accounts are given in reviews by Colby et al., 1979; Large, 1981; Anthony, 1982.

N-methyl compounds are oxidised to formaldehyde and ammonia by the successive removal of methyl groups. Formaldehyde is then either further oxidised to formate, or

assimilated into cellular material. The basic oxidation pathway is:



It is characteristic of the pathway that for each oxidative demethylation step, alternative enzyme systems are found in different microorganisms.

Trimethylamine oxidation can proceed by one of three methods: In some facultative methylotrophs such as Pseudomonas aminovorans (Boulton et al., 1974), the oxidation is mediated via two enzymes. The first is a mixed function oxygenase (trimethylamine monooxygenase) which requires NAD(P)H and produces trimethylamine N-oxide.

$$(CH_3)_3N + O_2 + NAD(P)H_2 \longrightarrow (CH_3)_3NO + H_2O + NAD(P) \quad (1)$$

The product of equation (1), trimethylamine N-oxide is then demethylated to dimethylamine and formaldehyde by trimethylamine N-oxide aldolase (demethylase).

$$(CH_3)_3NO \longrightarrow (CH_3)_2NH + HCHO \quad (2)$$

The second method of trimethylamine oxidation is catalysed by trimethylamine dehydrogenase, and has been described in Bacterium 4B6 and C2A1 (Colby and Zatman, 1973, 1974). The reaction is an anaerobic oxidative demethylation, catalysed by a flavoprotein containing enzyme.

$$(CH_3)_3N + \text{Flavoprotein} + H_2O \longrightarrow (CH_3)_2NH + HCHO + \text{Reduced Flavoprotein} \quad (3)$$

The third method of trimethylamine oxidation involves

the use of an NAD linked trimethylamine dehydrogenase and has been reported only in Mycobacterium vaccae (Loginova and Trotsenko, 1979).

$$(CH_3)_3N + H_2O + NAD \longrightarrow (CH_3)_2NH + HCHO + NADH_2 \quad (4)$$

Dimethylamine oxidation proceeds via one of two methods. The first method involves a dimethylamine monooxygenase, an example of which has been purified from Pseudomonas aminovorans.

$$(CH_3)_2NH + O_2 + NADH_2 \longrightarrow CH_3NH_2 + H_2O + NAD \quad (5)$$

The second method of dimethylamine oxidation has been described only in Hyphomicrobium X (Meiberg and Harder, 1978, 1979; Meiberg et al., 1980), and involves the use of a dye linked dimethylamine dehydrogenase.

$$(CH_3)_2NH + PMS + H_2O \longrightarrow CH_3NH_2 + HCHO + PMSH_2 \quad (6)$$

The oxidation of methylamine can take place via one of three separate oxidation pathways. The first mechanism involves the use of methylamine oxidase which at present has only been found in members of the genus Arthrobacter (Levering et al., 1981a; Loginova and Trotsenko, 1976).

$$CH_3NH_2 + O_2 + H_2O \longrightarrow HCHO + NH_3 + H_2O_2 \quad (7)$$

The amine oxidase of Arthrobacter P1 has been studied (van Vliet Smits et al., 1981), and is thought to belong to the copper containing amine oxidases. The hydrogen peroxide which is produced by the enzyme must be broken down by catalase before its level becomes detrimental to the organism. The importance of catalase in methylamine utilising Arthrobacter P1 has been demonstrated by the inhibition of growth caused by the inclusion of the catalase inhibitor aminotriazole in the growth medium.

Methylotrophic yeasts can use methylamine as sole nitrogen source, but not as sole source of carbon and energy. In all of the yeasts examined so far, the oxidation of methylamine is via an amine oxidase (Large et al., 1980).

The second method by which methylotrophs can oxidise methylamine is via methylamine dehydrogenase. This enzyme was first identified by Eady and Large (1968) in Pseudomonas AM1. The enzyme uses PMS as an electron acceptor in vitro, and it is thought that the prosthetic group of the enzyme is PQQ (de Beer et al., 1980).

$$CH_3NH_2 + H_2O + PMS \longrightarrow HCHO + NH_3 + PMSH_2 \quad (8)$$

The third method of methylamine oxidation is an indirect one involving the formation and subsequent reoxidation of N-methyl amino acids. The reactions forming the N-methyl amino acids are well defined:

$$\text{L-glutamate} + CH_3NH_2 \longrightarrow \text{N-methylglutamate} + NH_4^+ \quad (9)$$

$$\text{L-glutamate} + CH_3NH_2 + ATP \longrightarrow \gamma\text{-glutamylmethylamide} + ADP + Pi \quad (10)$$

$$\text{Pyruvate} + CH_3NH_2 + NADPH \longrightarrow \text{N-methylalanine} + NADP^+ + H_2O \quad (11)$$

$$\alpha\text{-ketoglutarate} + CH_3NH_2 \longrightarrow \text{5-hydroxy-N-methylpyroglutamate} + H_2O \quad (12)$$

However little is known about the subsequent oxidation of the N-methyl amino acids that are formed in the latter steps. An N-methylglutamate dehydrogenase has been found in Pseudomonas AM1 (Hersh et al., 1971, 1972). This enzyme catalyses the oxidation of N-methylglutamate, yielding

glutamate and formaldehyde.

$$\text{N-methylglutamate} + H_2O + \text{PMS} \longrightarrow \text{glutamate} + \text{HCHO} + PMSH_2 \quad (13)$$

A further type of N-methylglutamate dehydrogenase has been found in both the Pseudomonads and Hyphomicrobia. This enzyme is soluble and NAD linked.

$$\text{N-methylglutamate} + \text{NAD} + H_2O \longrightarrow \text{glutamate} + NADH_2 + \text{HCHO} \quad (14)$$

It has been reported that N-methyalanine can be oxidised to alanine and formaldehyde by N-methylglutamate dehydrogenase (Hersh et al., 1972; Bamforth and Large, 1977).

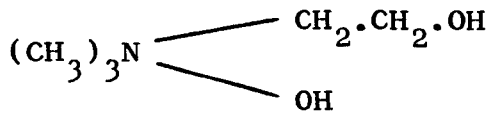
γ-Glutamylmethylamide can be converted to N-methylglutamate by an as yet uncharacterised enzyme.

$$\gamma\text{-glutamylmethylamide} \longrightarrow \text{N-methylglutamate} + NH_3 \quad (15)$$

The N-methylglutamate product of reaction (15) can be oxidised further by N-methylglutamate dehydrogenase as in reactions (13) or (14).

The microbial oxidation of choline

Choline [(β -hydroxyethyl) trimethylammonium hydroxide] has the chemical structure:



It is a compound found in many plants and animals and its breakdown has been extensively studied, especially in mammals. The oxidation of choline could proceed by one of two routes: either (1) the oxidation of choline to betaine, which is then

demethylated, or (2) the demethylation of choline, followed by the oxidation of the resulting amino ethanol (Goldstein, 1959; Shieh, 1964).

The enzymic oxidation of choline was first studied in the mammalian liver by Bernheim and Webster (1937), and has since been comprehensively examined in mammals. The initial oxidation of choline in mammals has been found to proceed via two steps. Initially the choline is oxidised by choline dehydrogenase (oxidase) to form betaine aldehyde; secondly, betaine aldehyde is oxidised to betaine by an NAD dependent betaine aldehyde dehydrogenase (Shieh, 1964). In the presence of the enzyme betaine-homocysteine transmethylase, betaine donates a methyl group to homocysteine, forming methionine and dimethylglycine. Dimethylglycine is then progressively demethylated through sarcosine to glycine, with the formation of 'C_1 units' (Shieh, 1964).

Kortstee (1970) confirmed the widespread ability of microorganisms to decompose choline when he found representatives of the genera Agrobacterium, Arthrobacter, Micrococcus, Pseudomonas, Rhizobium and Streptomycetes were able to grow in a medium containing choline as a sole source of carbon and nitrogen. All of the organisms used in this study were found to be able to utilise betaine, dimethylglycine and sarcosine as sole sources of carbon and nitrogen. However it was noted that 30% of the choline utilising organisms that were tested also grew on dimethylethanolamine and monomethylethanolamine. From the study indirect evidence was gained that aerobic choline oxidation in bacteria takes place in a similar way to that reported in mammals. Nevertheless, the observation that some of the organisms grew on methylated ethanolamines did not rule out the

possibility that choline could be degraded via these compounds.

Studies on the oxidation of choline by Achromobacter cholinophagum (Shieh, 1964), provided evidence that the oxidation proceeded via choline oxidase which yielded betaine aldehyde. This compound then being sequentially oxidised to betaine, and finally dimethylglycine. In this organism it was reported that betaine was directly demethylated to dimethylglycine, by a step not involving betaine - homocysteine transmethylase (Shieh, 1966). The dimethylglycine formed by the latter step was oxidised to sarcosine with the concomitant formation of formaldehyde, the sarcosine finally being oxidised to glycine again with the formation of formaldehyde (Shieh, 1965).

Studies on choline oxidation by Arthrobacter globiformis (Ikuta et al., 1977a, b), and Pseudomonas aeruginosa A16 (Nagasawa et al., 1975), showed that both organisms initiated degradation via an initial oxidation step, yielding betaine aldehyde. It was also reported that the choline oxidase of Arthrobacter globiformis was capable of converting betaine aldehyde to betaine.

Fungal oxidation of choline has been investigated using Cylindrocarpon didymum M1 (Tani et al., 1979; Mori et al., 1980 a, b), the pathway being similar to that established in mammals and bacteria:

Choline ⟶ Betaine Aldehyde ⟶ Betaine ⟶ Dimethylglycine ⟶ Sarcosine ⟶ Glycine

The Japanese research group have purified and characterised the enzymes choline oxidase, dimethylglycine oxidase, and sarcosine oxidase from the organism.

Studies on the degradation of choline by the facultative methylotroph Arthrobacter P1 (Levering et al., 1981b) have led to the proposal that C_1 assimilation pathways are involved in the degradation of choline in this organism. Choline oxidation in Arthrobacter P1 is reported to proceed via betaine aldehyde, betaine, dimethylglycine, sarcosine and glycine. During the demethylation reactions, formaldehyde is released. This toxic chemical must be dealt with by the organism before it reaches inhibitory levels. However, as Arthrobacter P1 contains no formaldehyde dehydrogenase, it has been suggested (Levering et al., 1981b) that formaldehyde enters the ribulose monophosphate cycle to be either assimilated or oxidised. Support for the theory comes from the fact that key enzymes of the ribulose monophosphate cycle are synthesised during growth on choline. It was also reported that enzymes characteristic of the serine pathway were produced during growth on choline, and this led to the proposal that the glycine end product of choline breakdown, together with formaldehyde from a demethylation step, could enter a serine pathway to be either assimilated or dissimilated (Figure 11).

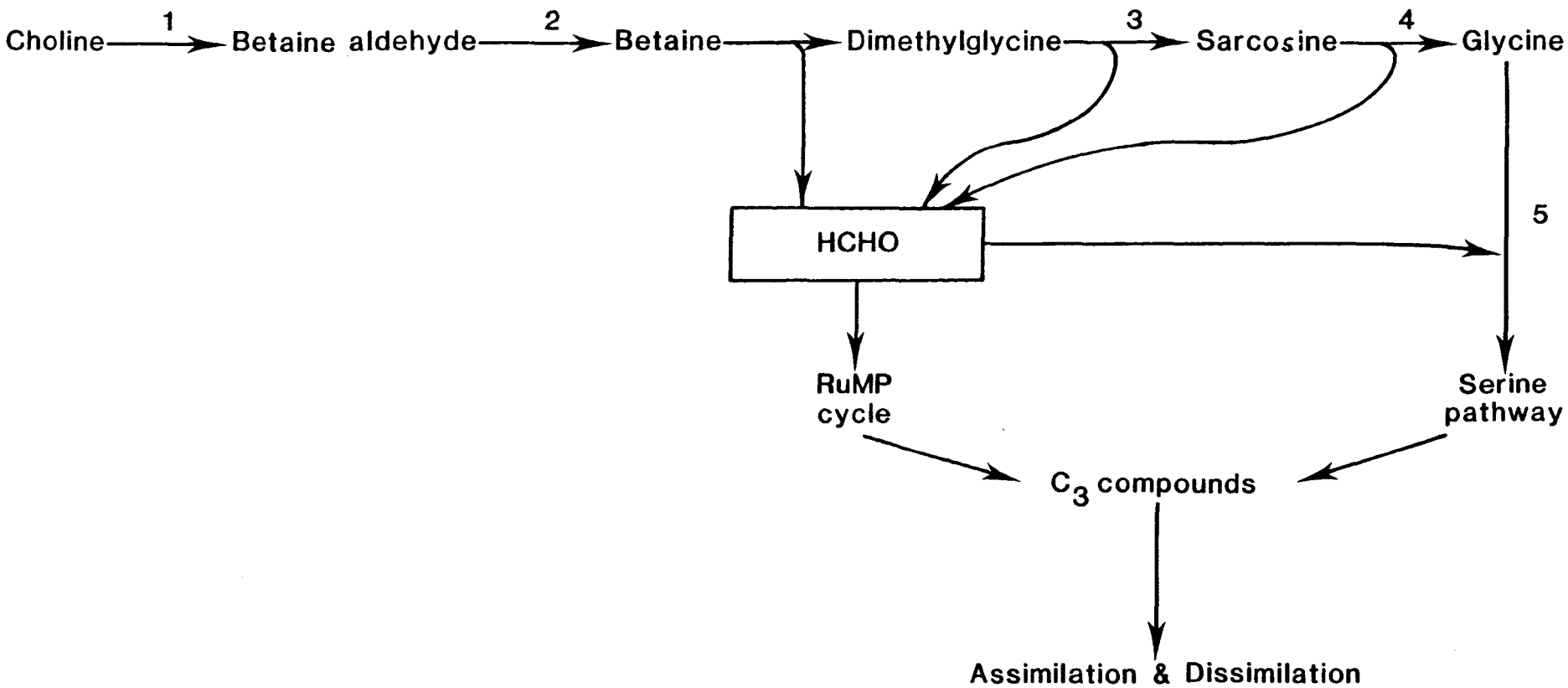
Arthrobacter 2B2

Arthrobacter 2B2 is a facultative methylotroph capable of growth on methylamine (Colby and Zatman, 1973), using the ribulose monophosphate cycle for the assimilation of C_1 compounds (Cox and Zatman, 1974). The initial studies of the organism at Sheffield were carried out by a final year student as an Honours project in order to construct a basic enzyme profile (Watson, 1979). These studies confirmed the work of Colby and Zatman, indicating that the organism used the ribulose monophosphate cycle during growth on methylamine, and further showed that the organism probably utilised the

Figure 11 Hypothosised metabolism of choline by Arthrobacter P1

Enzymes:

1. choline oxidase
2. betaine aldehyde dehydrogenase
3. dimethylglycine oxidase
4. sarcosine oxidase
5. serine transhydroxymethylase


Choline
1
Betaine aldehyde
2
Betaine
Dimethylglycine
3
Sarcosine
4
Glycine
5
HCHO
RuMP cycle
Serine pathway
C_3 compounds
Assimilation & Dissimilation

fructose bisphosphate aldolase cleavage variant of the cycle. The presence of a soluble amine oxidase was found and suggested to be the route of methylamine oxidation.

Further work by another final year student then concentrated on the fructose bisphosphate aldolase of the organism (Jermy, 1980), in order to see whether there was enzyme multiplicity in view of the different roles that this enzyme must perform during growth on methylamine, glucose and succinate. In the event the results of the work indicated that a single class II fructose bisphosphate aldolase (as defined by Rutter, 1964) was produced whether the organism was growing on methylamine, glucose or succinate.

Interest in Arthrobacter 2B2 was renewed after the isolation of a very similar organism, Arthrobacter P1, by Levering et al., (1981a). This bacterium is a facultative methylotroph, with amine oxidase as its methylamine oxidising enzyme, and using the ribulose monophosphate cycle as the C_1 assimilation pathway during growth on methylamine. However, despite high levels of hexulose phosphate synthase in cell-free extracts of the organisms, there was an apparent lack of phosphohexuloisomerase, one of the key enzymes of the ribulose monophosphate cycle. This unexpected finding contrasted with the results of Watson using Arthrobacter 2B2 and provided the starting point of the present investigation: firstly a careful rechecking of the enzyme profile of Arthrobacter 2B2 with special reference to the properties of the phosphohexuloisomerase, in either or both organisms.

CHAPTER 2

Materials and Methods

Source of organisms

The organisms used were *Arthrobacter* 2B2, and *Methylophilus methylotrophus*. *Arthrobacter* 2B2 was obtained from a freeze dried stock kept at Sheffield which originated from Dr. L. J. Zatman (Reading). *Methylophilus methylotrophus* was obtained from I.C.I. Billingham.

Maintenance

Arthrobacter 2B2 was maintained on slopes of an inorganic salts medium (see growth media section), containing 50 mM methylammonium chloride, 1 $\mu g l^{-1}$ biotin, and 1% (w/v) Oxoid Purified Agar. The slopes were incubated at 30°C for 24 hours, stored at 4°C, and transferred to fresh slopes every week.

Methylophilus methylotrophus was maintained on slopes of a mineral salts medium used by Beardsmore *et al.*, (1982) containing 0.5% (v/v) methanol, and 1% (w/v) Oxoid Purified Agar. The slopes were incubated at 37°C for 48 hours and transferred weekly to fresh slopes.

Growth Media

All the media used were prepared using glass distilled water.

Arthrobacter 2B2: the inorganic salts medium used for batch growth contained ($g l^{-1}$):

$NaH_2PO_4.2H_2O$	0.5
K_2HPO_4	1.55
$(NH_4)_2SO_4$	1.0
$MgSO_4.7H_2O$	0.2

To 1 l of this medium was added 0.2 ml of a trace element solution (Vishniac and Santer, 1957), and 1 μg biotin.

Carbon sources used were: betaine hydrochloride, choline chloride, dimethylglycine, ethylamine, glucose, glycine, methylammonium chloride, sarcosine, serine, and sodium acetate. The carbon source was added at a concentration of 50 mM, the pH was adjusted to 7.0, and the medium was sterilised by autoclaving for 20 minutes at 15 lb per sq. inch (1.05 kg cm^{-2}) at a temperature of 120°C.

When the organism was grown in chemostat continuous culture, the inorganic salts medium used was that detailed above. To that medium either 10 mM choline chloride, or 10 mM methylammonium chloride was added as a carbon source. The pH was then adjusted to 7.0 an sterilisation was carried out by autoclaving for 60 minutes at 15 lb per sq. inch (1.05 kg cm^{-2}), at a temperature of 120°C. After autoclaving biotin which had been previously sterilised by autoclaving for 15 minutes at 15 lb per sq. inch (1.05 kg cm^{-2}) at 120°C, was aseptically added to the medium.

Methylophilus methylotrophus: the medium used for batch growth was that described by Beardsmore et al (1982). The method of sterilisation was as described above. After sterilising methanol was added to the medium as a carbon source to give a final concentration of 0.5% (v/v). The methanol was sterilised by passage through a Seitz filter.

When the organism was grown in chemostat continuous culture the medium of Dawson and Jones (1981) was used. As above, methanol was filter sterilised and added to the previously autoclaved medium to give a final concentration of 0.25% (v/v).

Growth and Harvesting

Batch cultures were grown either in 2 l shake flasks

containing 700 ml of medium, or for large scale work in a Microferm fermentor (New Brunswick Scientific Co.,) with a working volume of 11 l. Arthrobacter 2B2 was grown at 30°C and Methylophilus methylotrophus at 37°C. Chemostat continuous culture was carried out in a 2.5 l laboratory fermentor (LHE CC 1500, L. H. Engineering Co., Stoke Poges, Bucks., U.K.), with an agitation rate of 300 rev. min^{-1}, and an aeration rate of 2.5 l of air min^{-1}. The culture was maintained at pH 7.0 ± 0.1 by the automatic addition of 2 M NaOH. Arthrobacter 2B2 was grown at a temperature of 30°C in the chemostat, while Methylophilus methylotrophus was grown at 40°C.

Cultures to be used for enzyme purification were harvested during mid- to late-exponential growth phase, and all cells were collected by centrifugation at 6300 g for 15 minutes. The cells were washed once in 20 mM sodium potassium phosphate buffer at pH 7.0 and either used immediately or deep frozen at -15°C until required.

Measurement of Growth

The growth of the cells was measured by determining the absorbance of cell suspensions at 610 nm, using a Pye Unicam SP6-250 spectrophotometer.

Chemicals

Chemicals available commercially

All the chemicals used except those listed below were of analytical grade and were purchased from BDH Chemicals Ltd., (Poole, England). The following chemicals, biochemicals, and enzymes were obtained from the sources shown in parenthesis:

Bovine serum albumin powder (fraction V from bovine plasma) (Armour Pharmaceuticals Co., Ltd.,); hydroxylapatite (Bio-gel HTP, Bio Rad Laboratories); acrylamide, N_1N^1-methylene bisacrylamide (Eastman Kodak Ltd.,); ammonium persulphate, methanol (Fisons Scientific Apparatus); 2-mercaptoethanol (Koch-Light Laboratories Ltd.,); cellulose-coated thin layer chromatography plates (Merck); Blue Dextran, Sephadex G25, Sephadex G150, Sephadex G200, and DEAE Sephacel bead formed, preswollen, cellulose anion exchanger (Pharmacia Fine Chemicals); Coomassie Brilliant Blue G250 (Serva Feinbiochemica, Heidelberg); betaine aldehyde, betaine hydrochloride, bismuth oxynitrate, choline chloride, cocarboxylase (thiamine pyrophosphate) erythrose 4-phosphate, α-glycerophosphate dehydrogenase, glyoxylic acid, α-ketoglutaric acid, phenylhydrazine, phosphoriboisomerase, pyridoxal 5-phosphate, ribose 5-phosphate, sedoheptulose 1, 7-bisphosphate, sodium dodecyl sulphate, N.N.N^1.N^1-tetramethylethylenediamine, tetrahydrofolic acid, transaldolase and transketolase (Sigma Chemical Company); diethylaminoethyl cellulose (DE.52) microgranular preswollen anion exchanger Whatman Biochemicals Ltd.,); dialysis tubing (Medicell International Ltd., London). All other enzymes and biochemicals used during the course of the work were obtained from the Boehringer Corporation (London) Ltd.

Special preparations

(1) Formaldehyde

Formaldehyde was prepared from paraformaldehyde by the method of Waites and Quayle (1981).

(2) D-*arabino* 3-hexulose 6-phosphate

A crude preparation of D-*arabino* 3-hexulose 6-phosphate

was prepared for use in the phosphohexuloisomerase assay. The method of preparation was enzymic and relied on the enzyme hexulose phosphate synthase. This enzyme is not available commercially and was purified in the following way from Arthrobacter 2B2.

(i) Crude cell free extract was prepared by resuspending 6 g of methylamine grown cell paste in 24 ml of 20 mM sodium potassium phosphate buffer pH 7.5 containing 2 mM $MgCl_2$ and 15% (v/v) methanol. This was sonicated for 5, one minute periods and the cell debris were removed by centrifugation at 38000 g for 20 minutes at $2^{o}C$. The supernatant was treated as the cell free extract.

(ii) The crude cell free extract was raised to a level of 50% saturation with the addition of solid $(NH_4)_2SO_4$, and the precipitated protein was removed by centrifugation. The resulting supernatant was treated by further fractionation with $(NH_4)_2SO_4$ to 80% saturation. The precipitated protein was collected by centrifugation, and redissolved in the sonication buffer.

(iii) The redissolved protein from stage (ii) was desalted by dialysis for three hours, against 100 volumes of sonication buffer. The dialysate was then applied to a DEAE cellulose column (6 x 1.5 cm) equilibrated with sonication buffer. After the unbound protein had been eluted from the column, a gradient of 0 - 250 mM sodium chloride made up in 400 ml of sonication buffer was applied. Fractions 65 to 110 containing activity were pooled, made up to a concentration of 5 μ mol ml^{-1} with respect to ribose 5-phosphate, and then had added 5 units of phosphoriboisomerase. The pooled fractions were then

ultrafiltered to a small volume.

(iv) The concentrated enzyme from step (iii) was desalted by dialysis for three hours, against 100 volumes of sonication buffer containing 5 μ mol ml^{-1} ribose 5-phosphate, and 5 units of phosphoriboisomerase. The dialysate was then applied to a hydroxylapatite column (4 x 1.5 cm) equilibrated with sonication buffer. A gradient of 20 - 150 mM sodium potassium phosphate buffer pH 7.5 containing 2 mM $MgCl_2$ and 15% (v/v) methanol was used to elute the column. Fraction numbers 8 to 50 containing activity were pooled and stored until required at -15°C.

In order to produce D-arabino 3-hexulose 6-phosphate the following mixture was made up in a total volume of 5 ml:

100 μ mol sodium potassium phosphate pH 7.2
25 μ mol $MgCl_2$
100 μ mol ribose 5-phosphate
20 units phosphoriboisomerase
100 μ mol formaldehyde
5 units purified hexulose phosphate synthase

The reaction mixture was incubated at 30°C for 45 minutes. The protein fraction was marked by the addition of Blue Dextran, before passage through a Sephadex G25 column (21.5 x 1.5 cm) equilibrated with 50 mM sodium potassium phosphate buffer pH 7.0. The column was washed with equilibration buffer and fractions (1.5 ml) were collected. The protein visualised by the presence of Blue Dextran was eluted from the column between fractions 8 and 12, the inorganic constituents including D-arabino 3-hexulose 6-phosphate, between fractions 14 and 24. The final concentration of the hexulose 6-phosphate produced was 3 mM. During kinetic studies involving the use

of hexulose 6-phosphate, a purified form was used which had been prepared by the method of Strøm et al., (1974) by Dr. A. J. Beardsmore.

(3) Potassium glycerate

This was produced by dissolving commercially produced calcium glycerate in distilled water, then adding a solution of KH_2PO_4 to the required concentration. The precipitate of calcium phosphate which forms was removed by centrifugation leaving a solution of potassium glycerate.

(4) Di-sodium tetrahydrofolate

The di-sodium salt of tetrahydrofolate was produced by mixing in a total volume of 50 ml:

3.5 mmol tetrahydrofolic acid

1.12 mmol sodium potassium phosphate pH 7.0

0.449 mmol sodium hydroxide

4.49 mmol mercaptoethanol

The mixture was repeatedly evacuated and flushed with hydrogen. It was then stored under hydrogen at -15°C.

(5) (2S)-4-malyl-coenzyme A was prepared by Mr. A. A. Hancock from (s)-(β hydroxysuccinyl)-N-octanoylcysteamine using the methanol described by Salem et al., (1973a).

Buffers

All buffers were prepared by the methods of Dawson et al., (1969), or Gomori, (1955).

Analytical Methods

Thin layer chromatography

Thin layer chromatography was carried out on pre-formed

cellulose plates (20 x 20 cm, 0.1 mm thickness). In all cases the running solvent used was methanol : butanol : water at a ratio of 10 : 10 : 5. The plates ran until the solvent was within 2 cm of the top. They were then removed, dried and sprayed with the developer.

Thin layer chromatography developers

(1) Choline

Choline was detected by spraying plates with the following reagents: (i) 1% (w/v) aqueous $K_4Fe(CN)_6$; then whilst still wet (ii) 0.5% (w/v) aqueous cobalt chloride. Choline is visualised as a green spot on a white background (Dawson et al., 1969). This method can be made quantitative by scraping the developed choline spots off the plate, solubilising the colour by placing in concentrated hydrochloric acid, and removing the cellulose debris by centrifugation. The absorbance of the resulting liquid was measured at 704 nm and related to a standard curve to quantify the choline present.

(2) Betaine

Betaine was detected using $KBiI_4$ (Dragendorff solution), Bregoff et al., 1953). The solution was sprayed onto the plate and betaine developed as an orange spot.

(3) Sarcosine

Sarcosine was detected by spraying the plate with a solution of 0.25% (w/v) ninhydrin in acetone. It develops as a purple spot after heating to 100°C. The ninhydrin solution reacts with most amino acids to give a purple colour. Consequently a sarcosine standard was always included on the plates to ensure that any sarcosine in the samples was correctly identified.

Protein estimations

Protein estimations were carried out by the method of Lowry *et al.*, (1951), using bovine serum albumin as standard.

Absorption Spectra

The ultra violet and visible wavelength absorption spectra of samples were determined by the use of a Pye-Unicam SP1800 double-beam recording spectrophotometer, set in the scanning mode.

Enzyme Assays

All continuous spectrophotometric assays were carried out in a Pye-Unicam SP1800 double-beam recording spectrophotometer at 30°C, in 1 ml quartz cuvettes with a 1 cm light path. In all assay systems the observed enzyme rate was initially linear and proportional to the amount of enzyme added. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μ mol product min^{-1}. Specific activities are defined as units $(mg\ protein)^{-1}$. The molar extinction coefficient (E) of reduced pyridine nucleotides was taken to be 6.22×10^{-3} $litre.mol^{-1}.cm^{-1}$.

Oxidase activities were determined polarographically using a Clark-type oxygen electrode (Rank Ltd.). For the oxidase one unit of enzyme activity corresponds to 0.5 μ mol of oxygen consumed per minute.

Hexulose phosphate synthase (D-*arabino* 3-hexulose 6-phosphate formaldehyde lyase) was assayed by two methods: (i) the discontinuous method of Ferenci *et al.*, (1974), based on the RuMP dependent removal of formaldehyde. The assay

contained in a volume of 1 ml:

50 μ mol sodium potassium phosphate pH 7.6

2.5 μ mol $MgCl_2$

5 μ mol formaldehyde

2 units phosphoriboisomerase

5 μ mol ribose-5-phosphate

The reaction mixture was preincubated at 30°C for 15 minutes, the reaction being initiated by adding a suitable amount of enzyme. Samples of 0.1 ml were removed at intervals over a two minute period and rapidly mixed with 1 ml of 10% (w/v) trichloroacetic acid. The concentration of formaldehyde in the withdrawn samples was determined colorimetrically by the method of Nash (1953).

(ii) The continuous enzymically coupled method of van Dijken et al., (1978), based on the RuMP and formaldehyde dependent production of hexulose phosphate. The assay contained in a volume of 1 ml:

50 μ mol sodium potassium phosphate pH 7.6

2.5 μ mol $MgCl_2$

1 unit glucose-6-phosphate dehydrogenase

1 unit phosphoglucoisomerase

0.4 μ mol NADP

2 units phosphoriboisomerase

1 unit phosphohexuloisomerase

After incubation at 30°C for several minutes, a sample of enzyme was added to the cuvette and any rate observed at 340 nm. This was followed by the addition of 5 μ mol Ri5P, and again any formaldehyde independent rate was noted. Finally the desired reaction was initiated by the addition of 5 μ mol formaldehyde. The rate after formaldehyde addition minus the

rate before the addition of formaldehyde was taken to be due to hexulose phosphate synthase activity. The phosphohexuloisomerase used in the assay was purified from Methylophilus methylotrophus by the method of Beardsmore et al., (1982).

Phosphohexuloisomerase (D-arabino-3-hexulose 6-phosphate 3,2-ketolisomerase) was assayed by a modification of the continuous enzymically coupled method of van Dijken et al., (1978). The assay contained in a volume of 1 ml:

50 μ mol tris-HCl pH 8.6

1 μ mol EDTA

1 unit glucose 6-phosphate dehydrogenase

1 unit phosphoglucoisomerase

0.5 μ mol NADP

0.7 μ mol HuMP

Any rate at 340 nm was observed before starting the reaction by the addition of a suitable amount of enzyme.

Hydroxypyruvate reductase (D-glycerate-NAD oxidoreductase EC 1.1.1.29) was assayed by a modification of the continuous method of Blackmore and Quayle (1970). The assay contained in 1 ml:

100 μ mol sodium potassium phosphate pH 6.0

0.4 μ mol NADH or NADPH

The mixture was pre-incubated for several minutes at 30^{o}C before the addition of the enzyme. The reaction was then initiated by the addition of 2 μ mol hydroxypyruvate and the rate of NADH/HADPH oxidation was followed at 340 nm.

Malate dehydrogenase (L-Malate : NAD^{+} oxidoreductase, EC 1.1.1.37) was assayed in the reverse direction as an oxaloacetate reductase using a similar assay mixture as that used for hydroxypyruvate reductase, but substituting 2 μ mol oxaloacetate as a substrate.

Formaldehyde dehydrogenase [formaldehyde : NAD oxidoreductase (glutathione-formylating), EC 1.2.11] was assayed by a modification of the method of Beardsmore et al., (1982). The assay contained in 1 ml:

100 μ mol tris-HCl pH 8.0

10 μ mol GSH

1 μ mol NAD

The mixture was pre-incubated for several minutes at 30°C before the addition of the enzyme, the reaction then being initiated by the addition of 20 μ mol formaldehyde.

Unmodified published procedures were adopted for assaying the following enzymes:

Betaine aldehyde dehydrogenase [betaine-aldehyde : NAD(P) oxidoreductase, EC 1.2.1.8] (Levering et al., 1981b); fructose bisphosphate aldolase [D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13] (Blostein and Rutter, 1963); glucose 6-phosphate dehydrogenase [D-glucose 6-phosphate-NAD(P) oxidoreductase, EC 1.1.1.49] (Hohorst, 1963); 6-phosphogluconate dehydrogenase [6-phospho-D-gluconate : NAD(P) 2-oxidoreductase, EC 1.1.1.43] (Beardsmore et al., 1982); transketolase [sedoheptulose 7-phosphate-D-glyceraldehyde 3-phosphate glycolaldehyde transferase, EC 2.2.1.1] (Strøm et al., 1974); transaldolase [sedoheptulose 7-phosphate-D-glyceraldehyde 3-phosphate dihydroxyacetone transferase, EC 2.2.1.2] (Levering et al., 1982); sedoheptulose 1,7-diphosphatase [sedoheptulose 1,7-diphosphate 1-phosphohydrolase, EC 3.1.3.66] (Racker, 1965); phosphoriboisomerase [D-ribose 5-phosphate ketol isomerase, EC 5.3.1.6] (Domagk and Doering, 1975); ribulose phosphate 3-epimerase [D-ribulose 5-phosphate 3-epimerase, EC 5.1.3.1] (Strøm et al., 1974); phosphofructo-kinase [ATP-D-fructose 6-phosphate 1-phosphotransferase,

EC 2.7.1.11] (van Dijken et al., 1978); fructose bisphosphatase [D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11] (Beardsmore et al., 1982); DCPIP linked formaldehyde dehydrogenase [no enzyme commission number] (Johnson and Quayle, 1964); formate dehydrogenase [formate : NAD oxidoreductase, EC 1.2.1.2] (Quayle, 1966); DCPIP-linked methanol dehydrogenase [alcohol : (acceptor) oxidoreductase EC 1.1.99.8] (Anthony and Zatman, 1964); 6-phosphogluconate dehydrase [6-phospho-D-gluconate hydrolase, EC 4.2.1.12] and 2-keto-3-deoxy-6-phosphogluconate aldolase [KDPG aldolase; 6-phospho-2-keto-3-deoxy-D-gluconate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.14] were assayed together (Wood, 1971); β galactosidase [β -D-galactoside galactohydrolase, EC 3.2.1.23] (Stephens and Gib Debusk, 1975); glycerate kinase [ATP : D-glycerate 3-phosphotransferase, EC 2.7.1.31] (Levering et al., 1981b); lactate dehydrogenase [L-lactate: NAD oxidoreductase, EC 1.1.27] (Stolzenbach, 1966); serine/ glyoxylate aminotransferase [L-Serine : glyoxylate amino-transferase, EC 2.6.1.45] (Levering et al., 1981b); serine/ α ketoglutarate aminotransferase [no enzyme commission number] (Levering et al., 1981b); serine transhydroxymethylase [5, 10-methylene-tetrahydrofolate : glycine hydroxymethyltransferase, EC 2.1.2.1] (Heptenstall and Quayle, 1970 as modified by Harder et al., 1973); catalase [hydrogen-peroxide : hydrogen peroxide oxidoreductase, EC 1.11.1.6] (Chance and Maehly, 1955): isocitrate lyase [threo-D-isocitrate glyoxylate-lyase, EC 4.1.3.1] (Dixon and Komberg, 1959); amine oxidase [EC 1.4.3] (Levering et al., 1981a); choline oxidase [choline : oxygen 1-oxidoreductase EC 1.1.3.17] (Levering et al., 1981a); dimethylglycine oxidase [no enzyme commission number] (Levering et al., 1981b); sarcosine oxidase [sarcosine : oxygen

oxidoreductase (demethylating), EC 1.5.3.1] (Levering et al., 1981b); serine dehydratase [L-serine hydrolyase, EC 4.2.1.13] (Levering et al., 1981b).

Techniques for enzyme purification

Unless otherwise stated all procedures were carried out between 0 and 4°C.

Preparation of crude cell free extracts

Extracts were produced by resuspending previously harvested cells in a suitable ice cold buffer (for details of the types of buffer and volumes used for particular enzymes see the relevant results section). The cold cell suspensions were then disrupted by sonication in an ultrasonic disintegrator (MSE model 150 W), for 5 periods of one minute interspersed with periods of cooling in ice. The cell debris was removed by centrifugation at 38000 g for 20 minutes at 2°C, the resulting supernatant is referred to as 'crude extract'.

Methods of enzyme separation

1. Protamine sulphate precipitation. This was performed by the slow addition of a 2% (w/v) solution of salmine sulphate into the crude extract to a final concentration of 1 mg salmine sulphate to 10 mg protein in the extract. The additions were made while the extract was continuously stirred at 0°C. After equilibration for 20 minutes the precipitated nucleic acid and protein were removed by centrifugation at 38000 g for 15 minutes at 2°C.

2. Ammonium sulphate fractionation. The stirred

extract was treated by the slow addition of solid ammonium sulphate at 0°C. The table of Dawson et al (1969) was used to compute the amount of $(NH_4)_2SO_4$ required. After equilibration for 20 minutes the precipitate was removed by centrifugation at 38000 g for 15 minutes at 2°C.

3. Heat treatment. A sample was heated by suspending a stirred beaker in a water bath of the required temperature for 10 minutes. The precipitate was removed by centrifugation at 38000 g for 15 minutes at 2°C.

4. Gel filtration. The column medium used was Sephadex G25, G150, or G200. It was pre-swollen in the sample application buffer, the fines were decanted and degassing was carried out according to the manufacturer's instructions (Gel filtration theory and practice, by Pharmacia Fine Chemicals). The method of sample application depended on the column being used. For G25 the sample was applied by running it down the side of the column onto the drained bed surface. The sample was allowed to run into the column and then washed into it with a small volume of buffer. Following this the column was refilled with buffer, connected to a reservoir and allowed to elute. When using a G200 column the sample was applied under the buffer at the column head to avoid disturbing the column surface. After sample application using a bent pipette, the column was attached to a reservoir of equilibration buffer. This was pumped through the column using an LKB Varioperpex peristaltic pump, at a flow rate of 10 ml h^{-1} until sample elution had occurred.

In all columns fractions (usually 4 - 5 ml) were collected using an LKB 7000 Ultravac fraction collector and

the protein in the eluate was monitored using an LKB 8300 Uvicord II at 280 nm.

5. DEAE-cellulose ion exchange chromatography. The material used was either Whatman DE 52 or DEAE Sephacel. Both are pre-swollen and just require equilibration in the buffer to be used for elution. Most work carried out using ion exchange utilised Whatman DE 52. Where DEAE Sephacel was used in a purification, it is named in the procedure concerned. In both cases, however, the material was equilibrated for a period of five to six hours during which the buffer was changed five times, the fines were decanted and the material degassed. It was then cooled to $4^{o}C$ prior to packing a column also at that temperature. The column was loaded in a similar manner to that described for Sephadex G25, and was initially eluted with the equilibration buffer. After the first peak of protein had been washed off the column (the protein that would not adhere to the column), a gradient of sodium chloride in the equilibration buffer was initiated and fractions of 4 to 5 ml were collected as described previously.

6. Hydroxylapatite adsorption chromatography. The column material was equilibrated in two changes of sample buffer, the fines being decanted off after each change. The material was then cooled to $4^{o}C$ and a column was loaded at that temperature. Sample application and elution followed a similar procedure as that used for DEAE-cellulose chromatography.

7. Dialysis. This procedure was used to remove inorganic molecules from samples, e.g. $(NH_4)_2SO_4$ from ammonium sulphate precipitations, and sodium chloride from gradient eluted column samples where it was thought that removal by

passage down a column of Sephadex G25 would be detrimental to the purification process. The dialysis tubing was soaked for 12 hours in the dialysis buffer. It was then filled with sample and the ends securely tied before being placed in 100 volumes of stirred dialysis buffer for between 1 and 3 hours.

8. Concentration by ultrafiltration. Concentration was carried out by passing the sample through an Amicon Diaflow PM10 ultrafilter at a pressure of 40 lb per in^2 (2.8 kg cm^{-2}), in an Amicon pressure filtration vessel.

Molecular weight determination

Molecular weights of the native enzymes were determined by passage down a column of Sephadex G200 using the method of Andrews (1964). The molecular weight standard used were: cytochrome c (M. Wt. 12400), bovine serum albumin (M. Wt. 65000-70000), lactate dehydrogenase (M. Wt. 130000-140000), catalase (M. Wt. 230000-250000), ovalbumin (M. Wt. 43000), aldolase (M. Wt. 158000), β galactosidase (M. Wt. 510000 - 530000).

Polyacryamide gel electrophoresis

Denaturing polyacrylamide gels were used to determine the subunit size of the purified proteins. The methods used were based on those of Laemmli (1970). Separation gels (7% w/v acrylamide) were prepared without stacking gels in glass tubes (7 x 0.5 cm). Samples were prepared in the buffer mixture of Laemmli (1970) and then heated to 90°C for 5 minutes, cooled, and applied to the gels. Samples were overlaid with electrophoresis buffer and run at 2mA per gel

for 2 - 3 hours. Gels were stained with 2% (w/v) Coomassie Brilliant Blue G250 in 7% (v/v) acetic acid for 1 hour, then destained in repeated changes of 7% (v/v) acetic acid containing 5% (v/v) methanol. The molecular weight standards used were: horse heart cytochrome c (M. Wt. 12500), soya bean trypsin inhibitor (21500), hen egg ovalbumin (45000), beef liver catalase subunit (60000), bovine serum albumin (68000), and the α, β and β' subunits of Escherichia coli RNA polymerase (α -39000, β -155000, β'-165000).

Non-denaturing polyacryamide gels were used to estimate the purity of enzyme purifications. This procedure was carried out at pH 8.8 with 7.5% (w/v) polyacrylamide gels as described by Davis (1964).

CHAPTER 3

Arthrobacter 2B2

Arthrobacter 2B2 when grown in batch culture on the medium described in the Materials and Methods section, exhibited two morphologies depending on the stage of the growth cycle attained.

During the exponential growth phase the cells were rods which completely transformed into coccoid forms at the end of the growth phase. This phenomenon was independent of any growth substrate tested. Transference of the coccoid cells into fresh growth medium had the effect of again turning the cells into the rod form. Cells in exponential phase grew as irregular clumps, with some V-forms also being present; no motility was observed on any of the growth substrates utilized. Growth of the organism in the presence of light induced the formation of a yellow pigment within the cells. The pigment which was produced independently of growth substrate could be extracted by deproteinising the cell free extract, by making up to 10% (w/v) with trichloroacetic acid, then extracting the pellet produced by centrifugation of the precipitate, with acetone. The absorption spectrum of the extracted pigment showed three peaks. The first at 422 nm had a shoulder at 400 nm; the second was at 450nm, and the third at 474 nm. It was noted that pigment production occurred in cells stored at $4^{o}C$ after growth in the dark at $30^{o}C$.

Discussion

Arthrobacter 2B2 exhibits a rod to coccus transition depending on whether it is in exponential or stationary growth phase. This is a characteristic of the genus Arthrobacter, as are the presence of complex V formations in exponentially growing cells (Buchanan and Gibbons, 1974).

It has previously been reported that the facultative methylotroph Arthrobacter P1 produces a yellow pigment when grown in the light (Levering et al., 1981a). It was suggested that the colour was due to a carotenoid like structure. As the absorption spectra of the pigments produced by both organisms were very similar, the presence of a comparable pigment is indicated in Arthrobacter 2B2.

CHAPTER 4

The Utilization of Methylamine as Sole Source of Carbon

Crude cell free extracts of methylamine grown Arthrobacter 2B2 were examined for the presence of three groups of enzymes using the assay systems previously described in the Materials and Methods section. 1. Enzymes involved in the oxidation of methylamine to yield formaldehyde; 2. those utilized in the fixation, cleavage and rearrangement phases of the RuMP cycle, and 3. enzymes used in the serine pathway of C_1 fixation. The activities obtained using cells grown on the C_1 substrate methylamine were compared to those obtained from glucose grown cells.

Table 2 represents a survey of such enzymes in Arthrobacter 2B2. The presence in methylamine grown cells of high activities of methylamine oxidase, together with the absence of methylamine dehydrogenase and methylglutamate dehydrogenase indicate that the substrate is directly oxidised to formaldehyde via the oxidase enzyme. The very low levels, or in some instances total lack of enzymes involved with the serine pathway, in conjunction with high activities of hexulose phosphate synthase and phosphohexuloisomerase, indicate that the pathway of carbon assimilation from methylamine is the RuMP cycle. The rearrangement phase using one molecule of glyceraldehyde 3-phosphate and two molecules of fructose 6-phosphate is accomplished by the enzymes transaldolase, transketolase, ribulose 5-phosphate 3-epimerase, and phospho-riboisomerase. These enzymes all have relatively high activities in Arthrobacter 2B2. The absence of sedoheptulose 1,7-diphosphatase together with the high activity of transaldolase in Arthrobacter 2B2 indicates that the rearrangement pattern is Transaldolase$^+$ Sedoheptulose 1,7-diphosphatase$^-$.

The presence of phosphofructokinase and fructose

TABLE 2 Specific activities of enzymes in cell free extracts of Arthrobacter 2B2

The preparation of crude cell free extracts and the assay procedures used are described in the Materials and Methods section. The assay method used for hexulose phosphate synthase was that of van Dijken et al., (1978).

Enzyme	Specific Activities (m units mg protein^{-1})	
	Methylamine grown	Glucose grown
Hexulose phosphate synthase	340	25
Phosphohexuloisomerase	240	5
Glucose 6-phosphate dehydrogenase		
NADP	95	12
NAD	n.d.	n.d.
6-phosphogluconate dehydrogenase		
NADP	290	150
NAD	n.d.	n.d.
Transketolase	950	120
Transaldolase	228	58
Sedoheptulose 1,7-diphosphatase	n.d.	n.d.
Phosphoriboisomerase	1790	240
Ribulose 5-phosphate 3-epimerase	737	152
6-phosphofructokinase	131	220
6-phosphogluconate dehydrase and 2-keto 3-deoxy 6-phosphogluconate aldolase)	n.d.	n.d.
Fructose 1,6-phosphate aldolase	71	45
Fructose 1,6-phosphatase	58	21
Formaldehyde dehydrogenase NAD	n.d.	n.d.
DCPIP	n.d.	n.d.

Enzyme		Specific Activities (m units mg protein^{-1}) Methylamine grown	Glucose grown
Formate dehydrogenase	NAD	1	n.d.
Methanol dehydrogenase		n.d.	n.d.
Methylamine oxidase		128	n.d.
Hydroxypyruvate reductase	NADH	13	n.d.
	NADPH	990	n.d.
Serine transhydroxymethylase		0.4	n.d.
Serine- α ketoglutarate aminotransferase		n.d.	n.d.
Glycerate kinase		n.d.	n.d.
Malyl CoA lyase		0.3	n.d.
Isocitrate lyase		8.4	n.d.

n.d. = not detected.

1,6-bisphosphate aldolase coupled with the lack of the enzymes of the Entner-Doudoroff cleavage system - 6-phosphogluconate dehydrase and 2-keto 3-deoxy 6-phosphogluconate aldolase, suggest that the route adopted for the net production of a C_3 moiety is the Embden-Meyerhof cleavage variant. Overall this indicates that the organism uses the fructose 1,6-bisphosphate aldolase$^+$, transaldolase$^+$ variant of the ribulose monophosphate cycle for the net production of dihydroxyacetone phosphate and regeneration of the acceptor molecule, RuMP.

One rather interesting result was that for hydroxypyruvate reductase. This enzyme has often been used as a diagnostic marker for the operation of the serine pathway of C_1 assimilation in methylotrophs. In Arthrobacter 2B2 there is a very high activity of hydroxypyruvate reductase during growth on methylamine. The enzyme is not constitutive as it is not present in glucose grown cells. However, the serine pathway appears to be inoperative as other key enzymes of the route are lacking.

The lack of NAD, and DCPIP-linked formaldehyde dehydrogenase coupled with the very low levels of NAD-linked formate dehydrogenase, indicate that a linear oxidation pathway of formaldehyde yielding CO_2 cannot be operative in this organism during growth on methylamine. Arthrobacter 2B2 did contain reasonable activities of NADP-linked 6-phospho-gluconate dehydrogenase, and NADP-linked glucose 6-phosphate dehydrogenase. This suggested that a cyclic pathway for the oxidation of formaldehyde may be operative in the organism.

Discussion

The key enzymes of the RuMP cycle, hexulose phosphate

synthase and phosphohexuloisomerase, have been located in Arthrobacter 2B2. Enzymes necessary for the regeneration of the acceptor moiety, RuMP, via this cycle are also present. The variant of the cycle adopted appears to be fructose 1,6-bisphosphate aldolase^{+}, transaldolase^{+}. Low activity of formaldehyde and formate dehydrogenases, coupled with higher activities of 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase, suggest the use of a cyclic formaldehyde oxidation route.

If it is assumed that the triose phosphate produced by the cycle is converted to pyruvate by established glycolytic steps, then the variant of the RuMP cycle utilized by this organism is the most energetically favourable of the four, with a net yield of one ATP.

The oxidation of methylamine is carried out via the enzyme methylamine oxidase. Such enzymes are widespread in eucaryotes such as some yeasts (Large, 1981) which can use methylamine as a nitrogen source but not as carbon source. However, at present only two other procaryotes have been shown to contain the enzyme, both of these belonging to the genus Arthrobacter (Loginova and Trotsenko, 1976), (Levering et al., 1981a). It is interesting to note that the use of methylamine oxidase as the mechanism of methylamine oxidation is in terms of ATP production, the least efficient of the methylamine oxidising methods as it by-passes electron transport phosphorylation completely. This is possible a reason why such enzymes have not proved to be widespread amongst methylotrophic bacteria.

CHAPTER 5

Purification and Characterisation of Hexulose Phosphate Synthase from Arthrobacter 2B2

Introduction

Hexulose phosphate synthases have been previously purified from several organisms, notably Methlyococcus capsulatus (Ferenci et al.,1974), Methylomonas M.15 (Sahm et al., 1976), Methylomonas aminofaciens (Kato et al., 1978), and Methylophilus methylotrophus (Beardsmore et al., 1982). Methylococcus capsulatus is a methane utilising methylotroph, and its hexulose phosphate synthase appears to be markedly different from the enzymes purified from the other three organisms, which are obligate methanol utilisers.

The hexulose phosphate synthase from the facultative methylotroph Arthrobacter 2B2 has been purified and characterised, in order to compare the properties of the enzyme from this facultative organism, with those of enzymes previously purified from an obligate methane utiliser (Methylococcus capsulatus), and obligate methanol utilisers (Methylomonas M.15, Methylomonas aminofaciens, and Methylophilus methylotrophus).

TABLE 3 Ammonium sulphate fractionation of hexulose phosphate synthase of methylamine grown Arthrobacter 2B2

Crude cell free extract prepared by sonicating a suspension of one part (wet weight) of cells with four parts (by volume) of 20 mM sodium potassium phosphate buffer pH 7.5 containing 5 mM $MgCl_2$ was subjected to ammonium sulphate fractionation using the procedures outlined in the Materials and Methods section.

Fraction	Total Activity (m units)	Specific Activity (m units mg protein^{-1})
Crude extract	12480	288
0-30% $(NH_4)_2SO_4$	2400	300
30-40% $(NH_4)_2SO_4$	660	73
40-50% $(NH_4)_2SO_4$	0	0
50-60% $(NH_4)_2SO_4$	3900	354
60-70% $(NH_4)_2SO_4$	2400	600
70-80% $(NH_4)_2SO_4$	1372	280

Crude cell free extracts of methylamine-grown Arthrobacter 2B2 were produced as described in the Materials and Methods section, using 20 mM sodium potassium phosphate buffer at pH 7.5. Initially cell free extracts were made in sodium potassium phosphate buffer in the presence or absence of 5 mM $MgCl_2$. The extract prepared in the presence of $MgCl_2$ had hexulose phosphate synthase activities in the range of 300 to 500 m units (mg protein)$^{-1}$, whilst that produced in buffer lacking $MgCl_2$ had activities of around 40 m units (mg protein)$^{-1}$. The results indicate that the hexulose phosphate synthase contained in cell free extracts of Arthrobacter 2B2 had a requirement for the presence of Mg^{2+} in order to remain fully active. All buffers subsequently used during the course of the purification of this enzyme contained $MgCl_2$.

Initial Purification Procedure

As an initial step in the purification process, the crude cell free extract was treated with ammonium sulphate in order to precipitate protein. Crude extract was raised to 30% saturation with solid $(NH_4)_2SO_4$, the resulting supernatant being subjected to sequential 10% $(NH_4)_2SO_4$ fractionations up to a saturation of 80%. All precipitated protein was redissolved in 20 mM sodium potassium phosphate buffer pH 7.5, and assayed for hexulose phosphate synthase activity. The results obtained are shown in Table 3.

After subjecting the crude extract to $(NH_4)_2SO_4$ fractionation, two peaks of hexulose phosphate synthase activity were observed. The first peak was between 0% and 40% saturation, the second between 50% and 80% saturation. This could be the result of: (1) the presence of two enzymes

catalysing the same reaction and precipitating at different percentages of $(NH_4)_2SO_4$; or, (2) the presence of a single enzyme which has some association with particulate matter within the cell. It could be envisaged that if the association between enzyme and particulate matter was a loose one, sonication would dislodge some but not all of the enzyme, thus accounting for the presence of the two peaks of activity observed during $(NH_4)_2SO_4$ fractionation. The 0% to 40% peak would be due to the enzyme bound to small particulate matter, which would not sediment during the relatively low speed centrifugation used to produce cell free extract, but would precipitate at low percentages of $(NH_4)_2SO_4$. The 50% to 80% peak would be due to free unbound enzyme.

As previous studies had produced no evidence to suggest the presence of two hexulose phosphate synthases in one organism, but there is at least one membrane-bound enzyme (Ferenci et al., 1974), the latter possibility was most favoured. In order to test this theory a crude cell free extract was produced and subjected to a high speed centrifugation at 100000 g. After centrifugation the supernatant was decanted and the pellet was resuspended in 20 mM sodium potassium phosphate buffer pH 7.5 containing 5 mM $MgCl_2$, and then both fractions were assayed for enzyme activity.

The resuspended pellet contained approximately 20% of the total activity included within the cell free extract, the remaining 80% being in the supernatant. This corresponds well to the 25% of total activity contained within the 0% to 40% $(NH_4)_2SO_4$ fraction.

A membrane solubilization technique (Ferenci et al., 1974) was subsequently employed on the resuspended high speed

pellet. This involved the addition of NaCl up to a final concentration of 1 M. This was allowed to stand for 1 hour before centrifugation at 100000 g for 2 hours. The supernatant was assayed for the enzyme, and contained approximately 50% of the activity present in the original high speed pellet.

This confirms that the hexulose phosphate synthase from Arthrobacter 2B2 probably has a loose association with particulate material in the organism. Sonication disrupts the organism releasing much of the activity. Some, however, remains attached to particulate matter and this becomes apparent during $(NH_4)_2SO_4$ fractionation, or on high speed centrifugation.

A 50% to 80% $(NH_4)_2SO_4$ fractionation was adopted as an initial step in the purification process, the loss of activity in the initial 0% to 40% fraction being considered acceptable.

The resulting protein after resuspension in 20 mM sodium potassium phosphate buffer pH 7.5 containing 5 mM $MgCl_2$ was applied to a column of Sephadex G150 (36 x 2.5 cm), equilibrated with resuspension buffer and eluted with the same buffer. This step had the effect of removing ammonium sulphate as well as fractionating proteins by size. Fractions containing activity were pooled, and applied to a column of DEAE cellulose anion exchanger (6.0 x 1.5 cm) equilibrated with resuspension buffer. The active fractions were pooled and then concentrated to a small volume by ultrafiltration.

During the course of the purification process, which was carried out within 15 hours, a dramatic decrease in enzyme activity occurred, culminating in the total inactivation of the enzyme after ultrafiltration. The results indicated

that the enzyme was highly unstable. To confirm that theory a sample of the pooled eluate from the DEAE column was allowed to stand at 4°C and assayed for activity at timed intervals. This resulted in a 50% loss of activity in 2.5 hours, and a total loss of activity within 18 hours.

Effects of Stabilisers and Activators

In order to continue with an effective purification of the enzyme it was necessary to find a stabilising agent capable of retaining the activity of the enzyme during the course of the purification process. Initially 2 mM reduced glutathione (GSH), 2 mM dithiothreitol (DTT), 10% (v/v) methanol, and an equilibrium mixture of ribose 5-phosphate and ribulose 5-phosphate (produced by incubating 5 μ mol ribulose 5-phosphate with 2 units of phosphoriboisomerase and adding to 1 ml of partially purified enzyme), were added to samples of partially purified enzyme stored at 4°C, and its activity assayed at timed intervals. Figure 12 shows the results obtained with reference to a sample of enzyme to which no additions had been made.

DTT and GSH caused an initial decrease in activity compared to the sample which had no addition made. There was then a levelling off of the decay up to 200 minutes, after which there was a sharp decrease in activity. The equilibrium mixture of ribose 5-phosphate and ribulose 5-phosphate also gave an initial decrease in activity, after which the decay continued, but at a considerably reduced rate compared to that which occurred in samples containing DTT, GSH or with no addition. Methanol caused an immediate increase in the activity of the enzyme, followed by decay,

FIGURE 12 Effects of stabilisers on partially purified hexulose phosphate synthase of methylamine grown Arthrobacter 2B2

The prospective stabiliser was added to the enzyme at the stated concentration. Samples were withdrawn at times intervals and assayed for hexulose phosphate synthase by the continuous assay procedure.

Key:

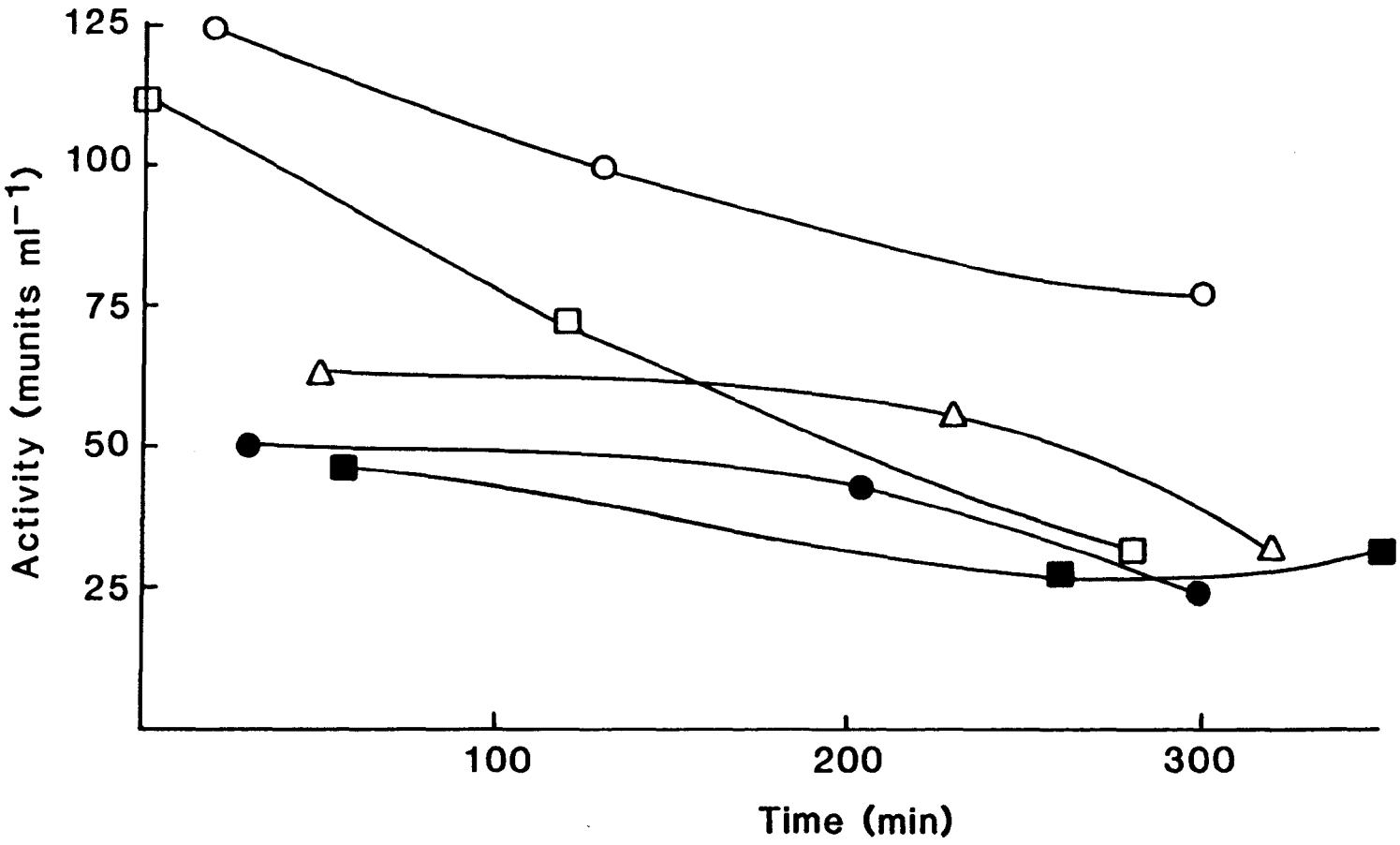
● 2 mM GSH

△ 2 mM DTT

○ 10% (v/v) Methanol

■ Ribose 5-phosphate/ribulose 5-phosphate mixture

□ None


125
100
75
50
25
Activity (munits ml^{-1})
100
200
300
Time (min)

again at a rate lower than that occurring in the other samples.

Samples of partially purified enzyme were next subjected to the addition of methanol plus an equilibrium mixture of ribose 5-phosphate and ribulose 5-phosphate, the effects of the addition being compared to those obtained with the additions of methanol, and the ribose 5-phosphate/ribulose 5-phosphate mixture singly. The results (Figure 13) confirmed that methanol activated the enzyme, whilst the ribose 5-phosphate/ribulose 5-phosphate mixture caused a decrease in activity, but also decreased the rate of decay. However, when methanol and the ribose 5-phosphate/ribulose 5-phosphate mixture were both added to the same portion of enzyme, a massive increase in activity, far higher than that observed using methanol alone, was observed. The mixture not only activated but also greatly stabilised the enzyme, the same activity being retained over a period of 18 days when stored at $4^{o}C$.

Tests were carried out on the effect of adding 10% (v/v) ethanol and 10% (v/v) glycerol to the partially purified enzyme to determine whether the activation could also be carried out by other alcohols. Results from the experiment indicated that the other alcohols could activate the enzyme in a similar manner to methanol. However, methanol had a marginally greater effect than either ethanol or glycerol. In order to determine what concentration of methanol gave the best activating effect, various concentrations were placed in samples of the partially purified enzyme and assayed at timed intervals. Figure 14 shows that 5% (v/v) methanol had little effect on activity. 10% (v/v) and 20%

FIGURE 13 Effects of ribose 5-phosphate/ribulose 5-phosphate and methanol on partially purified hexulose phosphate synthase of methylamine grown Arthrobacter 2B2

The stabilisers were added to the enzyme in the concentration stated. Samples were withdrawn at timed intervals and assayed for hexulose phosphate synthase by the continuous assay procedure.

Key:

△ 10% (v/v) methanol

● Ribose 5-phosphate/ribulose 5-phosphate mixture

○ 10% (v/v) methanol + ribose 5-phosphate/ribulose 5-phosphate mixture

□ None

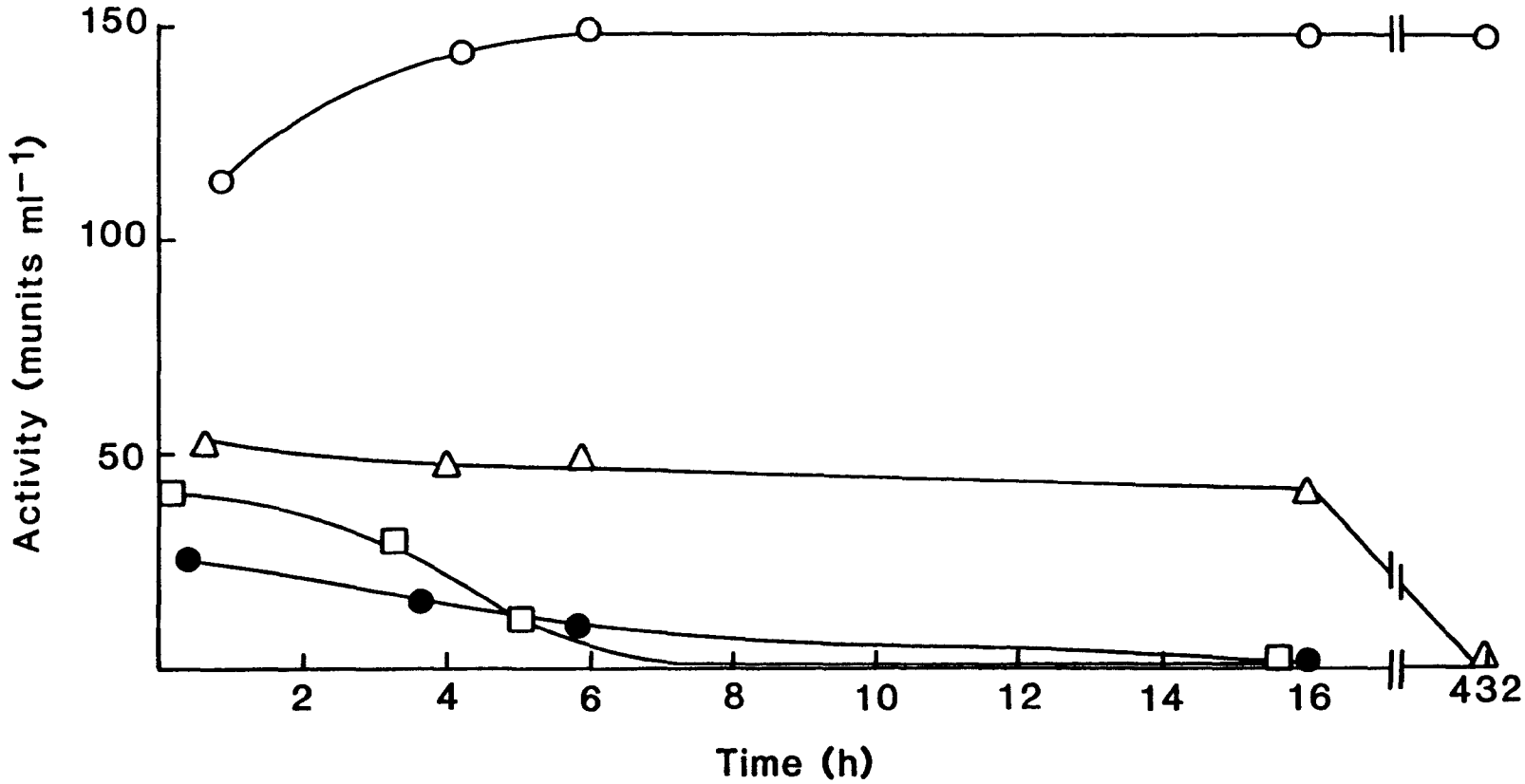

150
100
50
Activity (munits ml⁻¹)
2
4
6
8
10
12
14
16
432
Time (h)

FIGURE 14 Effects of methanol concentration o the activity and stability of partially purified hexulose phosphate synthase from methylamine grown Arthrobacter 2B2

Methanol was added to the enzyme at the concentration stated. Samples were withdrawn at timed intervals and assayed for hexulose phosphate synthase activity using the continuous assay procedure.

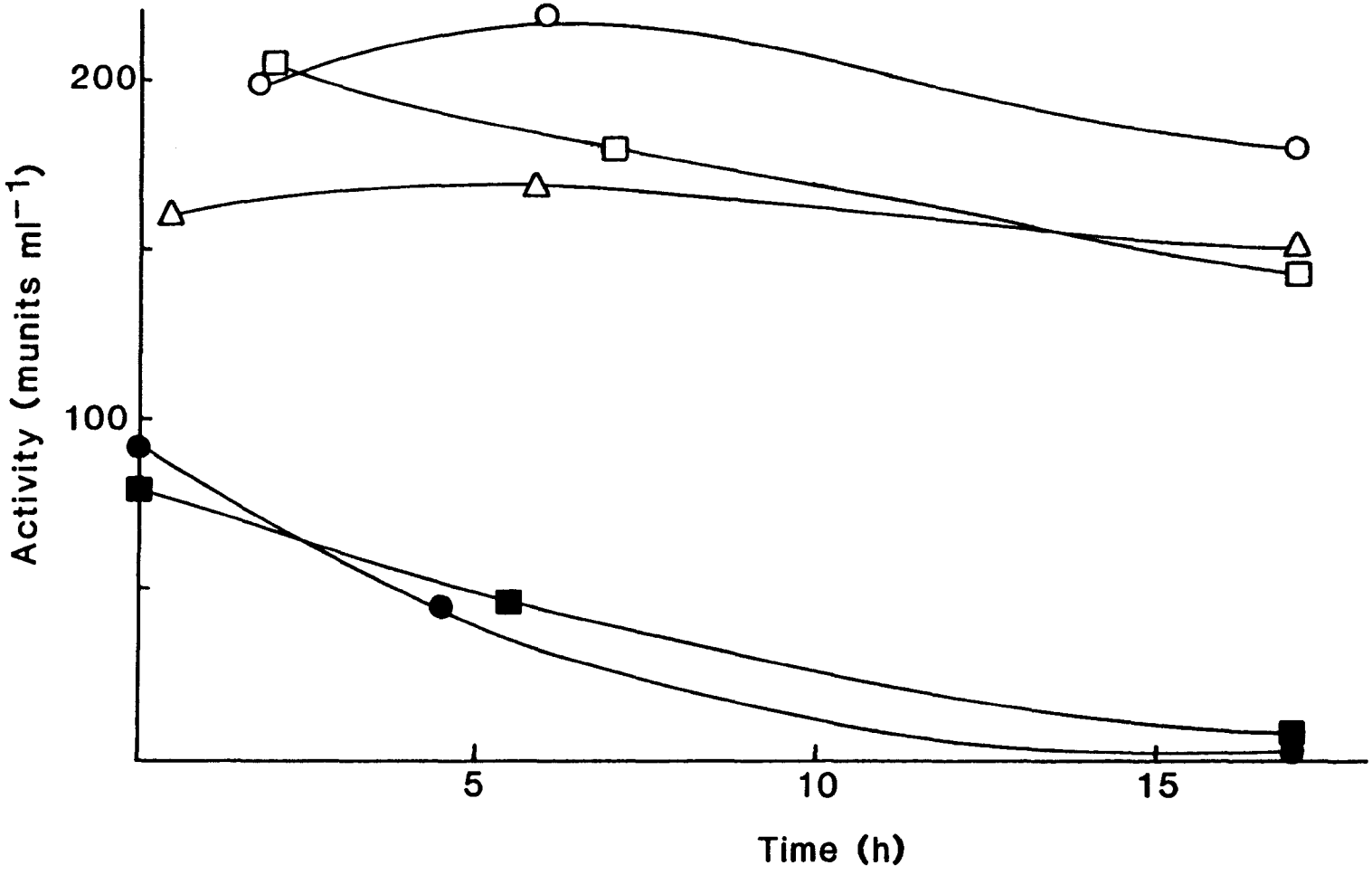
● 0% (v/v) methanol

■ 5% (v/v) methanol

△ 10% (v/v) methanol

○ 15% (v/v) methanol

□ 20% (v/v) methanol

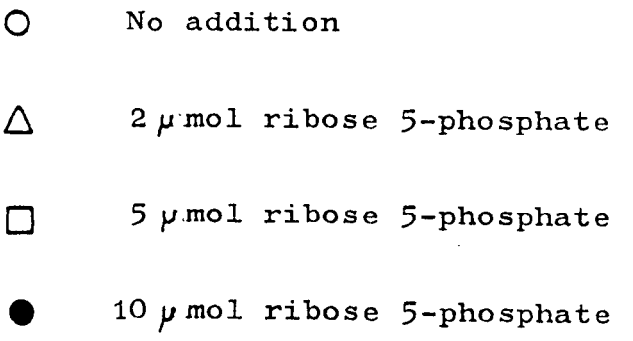

200
100
Activity (munits ml^{-1})
5
10
15
Time (h)

(v/v) methanol both act to a similar degree, but by far the best concentration was 15% (v/v) methanol. A similar experiment was carried out using differing concentrations of ribose 5-phosphate (all concentrations containing 2 units of phosphoriboisomerase). The results displayed in Figure 15 show that 2 μ mol, 5 μ mol and 10 μ mol ribose 5-phosphate plus 2 units of phosphoriboisomerase, when added to 1 ml of partially purified enzyme containing 15% (v/v) methanol gave a similar effect. However, 5 μ mol ribose 5-phosphate did prove to exhibit a marginally better result.

The ribose 5-phosphate/ribulose 5-phosphate mixture contains three components: ribose 5-phosphate, phosphoriboisomerase, and ribulose 5-phosphate which is formed by the reaction of the former with the latter. The stabilising effect of the mixture could reside in any one of these, or in any combination. To determine which component or components were responsible, an experiment was set up in which samples of partially purified enzyme containing 15% (v/v) methanol were incubated with: 1. phosphoriboisomerase, 2. ribose 5-phosphate, 3. ribose 5-phosphate plus phosphoriboisomerase (a mixture of ribose 5-phosphate, ribulose 5-phosphate and phosphoriboisomerase). Samples were withdrawn at timed intervals and assayed for activity, their activities being compared to those from a sample containing methanol alone. Figure 16 shows the results obtained. Phosphoriboisomerase gave a decay rate greater than that for methanol alone, and therefore could play no direct part in the stabilisation of the enzyme. Both ribose 5-phosphate and the mixture of ribose 5-phosphate and phosphoriboisomerase gave a similar level of activation above that obtained using methanol alone. This indicates one of

FIGURE 15 Effect of varying the ribose 5-phosphate concentration on the activity of hexulose phosphate synthase from methylamine grown Arthrobacter 2B2

The reagent was added to 1 ml of enzyme in the concentration stated. All samples contained 15% (v/v) methanol and 2 units of phosphoriboisomerase.



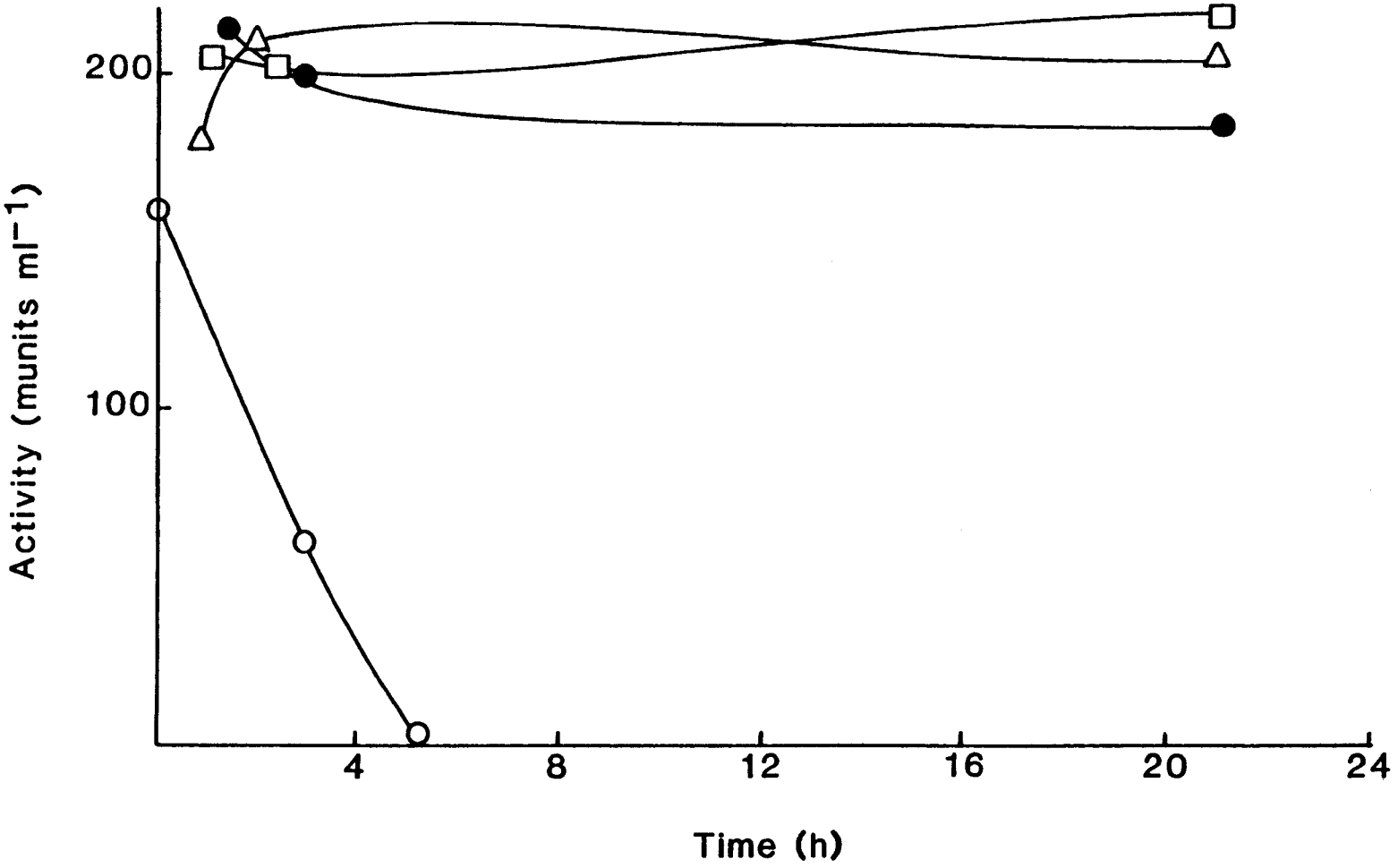

200
100
Activity (munits ml^{-1})
4
8
12
16
20
24
Time (h)

FIGURE 16 Effects of ribose 5-phosphate, phosphoriboisomerase and methanol on partially purified hexulose phosphate synthase of methylamine grown Arthrobacter 2B2

The reagents were added to the enzyme in the concentration stated and samples were withdrawn at timed intervals to be assayed by the continuous assay procedure.

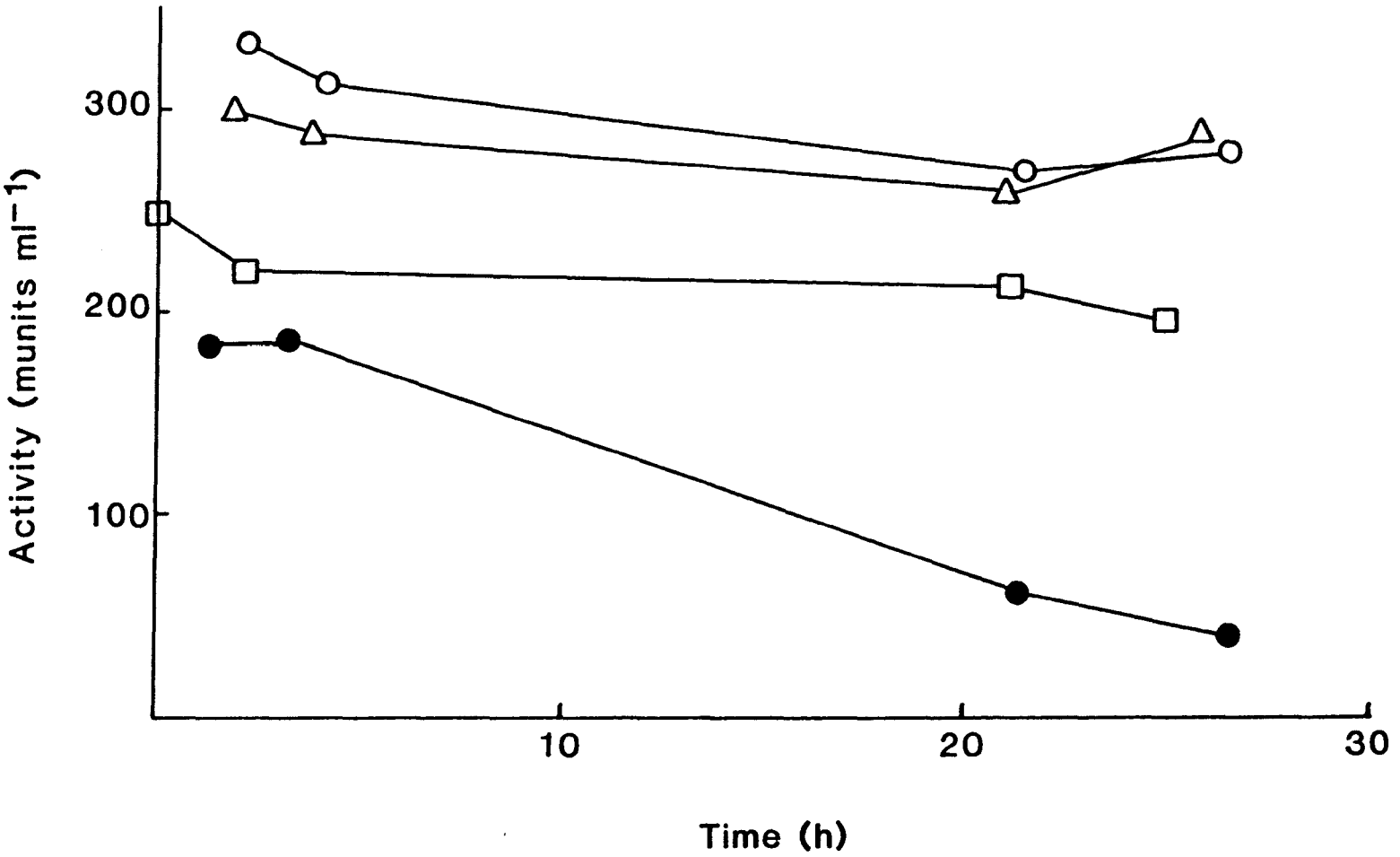
Key:

△ 5 mM ribose 5-phosphate + 15% (v/v) methanol

● 2 units phosphoriboisomerase + 15% (v/v) methanol

○ ribose 5-phosphate/ribulose 5-phosphate mixture (5 mM ribose 5-phosphate + 2 units phosphoriboisomerase) + 15% (v/v) methanol

□ 15% (v/v) methanol


Activity (munits ml⁻¹)
300
200
100
10
20
30
Time (h)

two options: 1. ribose 5-phosphate is the active reagent or, 2. ribulose 5-phosphate is the active reagent. For the latter to occur either the partially purified enzyme extract contains phosphoriboisomerase, thus converting ribose 5-phosphate to ribulose 5-phosphate, or the ribose 5-phosphate is contaminated with ribulose 5-phosphate. Assays were carried out on the partially purified extract to test for the presence of phosphoriboisomerase, none being found present. This indicated that the ribose 5-phosphate used might contain some ribulose 5-phosphate. The ribose 5-phosphate was assayed for the presence of ribulose 5-phosphate using the phosphoriboisomerase assay of Domagk and Doering (1975), which acts by detecting the presence of ribulose 5-phosphate. The assay had a positive result, showing the presence of ribulose 5-phosphate in the commercially obtained ribose 5-phosphate. The result still did not prove conclusively that ribulose 5-phosphate was the agent in the mixture responsible for its effects; the role of ribose 5-phosphate as the active agent has not been disproved. However, the former would appear to be the preferred option as ribulose 5-phosphate is the substrate for the enzyme and the observed increase in activity and stability could be explained by the effects of substrate stabilisation.

In order to stabilise and activate the enzyme during purification, methanol at a concentration of 15% (v/v) was used universally in all buffers, and an equilibrium mixture of ribose 5-phosphate/ribulose 5-phosphate produced by incubating 5 μ mol ml^{-1} ribose 5-phosphate with 2 units of phosphoriboisomerase, was added at selected stages to aid stabilisation and activation. The equilibrium mixture was

not used throughout the purification process, as its effects only became apparent after the enzyme had been partially purified. Experiments carried out on crude cell free extracts of the organism showed that methanol plus the equilibrium mixture gave a similar activation to that obtained using methanol alone. The reason for this could be explained by the presence of ribose 5-phosphate and ribulose 5-phosphate in the cells. These would be released into the cell free extracts during sonication and would aid activation of the enzyme on addition of methanol. However, as the purification proceeded the sugar phosphates would be removed in steps such as dialysis, and it would be at this point that the addition of the equilibrium mixture would exert its activating effect.

Final Purification Procedure

Initially the purification procedure used was that described previously, except that 15% (v/v) methanol was used in all of the buffers. However, two problems manifest themselves when this was carried out. First, it was evident that activity was being lost during the passage through the Sephadex G150 gel filtration column. As this did not happen before it was put down to a change in the properties of the column due to the presence of methanol in the buffers. Secondly, at certain points in the purification process where buffers were agitated, e.g. during the mixing of a gradient, a precipitate formed in the buffer. Further experimentation proved this to be due to the $MgCl_2$ which had been rendered less soluble in the buffer by the addition of methanol. To rectify these two points, 1. the Sephadex G150 gel filtration

step was omitted from the purification procedure, and 2. the $MgCl_2$ concentration in the buffers was reduced from 5 mM to 2 mM. This retained the activity of the enzyme but did not form a precipitate on agitation.

The final purification procedure used is detailed below.

(i) Preparation of Crude Cell Free Extract

Methylamine grown cell paste (6 g) was suspended in 24 ml of 20 mM sodium potassium phosphate buffer pH 7.5 containing 2 mM $MgCl_2$ and 15% (v/v) methanol, and sonicated as a single batch. The cell debris was removed by centrifugation at 38000 g for 20 minutes at $2^{o}C$. The pellet was discarded and the supernatant treated as the crude cell free extract.

(ii) Ammonium Sulphate Fractionation

The crude cell free extract was raised to 50% saturation by the addition of solid $(NH_4)_2SO_4$ and the precipitated protein was removed by centrifugation. The resulting supernatant was treated by further fractionation with $(NH_4)_2SO_4$ to 80% saturation. After the precipitated protein had been collected by centrifugation, the pellet was redissolved in sonication buffer.

(iii) DEAE Cellulose Chromatography

The redissolved protein from the ammonium sulphate fractionation was dialysed for three hours against 100 volumes of 20 mM sodium potassium phosphate buffer pH 7.5, containing 2 mM $MgCl_2$ and 15% (v/v) methanol. The dialysed extract was then applied to a DEAE cellulose column (6 x 1.5 cm) equilibrated with dialysis buffer. The column was then washed with 60 ml of the same buffer, before applying a gradient of 0-250 mM sodium chloride made up in 400 ml of sonication

TABLE 4 The purification of hexulose phosphate synthase from methylamine grown Arthrobacter 2B2

Fraction	Volume ml	Protein mg	Activity Units	Specific Activity Units mg protein^{-1}	Purification fold	Yield %
Crude cell free extract	24	139.2	200.64	1.44	1	100
$(NH_4)_2SO_4$ fractionation	24	91.2	180.8	1.94	1.7	90
DEAE cellulose chromatography	178	27.5	284.8	10.3	7.1	141
Hydroxylapatite chromatography	135	16.2	308.1	19.0	13	153

buffer. Activity eluting from the column was detected using the continuous spectrophotometric assay described in the Materials and Methods section. Fractions 65 to 110 containing activity were pooled and an equilibrium mixture of ribose 5-phosphate/ribulose 5-phosphate was added. The pooled fractions were then ultrafiltered to a small volume.

(iv) Hydroxylapatite Chromatography

The concentrated enzyme from step (iii) was desalted by dialysis for three hours against 100 volumes of 20 mM sodium potassium phosphate buffer pH 7.5 containing 2 mM $MgCl_2$, 15% (v/v) methanol, and an equilibrium mixture of ribose 5-phosphate/ribulose 5-phosphate. The dialysed enzyme was then applied to a hydroxylapatite column (4.0 x 1.5 cm) equilibrated with sonication buffer. A gradient of 20 to 150 mM sodium potassium phosphate buffer pH 7.5, containing 2 mM $MgCl_2$ and 15% (v/v) methanol was used to elute the column. Fractions were collected and numbers 8 to 50 containing enzyme activity were pooled and stored at $-15^{o}C$.

A table of the purification of hexulose phosphate synthase is given in Table 4. Figures 17 and 18 represent the DEAE cellulose and hydroxylapatite chromatography steps in the purification of the enzyme.

As shown, the enzyme was purified 13-fold with an apparent recovery of 153%. The reason for the apparent increase in yield is due to the activating effects of the ribose 5-phosphate/ribulose 5-phosphate mixture, added after passage through the DEAE cellulose column.

Using enzyme assay techniques, the partially purified enzyme was found to be free from detectable activities of phosphohexuloisomerase, phosphoglucoisomerase, glucose

FIGURE 17 DEAE cellulose chromatography of Hexulose Phosphate Synthase of methylamine grown Arthrobacter 2B2

Extract was applied to a DEAE cellulose column (6.0 x 1.5 cm), equilibrated with 20 mM sodium potassium phosphate buffer pH 7.5 containing 2 mM $MgCl_2$ and 15% (v/v) methanol. The elution pattern was followed as described in the Materials and Methods section.

Key:

○ = Extinction at 280 nm

△ = Hexulose phosphate synthase activity (m units ml^{-1})

____ = Sodium chloride gradient

Fraction volume = 4.0 ml.

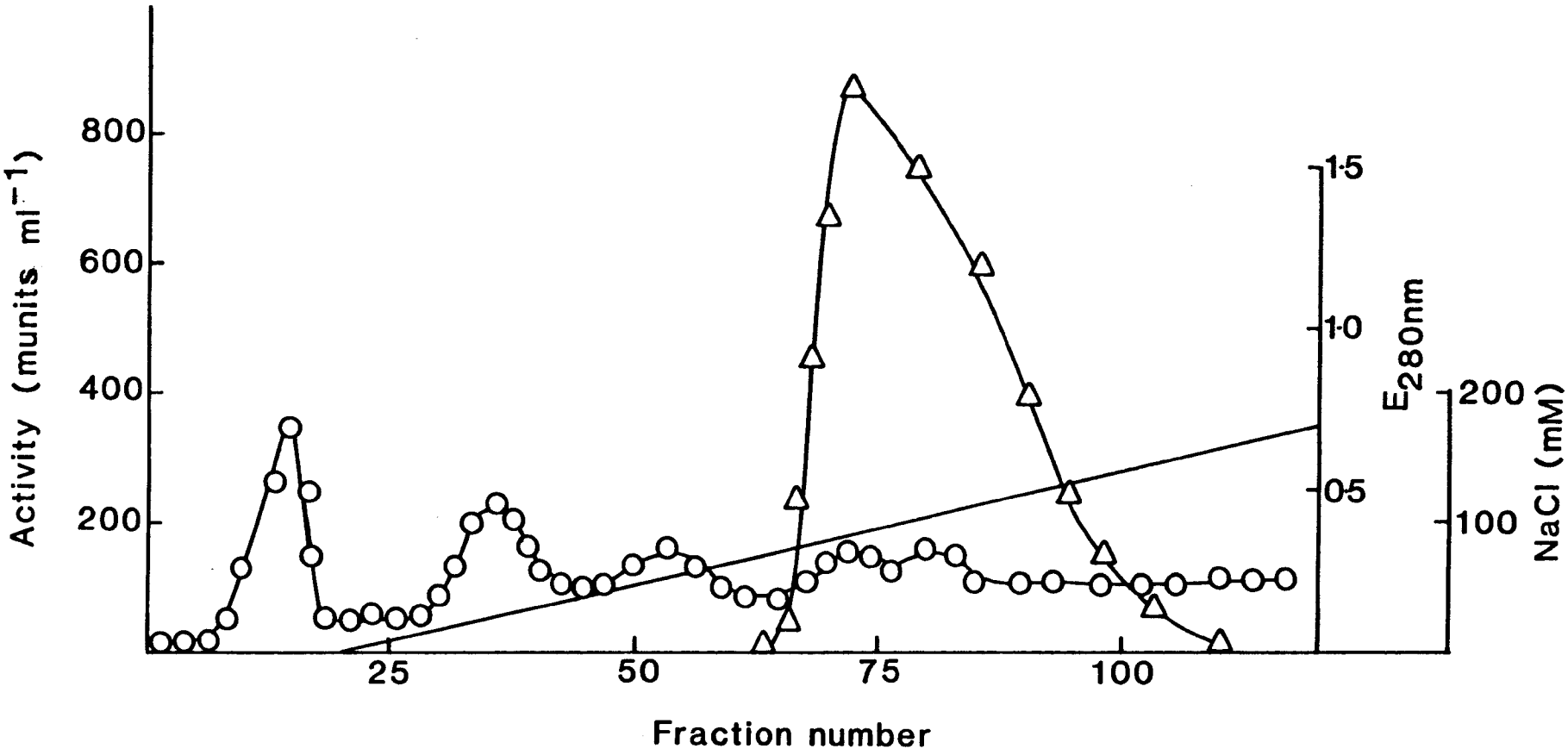

Activity (munits ml^{-1})
800
600
400
200
25
50
75
100
Fraction number
1·5
1·0
0·5
E_{280nm}
200
100
NaCl (mM)

FIGURE 18 Hydroxylapatite chromatography of hexulose phosphate synthase of methylamine grown Arthrobacter 2B2

Extract was applied to a hydroxylapatite column (4 x 1.5 cm), equilibrated with 20 mM sodium potassium phosphate buffer pH 7.5 containing 2 mM $MgCl_2$ and 15% (v/v) methanol. A gradient of 20 mM to 150 mM sodium potassium phosphate was initiated at fraction 6, and the elution pattern was followed as described in the Materials and Methods section.

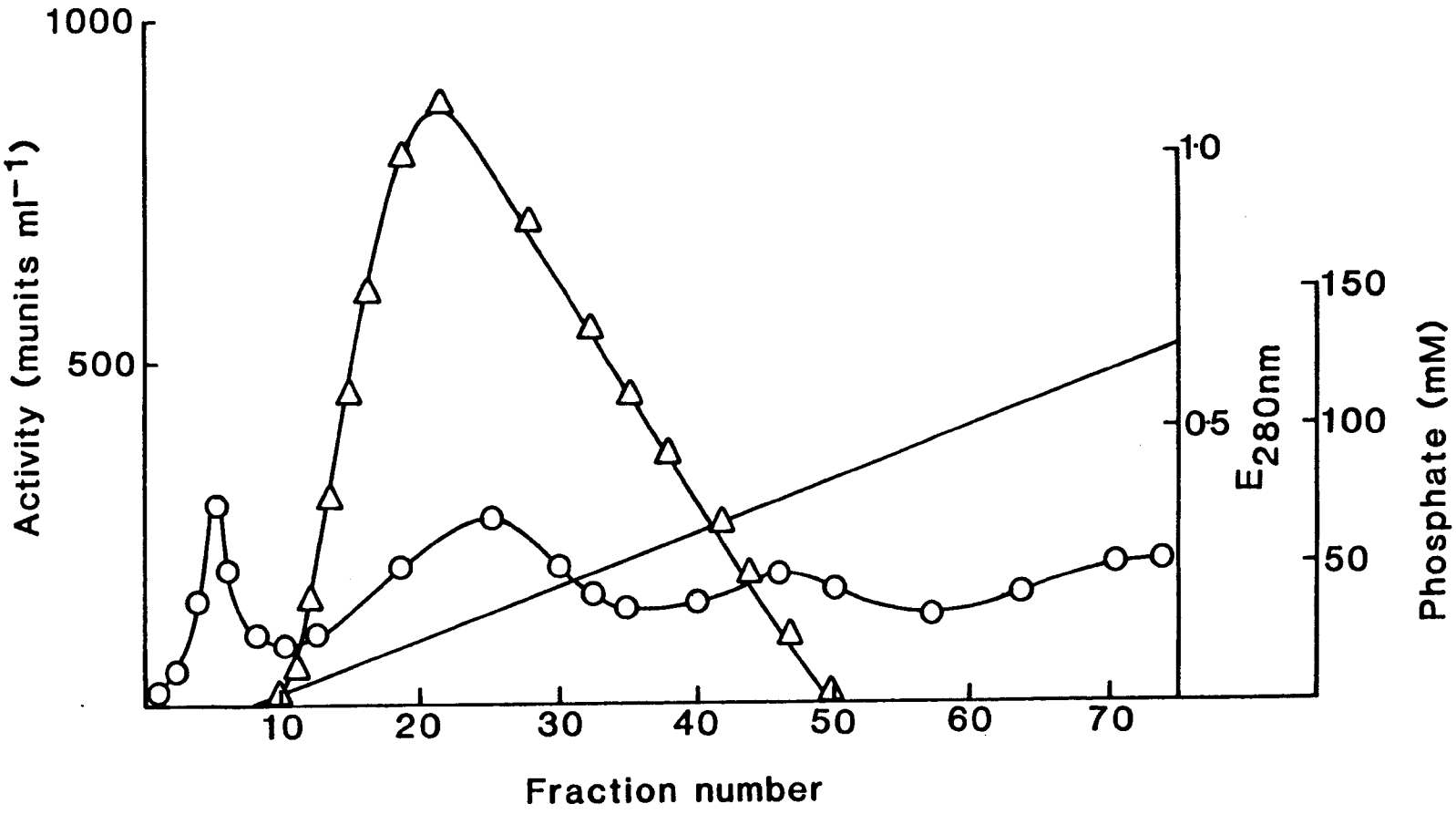
Key:

○ = Extinction at 280 nm

△ = Hexulose phosphate synthase activity (m units ml^{-1})

——— = phosphate gradient

Fraction volume = 3.2 ml.


1000
500
Activity (munits ml^{-1})
1·0
0·5
E_{280nm}
150
100
50
Phosphate (mM)
10
20
30
40
50
60
70
Fraction number

6-phosphate dehydrogenase, and phosphoriboisomerase. However, ribose 5-phosphate 3-epimerase was detected at an activity of 1.8 units (mg protein)$^{-1}$. This corresponds to a ratio of hexulose phosphate synthase to ribulose 5-phosphate 3-epimerase of about 11 : 1.

Non-denaturing polyacrylamide gel electrophoresis of the partially purified hexulose phosphate synthase was carried out after purification at pH 8.8 and 4^{o}C, adopting the procedure outlined in the Materials and Methods section. This provided the results shown in Figure 19. One major and four minor bands were observed. An activity stain was carried out on the completed gels, but no activity was detected, it being assumed that the enzyme had been inactivated during electrophoresis. It was presumed that the major band present on the electrophoresis gels was due to the enzyme hexulose phosphate synthase.

Properties of hexulose phosphate synthase

The properties of the hexulose phosphate synthase were established using samples of the partially purified enzyme.

Stability

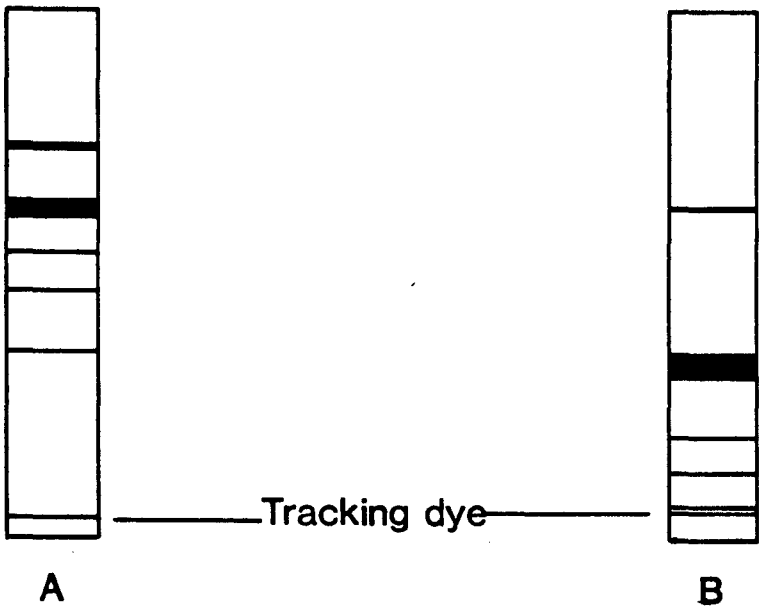
This has been discussed to some extent during the purification procedure for the enzyme. It was extremely unstable when stored without the presence of stabilisers in the buffers. Storage of the unstabilised enzyme at 0^{o}C caused a 100% loss of activity within 18 hours. If, however, the previously described full stabilising mixture of methanol and ribose 5-phosphate/ribulose 5-phosphate was included in the buffers containing the enzyme, the activity remained constant even after 18 days storage at 0^{o}C.

FIGURE 19 Polyacrylamide gel electrophoresis of partially purified hexulose phosphate synthase of methylamine grown Arthrobacter 2B2

The polyacrylamide gel electrophoresis of the purified enzyme was performed at pH 8.8 and 4°C using the method described in the Materials and Methods section. The diagram shows the actual length of the gel but the width has been increased 2-fold for clarity. The proteins were stained using Coomassie Brilliant Blue.

A protein bands stained in a 7% acrylamide gel

B protein bands stained in a 5% acrylamide gel


Tracking dye
A
B

On storage at -15°C the stabilised enzyme retained virtually all of its activity after 6 months. Repeated freezing and thawing reduced its activity only marginally.

To determine the stability to raised temperatures a sample of the stabilised enzyme was taken and placed at a temperature of 50°C. Samples were withdrawn at timed intervals and assayed for activity. Figure 20 shows the results obtained. The enzyme appears relatively unstable at this elevated temperature losing 50% of its activity after 60 minutes. There was an initial 33% loss of activity within the first 5 minutes of storage at this temperature which precluded the use of any heat step during purification procedure.

All steps in the purification were carried out at pH 7.5 and at this value the enzyme was very stable. Activity was retained on raising the pH to 8.5. Higher levels than this were not, however, tested. Lowering the pH to 5 reduced the activity of the enzyme to 20% of its former value within 5 minutes. The activity was not regained on increasing the pH back to 7.5.

pH optimum

The buffers used were 20 mM sodium potassium phosphate over the pH range 7.0 to 7.9, and 20 mM triethanolamine-sodium hydroxide over the pH range 7.3 to 8.2. The continuous spectrophotometric assay described in the Materials and Methods section was used throughout. The pH of the buffer was checked before each assay and the pH of the complete assay mixture was tested afterwards to verify that the pH had remained the same throughout. The linkage enzymes phosphoglucoisomerase and glucose 6-phosphate dehydrogenase

FIGURE 20 Effect of heat on partially purified hexulose phosphate synthase of methylamine grown Arthrobacter 2B2

A sample of partially purified enzyme contained in a stabilising buffer containing 15% (v/v) methanol and the ribose 5-phosphate/ribulose 5-phosphate mixture was exposed to a temperature of 50°C. Samples were withdrawn at timed intervals and assayed for activity.

Key:

○ = Sample at 50°C

△ = Sample at 4°C

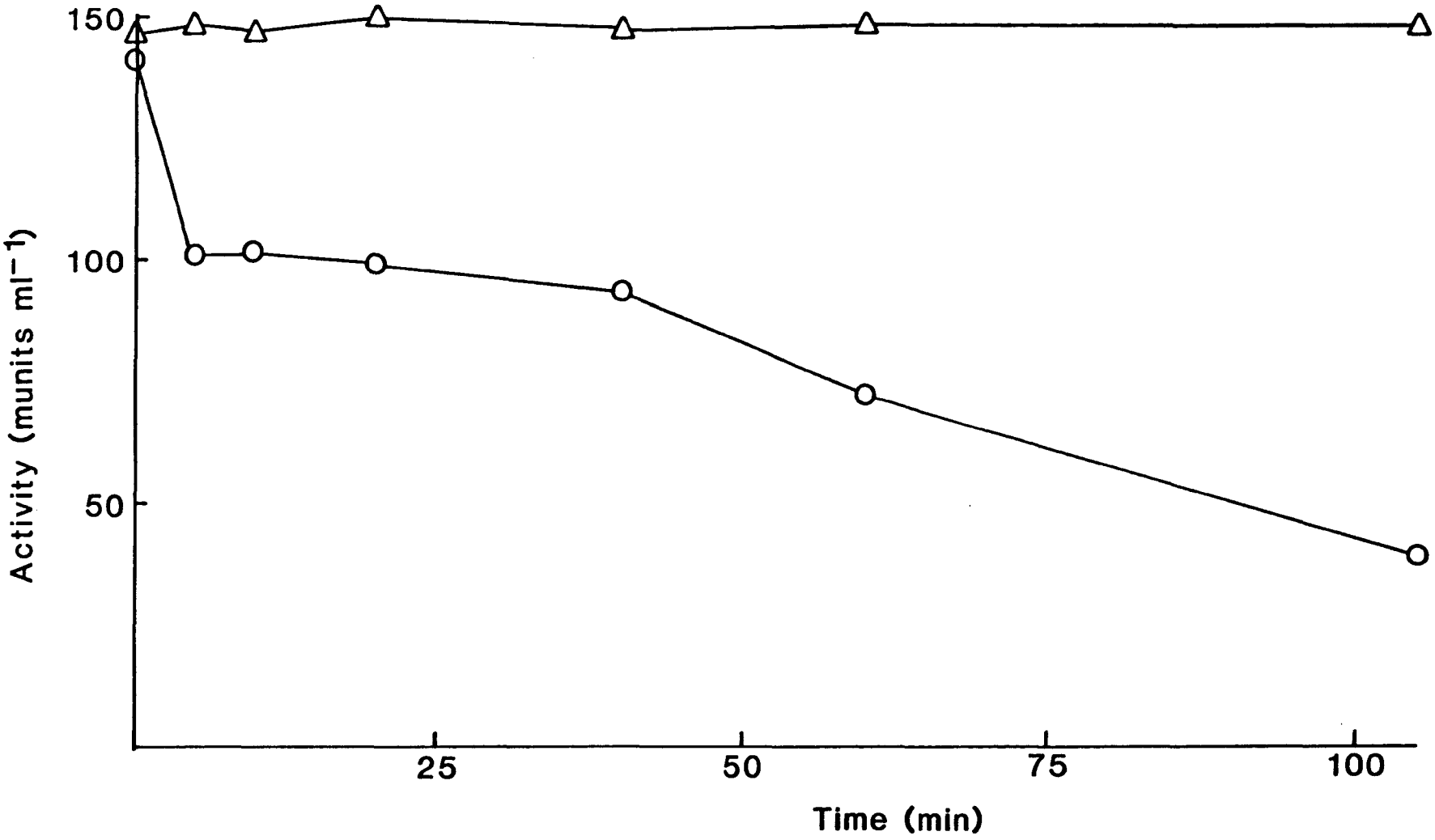

150
100
50
Activity (munits ml^{-1})
25
50
75
100
Time (min)

TABLE 5 Activity of hexulose phosphate synthase of methylamine grown Arthrobacter 2B2 in the presence of divalent metal ions.

The activity of the enzyme in the presence of the cation is expressed as a percentage of the activity measured in the presence of $MgCl_2$.

Metal ion	Concentration (mM)	Activity (%)
Mg	1	100
Mn	1	100
Zn	0.5	50
Co	1	43
Cu	1	0
Hg	0.5	0
Ca	1	51
None	-	0

TABLE 6 Inhibition of hexulose phosphate synthase of methylamine grown Arthrobacter 2B2 by divalent metal ions.

The inhibition of the enzyme was determined after the addition of the divalent cation to the standard assay which contained 1 mM $MgCl_2$. The inhibition is expressed as a percentage of the rate measured in the presence of 1 mM $MgCl_2$ alone.

Metal ion	Concentration (mM)	Inhibition (%)
Mg	1	0
Mn	1	25
Zn	0.5	41
Co	1	33
Cu	1	100
Hg	0.5	100
Ca	1	20

were in excess over the pH range used. The results are shown in Figure 21.

The optimum pH for hexulose phosphate synthase activity was 7.6. The results suggest that the enzyme was slightly inhibited by triethanolamine-sodium hydroxide buffers when compared to the use of sodium potassium phosphate buffer at the same pH.

Divalent metal ions

The assay used for these determinations was the discontinuous method involving the use of the Nash reagent to determine the disappearance of formaldehyde, as described in the Materials and Methods section. This procedure was adopted because the effect of the various ions on the linkage enzymes used in the continuous assay was unknown. 20 mM sodium potassium phosphate buffers at pH 7.5 were used throughout as some other buffer systems had proved to be slightly inhibitory to the enzyme. However, some of the metal ions do form insoluble precipitates with sodium potassium phosphate buffer if their concentrations are too great. This problem was overcome by reducing the concentrations of the metal ions concerned to a level where precipitation did not occur.

For the measurement of the activity of the partially purified enzyme in the presence of divalent metal ions (Table 5), the metal ions at a concentration of 0.5 mM or 1 mM were substituted for Mg^{2+} in the assay mixture. The results are expressed as a percentage of those determined in the presence of 1 mM $MgCl_2$.

Inhibitory effects (Table 6) were determined by the addition of the divalent cations at a concentration of 0.5 mM

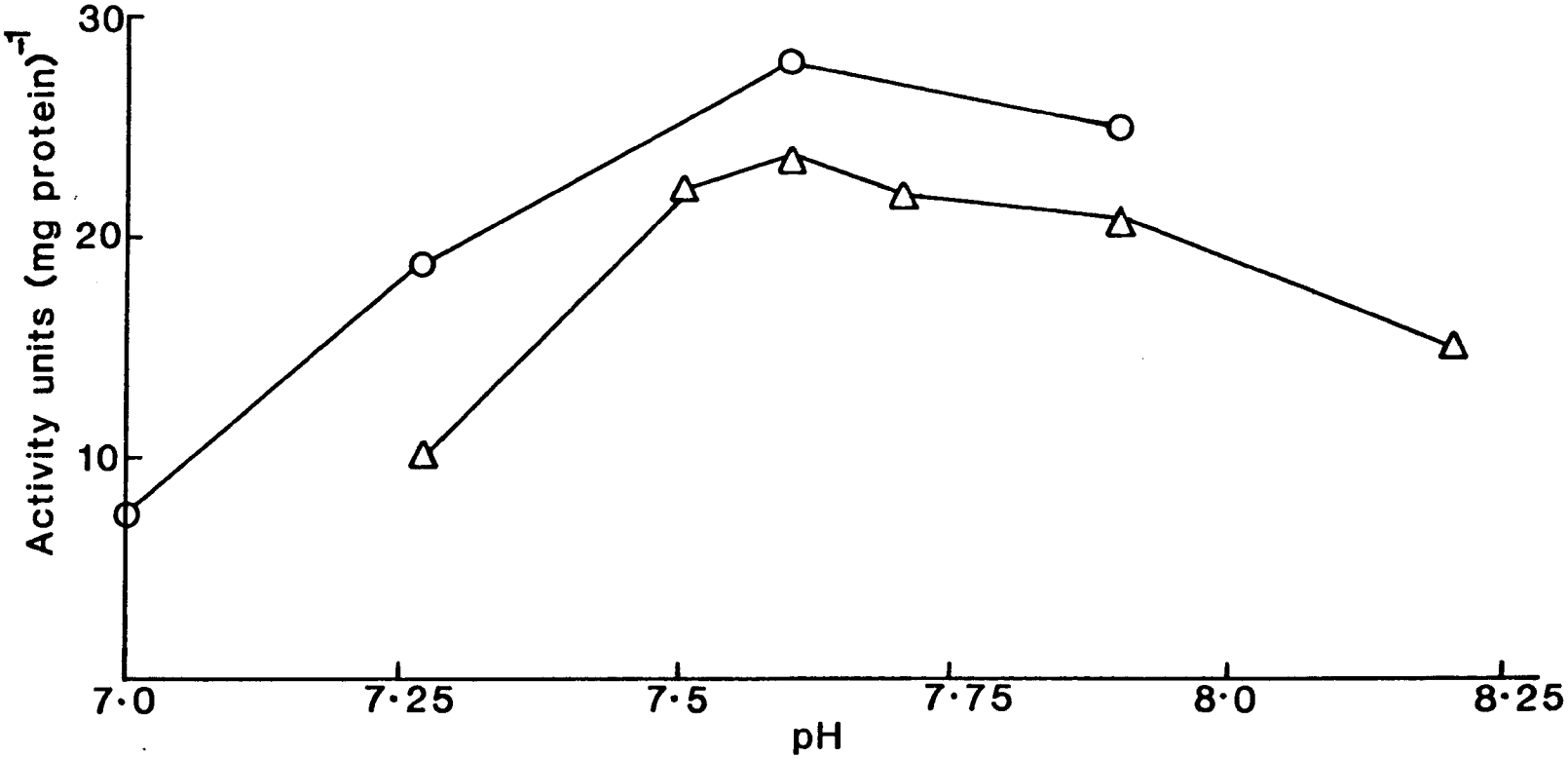
FIGURE 21 pH optimum of hexulose phosphate synthase of methylamine grown Arthrobacter 2B2

The assay used was the continuous spectrophotometric assay described in the Materials and Methods, except the buffers used were 50 mM sodium potassium phosphate and 20 mM triethanolamine-sodium hydroxide.

Key:

○ sodium potassium phosphate

△ triethanolamine-sodium hydroxide


Activity units (mg protein)$^{-1}$
30
20
10
7·0
7·25
7·5
7·75
8·0
8·25
pH

or 1 mM to the standard assay mixture which included 1 mM $MgCl_2$. The results are expressed as a percentage of the rate measured in the presence of 1 mM $MgCl_2$ alone.

The results for the activity of the enzyme exhibited in Table 5 show that $MnCl_2$ acts similarly to $MgCl_2$ giving 100% activity. $ZnSO_4$, $CoSO_4$ and $CaCl_2$ all gave between 40% and 50% of the activity present with $MgCl_2$, while $CuCl_2$ and $HgCl_2$ reduced the activity of the enzyme to zero. If no divalent metal ions were included in the assay, no activity was recorded. The results in Table 6 show the effects of inhibition by divalent metal ions. $CuCl_2$ and $HgCl_2$ totally inhibit the activity of the enzyme. $ZnSO_4$ and $CoCl_2$ give an inhibition between 30% and 40%,while $CaCl_2$ gives a value of only 20%. Interestingly, $MnCl_2$ gives a 25% inhibition of the enzyme activity, although as Table 5 shows, it can replace $MgCl_2$ as a divalent ion giving 100% activity.

Effects of possible modulators on hexulose phosphate synthase activity

The effects of the compounds were tested by including them in the continuous assay procedure described in the Materials and Methods section. The use of the continuous assay meant of course that the linkage enzymes phosphoglucoisomerase, glucose 6-phosphate dehydrogenase and phosphohexuloisomerase were exposed to the actions of the compounds and their activities could be altered giving misleading results. Initially tests were carried out to assess the effect of the compounds on the linkage enzymes. Glucose 6-phosphate dehydrogenase was found to be inhibited by about 25% by 0.2 mM NADH or 0.1 mM NADPH. However, under the conditions present during the assay, the glucose 6-phosphate dehydrogenase was always present in sufficient excess for the inhibitory effects to be disregarded. ATP, ADP and

TABLE 7 The effect of various compounds on HuMP synthase of methylamine grown Arthrobacter 2B2

Compound	Concentration (mM)	Activity (%)
AMP	1	100
ADP	1	100
ATP	1	100
NAD	1	100
NADH	0.1	100
NADP	1	100
NADPH	0.1	100
6-phosphogluconate	1	100
Pyruvate	1	100
EDTA	1	0

TABLE 8 Substrate specificity of HuMP synthase of methylamine grown Arthrobacter 2B2

The enzyme was tested for its specificity towards a formaldehyde acceptor molecule using the discontinuous assay procedure.

Substrate	Concentration (mM)	Activity (%)
Ribose 5-phosphate + 2 Units PRI	5	100
Xylulose 5-phosphate	1	62
"Sigma" ribulose 5-phosphate	5	27
Glucose 6-phosphate	10	0
Fructose 6-phosphate	10	0
Fructose diphosphate	5	0
Ribose 5-phosphate	5	0
Erythrose 4-phosphate	1	0
Dihydroxyacetone phosphate	1	0

AMP at 1 mM concentrations had no effect on any of the linkage enzymes present in the assay system.

Table 7 shows the results obtained. The only compound tested showing any effect was EDTA; this totally inhibited the enzyme. The reason for this inhibition was probably due to chelation of the Mg^{2+} in the assay by the EDTA, as was shown previously, the lack of free metal ions in the assay totally inhibiting the enzyme.

Substrate specificity of hexulose phosphate synthase

The substrate specificity was tested by placing a sample of partially purified enzyme in the discontinuous assay mixture. The discontinuous assay was employed because some of the compounds tested were substrates for the linkage enzymes in the continuous assay. Table 8 shows the results obtained from the investigation expressed as percentages of the activity present when ribulose 5-phosphate (generated *in situ* from ribose 5-phosphate and phosphoriboisomerase) is used as a substrate. The only substances utilised by the partially purified enzyme as substrates were the *in situ* generated ribulose 5-phosphate, commercially available ribulose 5-phosphate (Sigma Chemical Company), and xylulose 5-phosphate. The ribulose 5-phosphate purchased from Sigma showed only 27% of the activity of that generated *in situ*. This effect of the commercially available ribulose 5-phosphate has been previously noted in the case of the hexulose phosphate synthases from *Methylococcus capsulatus* (Kemp, 1972), and *Methylophilus methylotrophus* (Beardsmore et al., 1982). This may be due to the presence of an inhibitory impurity in commercial ribulose 5-phosphate which is only claimed to be 80-90% pure. The

activity observed using xylulose 5-phosphate is interesting. However, it does not prove that the enzyme can use this as a substrate as it has been previously noted that the partially purified enzyme contains ribulose 5-phosphate 3-epimerase activity. This could convert xylulose 5-phosphate to ribulose 5-phosphate, which would act as a substrate for the enzyme giving a false positive result when xylulose 5-phosphate was added.

The molecular weight determination of hexulose phosphate synthase

The native molecular weight of the partially purified enzyme was determined using Sephadex G200 gel filtration chromatography. The packing material was pre-swollen in 20 mM sodium potassium phosphate buffer pH 7.5 containing 2 mM $MgCl_2$ and 15% methanol, and a column (80 x 2.5 cm) packed and maintained at $2^{o}C$. Proteins of known molecular weight were loaded onto the column and eluted with equilibration buffer which was pumped through the column at a flow rate of 10 ml h^{-1}. The standard proteins used were cytochrome c (M. Wt. 12500), bovine serum albumin (M. Wt. 65000-70000), lactate dehydrogenase (M. Wt. 130000-140000), catalase (M. Wt. 230000-250000) and β galactosidase (M. Wt. 510000-530000). Partially purified hexulose phosphate synthase (7 units) was then applied to the column and eluted with equilibration buffer pumped through at a flow rate of 10 ml h^{-1}. The standard proteins were detected either by their absorbance (cytochrome c at 412 nm, bovine serum albumin at 280 nm), or by their enzymic assay as described in the Materials and Methods section. Hexulose phosphate synthase activity was

determined using the continuous assay procedure. After an initial run no hexulose phosphate synthase activity could be detected in the fractions eluted from the column. This could be due to one or a combination of the following:
1. the column material behaving in a similar manner to the Sephadex G150 column which was used during the early attempts at purification and in which a great loss of activity occurred; or, 2. the enzyme losing the ribose 5-phosphate/ribulose 5-phosphate stabilising mixture due to the molecular sieve action of the column. The loss of the stabiliser together with the length of time taken for the enzyme to come off the column (about 20 hours) would be enough to inactivate the enzyme.

In an attempt to prolong the activity of the enzyme for long enough to identify its elution volume via enzyme assays, the tubes used to collect the fractions were preloaded with 0.05 ml of an equilibrium mixture of ribose 5-phosphate/ ribulose 5-phosphate (produced by incubating 10 ml of 0.1 M ribulose 5-phosphate with 10 units of phosphoriboisomerase). A Sephadex G200 column (80 x 2.5 cm) was equilibrated and eluted as described previously using the same standards but incorporating the stabilising mixture in the collecting tubes. The hexulose phosphate synthase activity was detected and peaked at an elution volume of 277 ml.

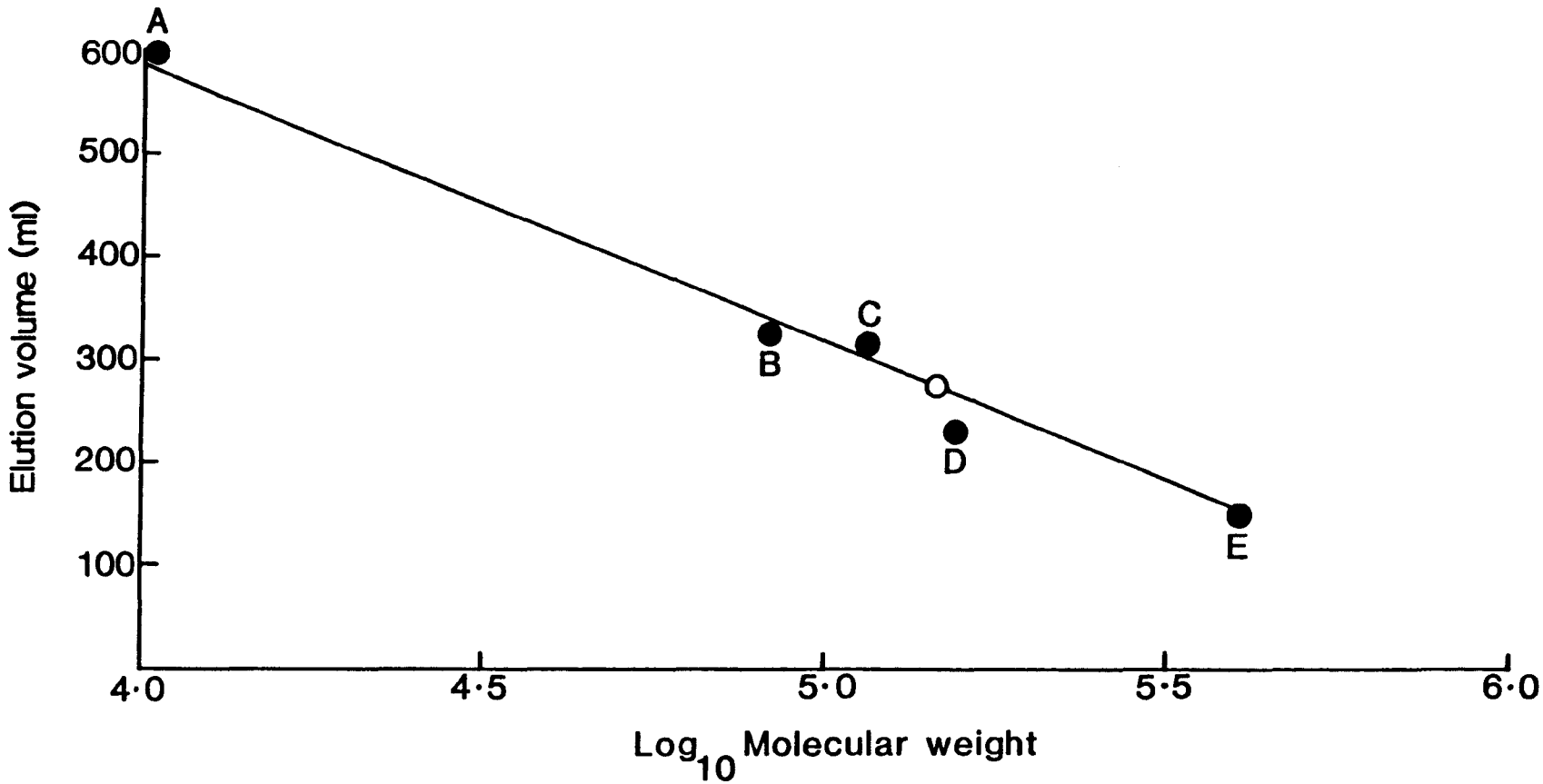
The determination of the molecular weight of hexulose phosphate synthase is shown in Figure 22. The molecular weight of the purified enzyme appears to be about 155000.

SDS-polyacrylamide gel electrophoresis of hexulose phosphate synthase

Using the procedures described in the Materials and

FIGURE 22 Molecular weight determination of the hexulose phosphate synthase of methylamine grown Arthrobacter 2B2 by gel filtration chromatography.

A Sephadex G200 column (80 x 2.5 cm) was equilibrated with 20 mM sodium potassium phosphate buffer pH 7.5 containing 2 mM $MgCl_2$ and 15% (v/v) methanol. The column was calibrated with cytochrome c (A), bovine serum albumin (B), lactate dehydrogenase (C), catalase (D) and β galactosidase (E). Partially purified hexulose phosphate synthase (O) was eluted through the column at a fraction volume corresponding to a molecular weight of 155000. The volume of each fraction was 3.2 ml and the flow rate was 10 ml h^{-1}.


A
600
500
400
300
200
100
Elution volume (ml)
B
C
D
E
4·0
4·5
5·0
5·5
6·0
Log10 Molecular weight

Methods section for SDS-polyacrylamide disc-gel electrophoresis, the subunit size of the hexulose phosphate synthase was determined. The molecular weight standards were run on separate gels as were the samples of denatured hexulose phosphate synthase. The results (Figure 23) show that the molecular weight of the SDS treated hexulose phosphate synthase was determined to be about 82000. There was a single protein band present on the gels containing the enzyme. This result, together with the apparent molecular weight of 155000 as determined by Sephadex G200 chromatography, suggests that the enzyme is a dimer made up of two identical subunits each of molecular weight 82000.

The determination of the Michaelis constants of hexulose phosphate synthase

These constants were determined by the direct linear plot of Eisenthal and Cornish-Bowden (1974).

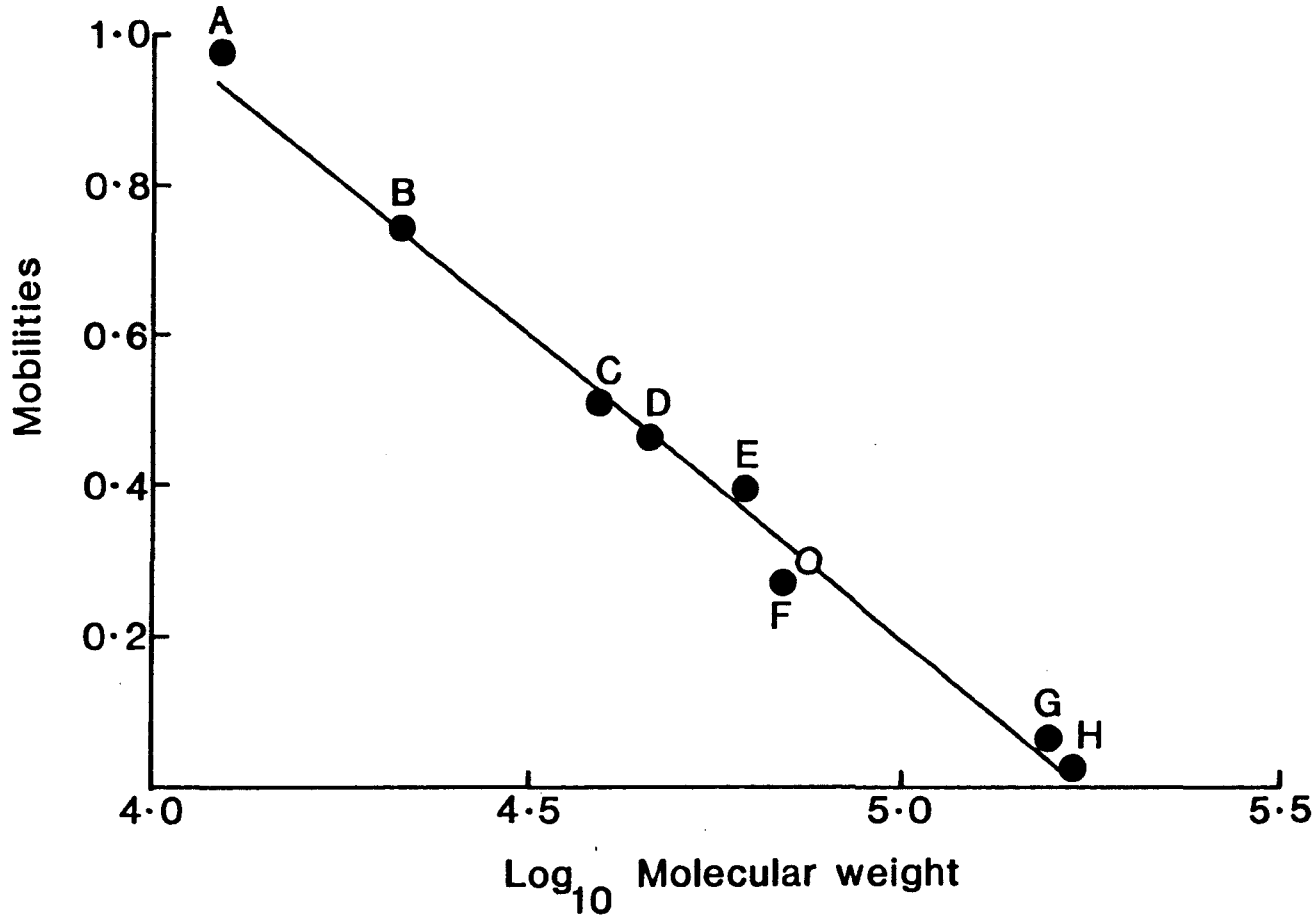
For the determination of the Km for ribulose 5-phosphate it was initially necessary to manufacture the ribulose 5-phosphate by the action of phosphoriboisomerase on ribose 5-phosphate. Differing concentrations of the ribulose 5-phosphate produced were then added to the continuous assay mixture and the activities recorded.

The ribulose 5-phosphate was produced in the following manner: 0.2 ml of 0.1 M ribose 5-phosphate was incubated with 2 units of phosphoriboisomerase at 37°C for 15 minutes. This mixture was then heated to 90°C to inactivate and precipitate the enzyme which was removed by centrifugation. The concentration of the ribulose 5-phosphate solution was determined by adding a known volume of it to the continuous

FIGURE 23 SDS polyacrylamide gel electrophoresis of hexulose phosphate synthase of methylamine grown Arthrobacter 2B2

The method used is described in the Materials and Methods section. The calibration proteins used were:

A	Cytochrome c	(M.Wt. 12500)
B	Trypsin inhibitor	(M.Wt. 21500)
C	RNA polymerase α chain	(M.Wt. 39000)
D	Ovalbumin	(M.Wt. 45000)
E	Catalase	(M.Wt. 60000)
F	Bovine serum albumin	(M.Wt. 68000)
G	RNA polymerase β chain	(M.Wt. 155000)
H	RNA polymerase β' chain	(M.Wt. 165000)
O	Hexulose phosphate synthase	


1·0
0·8
0·6
0·4
0·2
Mobilities
A
B
C
D
E
O
F
G
H
4·0
4·5
5·0
5·5
Log10 Molecular weight

spectrophotometric assay mixture, minus phosphoriboisomerase and ribose 5-phosphate. The concentration of the substrate could then be calculated from the total change in optical density obtained. The Km was determined by varying the ribulose 5-phosphate concentration in the continuous assay mixture from which phosphoriboisomerase and ribose 5-phosphate had been omitted. The formaldehyde concentration was kept constant throughout at 5 mM.

The determination of the Km for formaldehyde was carried out by varying the concentration of formaldehyde in the assay and adding a constant 5 mM concentration of ribose 5-phosphate, then converting it to ribulose 5-phosphate *in situ* using 2 units of phosphoriboisomerase.

The results are shown in Figures 24 and 25. The apparent Km for ribulose 5-phosphate was determined to be 1.2×10^{-4} M, and that for formaldehyde as 5.7×10^{-4} M.

Vmax values were calculated as being 7.9 units (mg protein)$^{-1}$ for ribulose 5-phosphate, and 8.58 units (mg protein)$^{-1}$ for formaldehyde.

Summary of results

The hexulose phosphate synthase from *Arthrobacter* 2B2 has been purified 13 fold. It appears in the soluble fraction of broken cells. However, there is some evidence to suggest that it has a loose association with some particulate matter within the cell. The partially purified enzyme has a molecular weight of 155000, and was found to be dimeric using SDS polyacrylamide gel electrophoresis consisting of two apparently identical subunits of molecular weight 82000. The enzyme has an absolute requirement for divalent metal

FIGURE 24 The determination of the apparent Km of the hexulose phosphate synthase from methylamine grown Arthrobacter 2B2, for ribulose 5-phosphate using a Cornish-Bowden linear plot.

The assay procedure used was the continuous spectrophotometric method outlined in the Materials and Methods section.

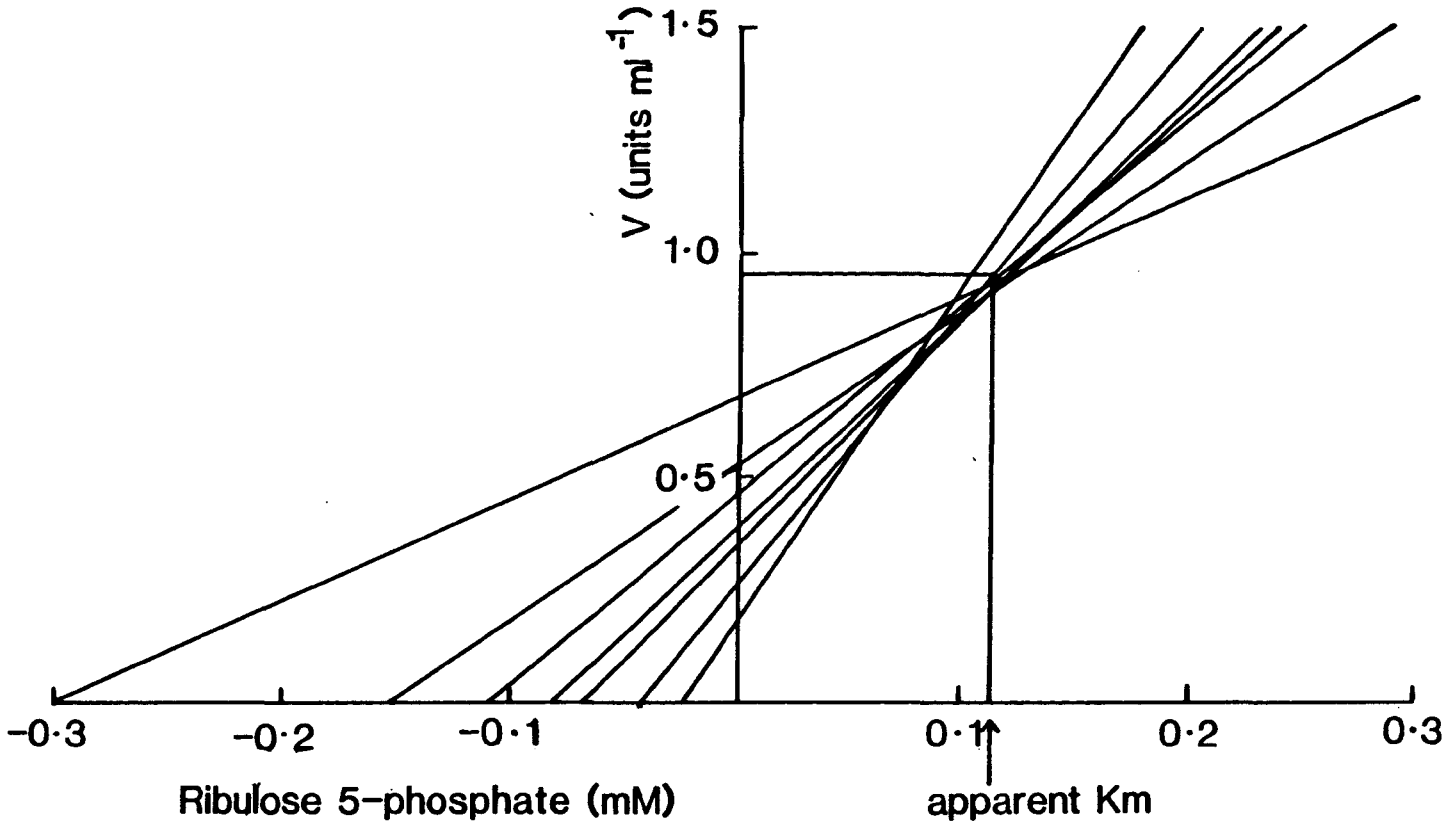
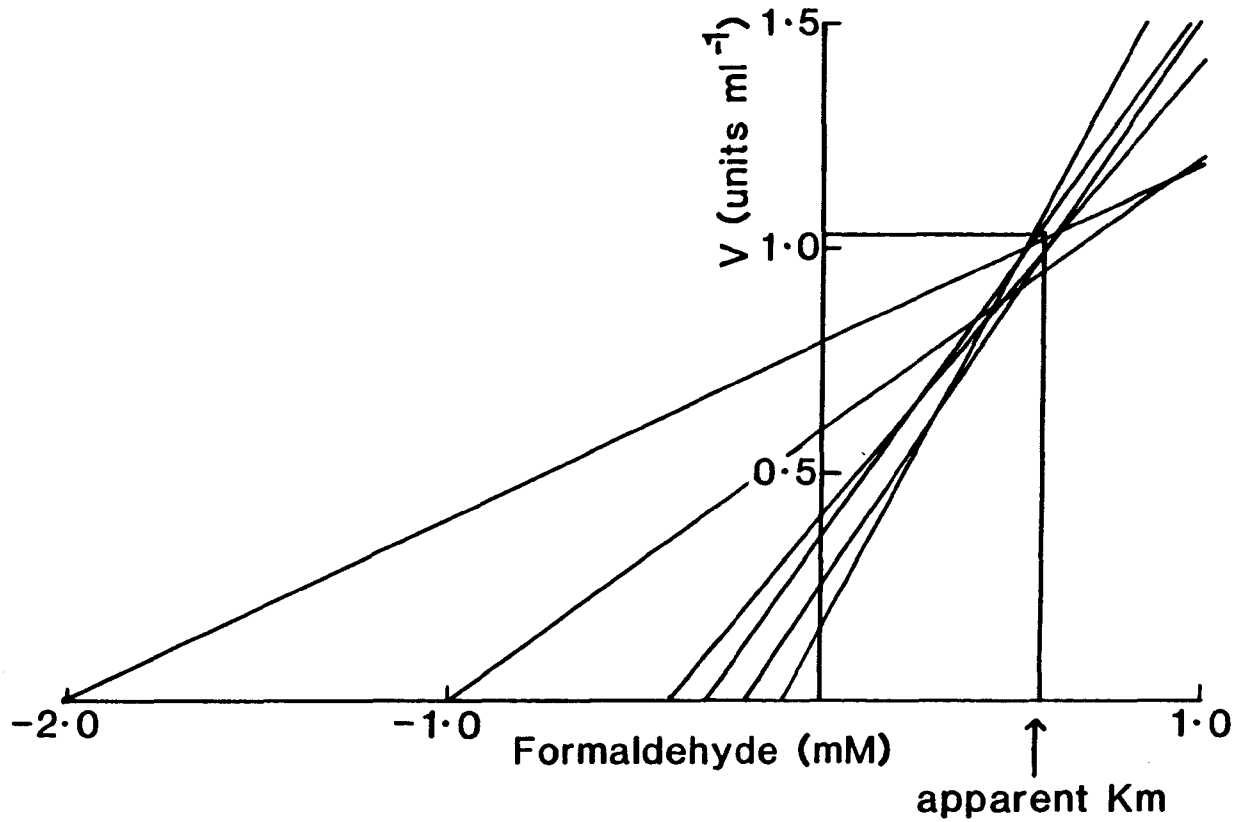

V (units ml-1)
1·5
1·0
0·5
-0·3
-0·2
-0·1
0·1
0·2
0·3
Ribulose 5-phosphate (mM)
apparent Km

FIGURE 25 Determination of the apparent Km of hexulose phosphate synthase of methylamine grown Arthrobacter 2B2 for formaldehyde using a Cornish-Bowden linear plot.

The continuous assay procedure outlined in the Materials and Methods section was used throughout the determination.


V (units ml^{-1})
1·5
1·0
0·5
−2·0
−1·0
Formaldehyde (mM)
1·0
apparent Km

ions for stability and activity. If no specific stabilisers are included in buffers containing the enzyme, it rapidly loses activity. It is stabilised and activated by alcohols with methanol proving to be marginally the best for this purpose. Inclusion of a mixture of ribose 5-phosphate and ribulose 5-phosphate in enzyme fractions already containing an alcohol increases the activity still further and provides an exceptionally good stabilising solution. The stabilising effect of the ribose 5-phosphate/ribulose 5-phosphate mixture is thought to be due to ribulose 5-phosphate, the substrate of the enzyme. A similar stabilisation has been attributed to this compound by Beardsmore et al (1982), after a study of the hexulose phosphate synthase from the obligate methylotroph Methylophilus methylotrophus. The enzyme is highly stable when stored in its stabilisers at 0 C and at -15°C. However, if the temperature is raised to 50°C, activity is lost quite rapidly. The enzyme appears to be specific for ribulose 5-phosphate as its acceptor molecule for formaldehyde. The pH optimum for the enzyme has been determined as being 7.6.

The absence of any form of modulation of enzyme activity by adenine or pyridine nucleotides suggests that regulatory control occurs either via some other compound acting on the enzyme, or elsewhere in the ribulose monophosphate cycle.

Discussion

A comparison of the properties of the hexulose phosphate synthase purified from the facultative methylotroph Arthrobacter 2B2, with those of enzymes previously purified from the obligate methylotrophs: Methylococcus capsulatus (obligate methane or methanol utiliser) (Ferenci et al.,1974); Methylomonas M15 (obligate methanol utiliser) (Sahm et al.,1976); Methylomonas aminofaciens (obligate methanol utiliser) (Kato et al.,1977,1978) and Methylophilus methylotrophus (obligate methanol utiliser) (Beardsmore et al., 1982), reveals many similarities. They are all specific with respect to ribulose 5-phosphate, formaldehyde and hexulose 6-phosphate as substrates, the pH optima for the enzymes occur in the range 7 to 8 and they are dependent on Mg or Mn ions for activity and stability. However the molecular weight of the enzyme from Arthrobacter 2B2 is unlike any of those reported for the other organisms. The enzyme from the obligate methane or methanol utiliser Methylococcus capsulatus has a molecular weight of 310000 consisting of six identical subunits, whilst the enzymes from the obligate methanol utilisers mentioned previously, are all dimers with total molecular weights of 40000 to 47000.

It therefore appears that the structure of the enzyme from the facultative organism Arthrobacter 2B2 does not resemble that from the methane/methanol utiliser Methylococcus capsutatus, or those from the obligate methanol utilisers. The purified enzyme from Arthrobacter 2B2, also has a much lower final specific activity than any of those reported for the other organisms.

CHAPTER 6

The Purification and Characterisation of Phosphohexuloisomerase from Arthrobacter 2B2

Introduction

Interest in the phosphohexuloisomerase from Arthrobacter 2B2 was stimulated when it was reported that a similar organism, Arthrobacter P1 had an apparent lack of this characteristic enzyme of the ribulose monophosphate cycle, although it contained hexulose phosphate synthase another characteristic enzyme of the ribulose monophosphate cycle (Levering et al., 1981a). The enzyme profile of Arthrobacter 2B2 detailed in Chapter 4, clearly shows the presence of phosphohexuloisomerase in this organism.

The phosphohexuloisomerase from Arthrobacter 2B2 was purified and characterised; firstly in order to see if the properties of the enzyme in this organism could explain its apparent lack in the very similar organism Arthrobacter P.1; and secondly to compare the properties of the enzyme, with those of enzymes previously purified from Methylococcus capsulatus (Ferenci et al., 1974), and Methylophilus methylotrophus (Beardsmore et al., 1982).

Crude cell free extracts of methylamine grown Arthrobacter 2B2 were prepared as described in the Materials and Methods section using 20 mM sodium potassium phosphate buffer at pH 7.0. Initial tests for phosphohexuloisomerase activity were carried out using the continuous enzymically coupled method of hexulose phosphate synthase assay, modified by omitting phosphohexuloisomerase from the assay mixture. This procedure was carried out in order to establish whether the results obtained using cell free extracts of Arthrobacter P1 (Levering et al., 1981a), were also noted in Arthrobacter 2B2. In the former organism the hexulose phosphate synthase assay demonstrated activity in crude extracts only if a partially purified preparation of phosphohexuloisomerase was added to it as a linkage enzyme, but not if the phosphohexuloisomerase was omitted.

Crude extracts were prepared from methylamine-grown Arthrobacter 2B2 in buffer containing 5 mM $MgCl_2$ in order to retain maximal hexulose phosphate synthase activity. When such extracts were assayed using the modified hexulose phosphate synthase procedure, no activity was detected. Preparation of a further sample of cell free extract was carried out omitting $MgCl_2$ from the buffer in case the phosphohexuloisomerase was inhibited by Mg^{2+}. The result was similar to that found previously, no activity being detected in the modified assay.

The results obtained confirm that cell free extracts of Arthrobacter 2B2, like those of Arthrobacter P1, exhibit no activity when placed in a modified hexulose phosphate synthase assay from which phosphohexuloisomerase has been omitted as a linkage enzyme.

Cell free extracts of the methylamine-grown organism were subsequently tested in the phosphohexuloisomerase assay detailed in the Materials and Methods section. This assay called for the production of the substrate for the enzyme hexulose 6-phosphate which was manufactured by the procedure described in the Materials and Methods. This assay gave activities of approximately 0.4 units (mg protein)$^{-1}$ using samples of cell free extract prepared both with and without $MgCl_2$ in the sonication buffer.

One difference in conditions between the modified hexulose phosphate synthase assay and the phosphohexuloisomerase assay was that the former was carried out at pH 7.6, while the latter was performed at pH 8.6. It was thought possible that this pH differential could result in the different activities observed using the two assay systems. In order to test this theory two cell free extracts were produced, one containing $MgCl_2$ in the sonication buffer, the other without. The two extracts were then assayed in the phosphohexuloisomerase assay at pH 8.6 and at pH 7.6. The results confirm that the presence or absence of $MgCl_2$ in the sonication buffer made no difference to the observed activity. However, assays carried out at pH 8.6 increased the activity of the enzyme by 150% compared to assays carried out at pH 7.6. It appeared that the pH of the assay considerably affected the activity of the enzyme, nevertheless even the large change in activity observed using different pH values, cannot account for the total lack of activity occurring in the modified hexulose phosphate synthase assay.

As the phosphohexuloisomerase activity was affected by the assay pH, the conditions of pH present during

sonication might also have had an effect on it. To verify this cell free extracts were produced in sodium potassium phosphate buffer at pH 7.0, and in tris-HCl at pH 8.6. Both extracts were assayed using the phosphohexuloisomerase assay at pH 8.6, the results showing little difference in activity.

The initial work on the phosphohexuloisomerase has shown that cell free extracts placed in a hexulose phosphate synthase assay from which external phosphohexuloisomerase has been omitted as a linkage enzyme will exhibit no activity. However, a phosphohexuloisomerase assay using authentic hexulose 6-phosphate as substrate displays quite high activities of the enzyme, confirming its presence. The inclusion of $MgCl_2$ either in the sonication buffer or in the phospho-hexuloisomerase assay itself has no effect on the activity of the enzyme. The pH of the sonication buffer does not affect the activity of the phosphohexuloisomerase assayed in the cell free extract. However, the pH of the assay does, an assay pH of 8.6 resulting in a 150% increase in activity over a pH of 7.6. In all subsequent work cell free extracts were produced by resuspending cell paste of the organism in two volumes of 20 mM sodium potassium phosphate buffer at pH 7.0, and the activity of the enzyme was assayed using the phosphohexuloisomerase assay at pH 8.6.

Initial purification of Phosphohexuloisomerase

In crude extracts of Arthrobacter 2B2 phosphohexulo-isomerase activity was found to be predominantly contained in the supernatant fraction after removal of the cell wall debris by centrifugation.

TABLE 9 Ammonium sulphate fractionation of phosphohexuloisomerase of methylamine grown Arthrobacter 252

Crude cell free extract was subjected to $(NH_4)_2SO_4$ fractionation using the procedure outlined in the Materials and Methods section.

Fraction	Total Activity (m units)
Crude extract	2668
0-30% $(NH_4)_2SO_4$	0
30-40% $(NH_4)_2SO_4$	0
40-50% $(NH_4)_2SO_4$	104
50-60% $(NH_4)_2SO_4$	940
60-70% $(NH_4)_2SO_4$	144
70-80% $(NH_4)_2SO_4$	0

Initially the crude cell free extract was treated with protamine sulphate in order to precipitate basic proteins, 1 mg of protamine sulphate being added for every 7 mg of protein in the crude extract. This was allowed to equilibrate at 20°C for 20 minutes before removing the precipitated material by centrifugation at 38000 g for 15 minutes. When the results from the protamine sulphate step were examined it was noted that there had been a 33% loss in total activity and a 30% loss in specific activity. This was considered a totally unacceptable loss of activity.

The crude cell free extract was next subjected to ammonium sulphate fractionation, the extract initially being raised to 30% saturation and after removal of the precipitated protein sequential 10% fractions were made up to a level of 70% $(NH_4)_2SO_4$. The results (Table 9) show that the enzyme precipitates out between 40% and 70% $(NH_4)_2SO_4$ saturation, but that 35% of the total activity of the enzyme was lost in this step. The loss of activity was deemed unacceptable and this fractionation was not included in further purification attempts.

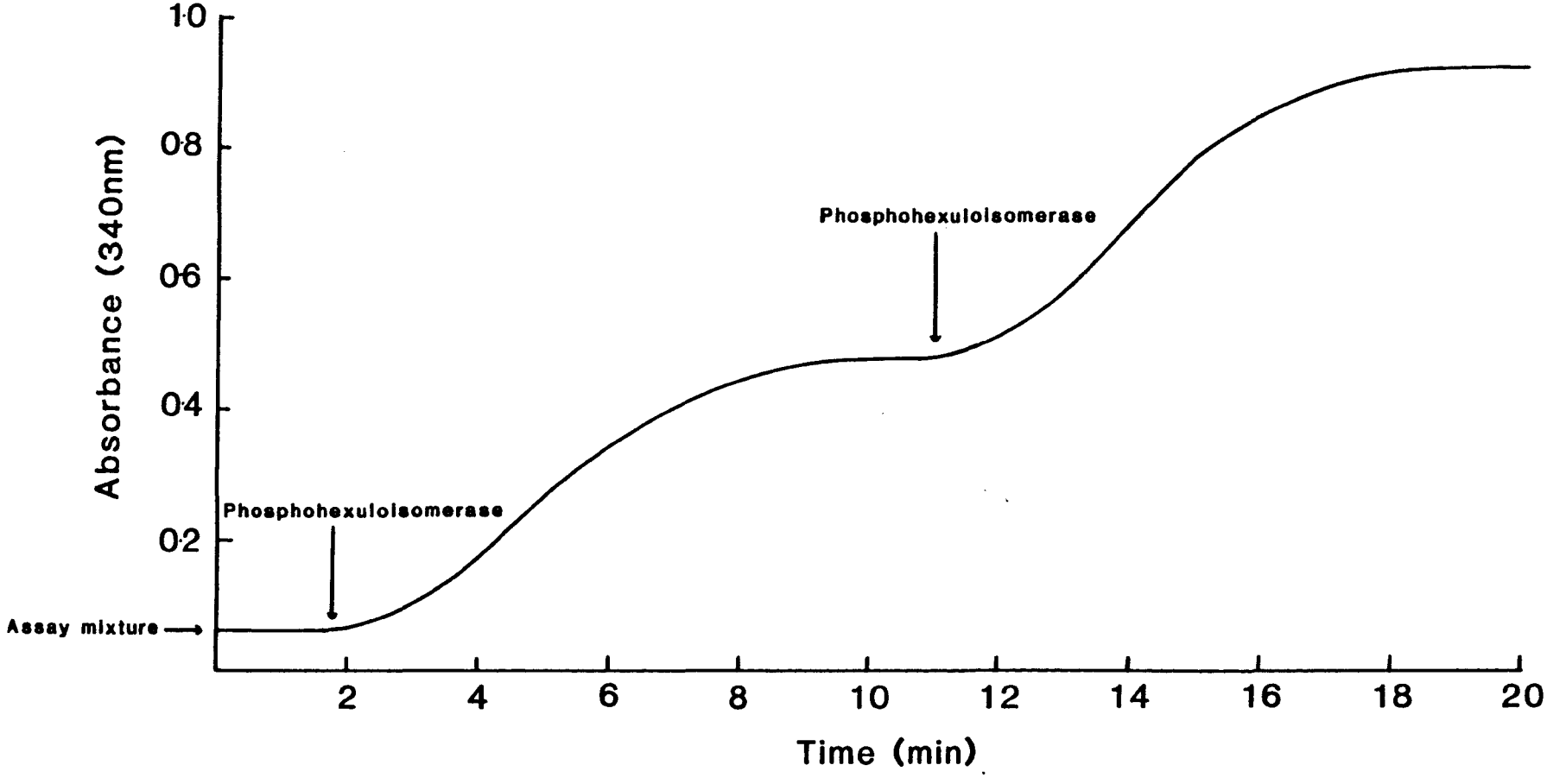
Some preliminary work had indicated that a good purification step would be the use of a DEAE cellulose ion exchange column. A DEAE cellulose column (13 x 1.6 cm) was equilibrated with 20 mM sodium potassium phosphate buffer pH 7.0 containing 5 mM $MgCl_2$, and a sample of crude cell free extract was loaded onto it. Non adhering protein was washed through the column with the same equilibration buffer. Enzyme assay demonstrated the presence of phosphohexuloisomerase activity in the initial wash. Results from the column show that a 100% yield was obtained, together with a 10-fold

purification. This proved to be a good purification step as samples of the pooled DEAE cellulose column eluate placed on non denaturing polyacrylamide gels gave only two protein bands on staining with Coomassie Blue. However, analysis by enzyme assay revealed the presence of low activities of contaminating hexulose phosphate synthase.

It was at this stage in the purification process that the stability problem of the enzyme was noted. In crude cell free extracts the enzyme was relatively stable at $0^{o}C$. However, the partially purified DEAE cellulose eluate proved to be considerably less stable. Work carried out with the enzyme assay system disclosed that different enzymic rates were recorded depending on at what stage of the assay the extract was added. The highest rates occurred when the extract was the final addition to the assay mixture. This resulted in an initial linear rate. However, after a few minutes the rate gradually began to decay, eventually completely levelling off. This effect could have been due to either one of the substrates becoming exhausted, or the enzyme decaying during the assay. The addition of further samples of the substrates did not initiate any further activity. However, addition of a further sample of enzyme resulted in an immediate increase in rate to its previous value, before again decaying to zero (Figure 26). This result adds substance to the theory that the enzyme was decaying during the assay; to confirm it a method developed by Selwyn (1965) was employed. This procedure calls for assays to be carried out using differing quantities of enzyme. The results obtained are plotted as the concentration of product, versus the volume of enzyme multiplied by time.

FIGURE 26 The Decay of Phosphohexuloisomerase of methylamine grown Arthrobacter 282 during assay

The assay was initiated by the addition of phosphohexuloisomerase to the complete assay mixture described in the Materials and Methods section. After several minutes the rate decays to zero. When a further addition of phosphohexuloisomerase is then made to the assay, the rate increases to its original value, before again decaying to zero.


1·0
0·8
0·6
0·4
0·2
Absorbance (340nm)
Phosphohexuloisomerase
Phosphohexuloisomerase
Assay mixture
2
4
6
8
10
12
14
16
18
20
Time (min)

If the enzyme does not decay during the assay all the points plotted would lie on one line; if however the enzyme does undergo decay, then the observations for different concentrations of enzyme fall on different curves. The procedure was carried out for the partially purified phosphohexuloisomerase using 0.1 ml, 0.15 ml and 0.2 ml of enzyme in the assay. Figure 27 shows the results obtained, the points lying on three distinct curves, thus confirming that the enzyme was undergoing decay during the assay procedure.

The decay curve seen in the assay using partially purified enzyme was reduced when crude cell free extract was used in the assay. This effect was anticipated to be due to the concentration of protein in the assay. The higher level of protein present in the cell free extract would stabilise the enzyme more than the lower level present in the partially purified sample. The effect was tested by incubating a sample of partially purified enzyme with 10 mg ml^{-1} of bovine serum albumin for several hours and assaying for activity, the results being compared to a sample not containing bovine serum albumin. The activity in the sample containing bovine serum albumin was 20% higher than in the sample without, confirming that the enzyme was more stable when in solutions containing high levels of protein. This is probably a contributory factor to inactivation of the enzyme during the assay as the assay mixture contained only a very low protein concentration. It also provides a possible explanation for the absence of activity in the modified hexulose phosphate synthase assay. Any cell free extract placed in the assay would be in a low protein environment and the phosphohexuloisomerase contained in it

FIGURE 27 A Selwyn plot for phosphohexuloisomerase of methylamine grown Arthrobacter 2B2

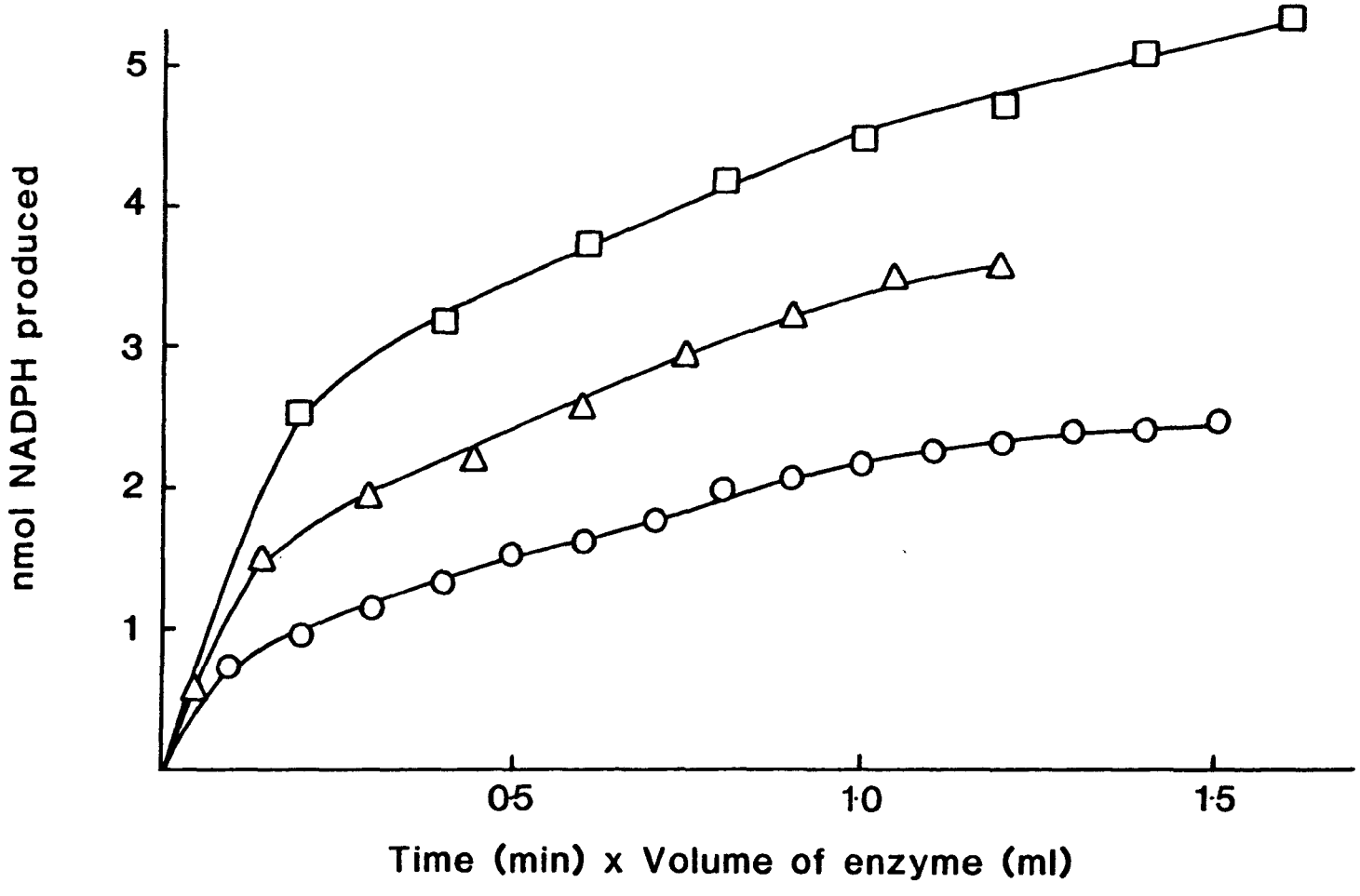
Different volumes of the partially purified enzyme were placed in the standard phosphohexuloisomerase assay described in the Materials and Methods section. Results from the assays were plotted as the concentration of product versus the volume of enzyme used in the assay multiplied by the time.

Key:

○ 0.1 ml phosphohexuloisomerase

Δ 0.15 ml phosphohexuloisomerase

□ 0.2 ml phosphohexuloisomerase


nmol NADPH produced
5
4
3
2
1
0·5
1·0
1·5
Time (min) x Volume of enzyme (ml)

would decay. As the cell free extract was placed in the curette early in the assay the phosphohexuloisomerase would be inactive by the time the substrate was added, giving no activity. However, when an external phosphohexuloisomerase was added as a linkage enzyme, the assay would be able to operate giving an enzymic rate.

Although the concentration of protein plays a role in stabilising the enzyme, another important factor could be stabilisation by the substrate hexulose 6-phosphate. However, such a theory is difficult to prove with this enzyme.

The results led to an alteration in the sequence of addition to the phosphohexuloisomerase assay, the extract containing the enzyme activity being the last addition to the assay thus being used to initiate the reaction. In this way the activity of the enzyme could be measured before the effects of inactivation came into play. Due to the effect of high protein concentration on the stability of the enzyme, further attempts at purification were carried out in an environment containing a high protein concentration whenever possible. This meant that pooled column eluants was rapidly concentrated by ultrafiltration after being washed off the column, and that to any extract intended for long term storage was added bovine serum albumin to a final concentration of 10 mg ml^{-1}.

In an attempt to remove hexulose phosphate synthase activity during the course of the next purification attempt the sonication buffer was altered to 20 mM sodium potassium phosphate buffer pH 7.0 containing no $MgCl_2$ as it was known that Mg^{2+} ions were required for the stability of the contaminating enzyme. Crude cell free extracts produced

using the Mg^{2+} free buffer were loaded directly onto a DEAE cellulose column equilibrated with, and eluted using the sonication buffer. Unlike previous DEAE cellulose columns in which the enzyme was washed off with the unbound protein, here the enzyme adhered to the column and had to be removed using a 0 mM to 500 mM sodium chloride gradient. Fractions containing activity were pooled and assayed. The pooled eluant was found to contain far more protein than had been previously encountered when the enzyme did not bind to the column, the purification being only 2 fold. However, no hexulose phosphate synthase could be detected in the eluate. The reason for the adherence of the enzyme to the column was anticipated to be due to the reduction in chloride ions owing to the absence of $MgCl_2$ in the buffers. To rectify the situation and retain the advantage of the removal of hexulose phosphate synthase from the eluant, chloride ions were replaced in the sonication, equilibration and elution buffers, their source being sodium chloride. When a DEAE cellulose column was loaded with cell free extract and eluted with 20 mM sodium potassium phosphate buffer pH 7.0 containing 10 mM sodium chloride, the enzyme was washed off the column with the unbound protein, and a 10 fold purification similar to that obtained in columns eluted with $MgCl_2$ containing buffers resulted. The pooled active fractions from the column contained no hexulose phosphate synthase activity.

The pooled DEAE cellulose eluant was concentrated by ultrafiltration and then reapplied to a second DEAE cellulose column in order to see if the reduction in protein which occurred on passage through the first column had affected the characteristics of the enzyme on the column. It was initially

washed with 20 mM sodium potassium phosphate buffer pH 7.0 containing 10 mM sodium chloride. The unbound protein eluted from the column contained no phosphohexuloisomerase activity, therefore a linear 10 mM to 250 mM sodium chloride gradient was applied to the column to remove activity. The second column gave a further 3 fold purification of the enzyme. The volume of the pooled fractions was reduced by ultrafiltration and stored containing 10 mg ml^{-1} bovine serum albumin at -15°C.

Final purification procedure

(i) Preparation of crude cell free extract

The crude cell free extract was prepared by the resuspension of 4 g of methylamine grown cell paste in 8 ml of 20 mM sodium potassium phosphate buffer pH 7.0 containing 10 mM sodium chloride. The suspension was sonicated in one batch and the cell debris was removed by centrifugation at 38000 g for 20 minutes at 2°C. The pellet was discarded and the supernatant treated as the crude cell free extract.

(ii) First DEAE cellulose chromatography

The crude cell free extract was applied directly onto a DEAE cellulose column (13 x 1.5 cm) equilibrated with sonication buffer, phosphohexuloisomerase activity was eluted from the column with the unbound protein fraction, the activity being detected by the spectrophotometric assay described in the Materials and Methods section. Fractions 10 to 38 were pooled and ultrafiltered to a small volume.

(iii) Second DEAE cellulose chromatography

The ultrafiltered material from step (ii) was applied directly to a second DEAE cellulose column (14 x 1.5 cm)

TABLE 10 The purification of phosphohexuloisomerase from methylamine grown Arthrobacter 2B2

Fraction	Volume ml	Protein mg	Activity Units	Specific Activity Units mg^{-1}	Purification fold	Yield %
Crude cell free extract	9	88	21.6	0.224	1	100
First DEAE cellulose chromatography	127	9	22.352	2.514	10	103
Second DEAE cellulose chromatography	75	0.6	4.8	8.0	35	22

equilibrated with sonication buffer. Unbound protein was washed off the column with equilibration buffer, and as no phosphohexuloisomerase activity could be detected in this peak, a linear gradient of 10 mM to 250 mM sodium chloride made up in 200 ml of equilibration buffer was applied to the column to wash off the activity. Active fractions were determined as before and fractions 40 to 55 were pooled and ultrafiltered to a small volume. The ultrafiltered material had 10 mg ml^{-1} of bovine serum albumin added to it and was stored at $-15^{o}C$ until required.

Table 10 shows the purification of phosphohexuloisomerase. The enzyme was purified 35 fold with a recovery of 22%. Figures 28 and 29 show the two successive DEAE cellulose chromatography stages, the initial column requiring no sodium chloride gradient to wash off the enzyme activity, the second column requiring a 10 mM to 250 mM sodium chloride gradient. The activity was washed off the column soon after the sodium chloride gradient was established.

The purified enzyme was examined for purity by enzymological study and polyacrylamide gel electrophoresis. It was found to be free from detectable activities of glucose 6-phosphate dehydrogenase, phosphoglucoisomerase and NAD and NADP linked 6-phosphogluconate dehydrogenases. Phospho-riboisomerase was present at a level of 5% relative to phosphohexuloisomerase activity. Adopting the procedure outlined in the Materials and Methods section, polyacrylamide gel electrophoresis of the purified phosphohexuloisomerase gave a single protein band when stained with Coomassie Blue.

The Properties of Phosphohexuloisomerase

FIGURE 28 First DEAE-cellulose chromatography of the Phosphohexuloisomerase of methylamine grown Arthrobacter 2B2

Crude cell free extract was applied to a DEAE-cellulose anion exchange column (13 x 1.5 cm) and eluted using a 20 mM sodium potassium phosphate buffer pH 7.0 containing 10 mM NaCl which had been used previously to equilibrate the column.

Key:

△ Phosphohexuloisomerase activity (units ml^{-1})

○ Extinction at 280 nm.

Volume of each fraction = 4.2 ml.

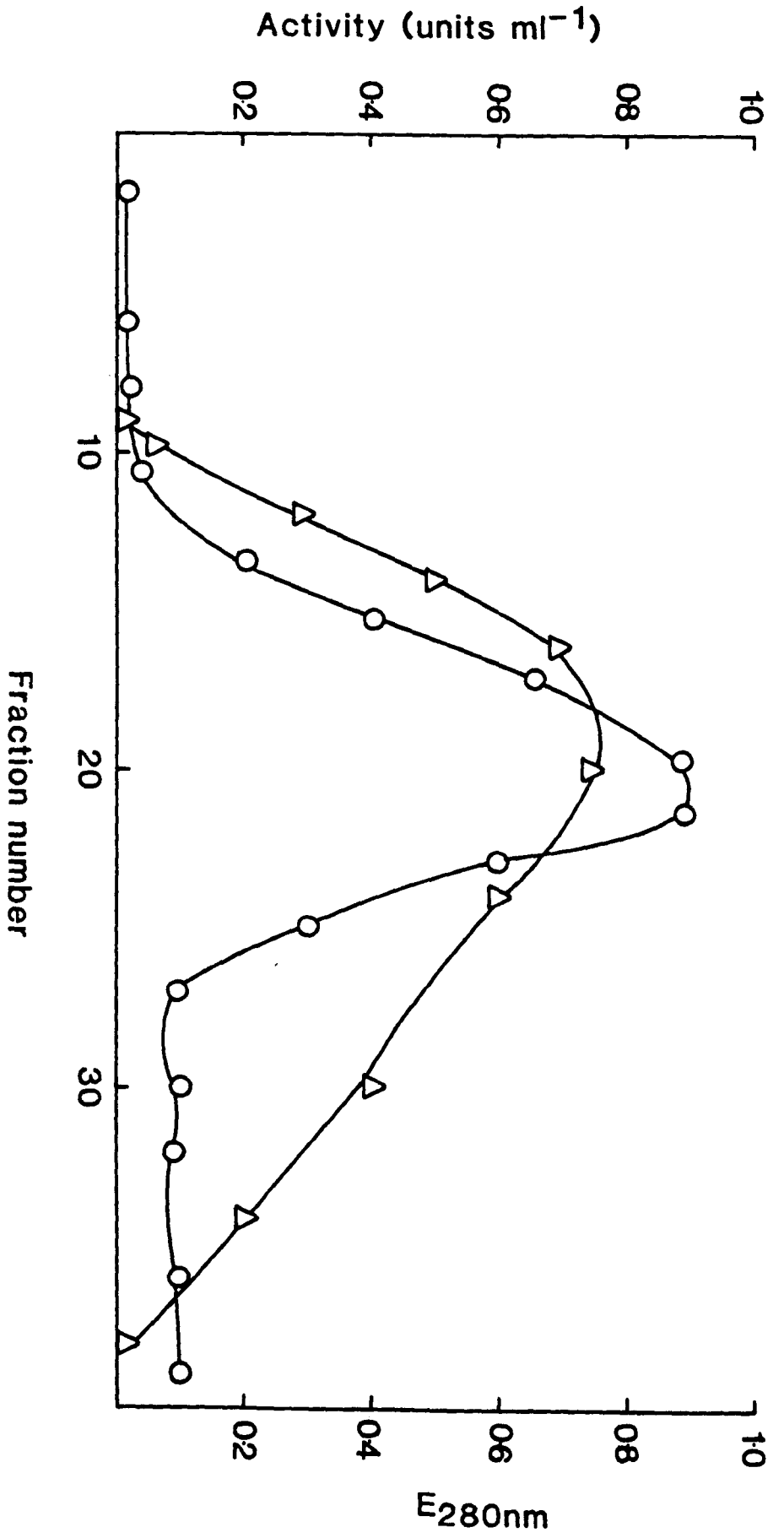

Activity (units ml^{-1})
0·2
0·4
0·6
0·8
1·0
10
20
30
Fraction number
E_{280nm}

FIGURE 29 Second DEAE-cellulose chromatography of the Phosphohexuloisomerase of methylamine grown Arthrobacter 2B2

Ultrafiltered protein after DEAE-cellulose chromatography was applied to a second DEAE-cellulose anion exchange column (14 x 1.5 cm) which had been equilibrated with 20 mM sodium potassium phosphate buffer pH 7.0 containing 10 mM NaCl.

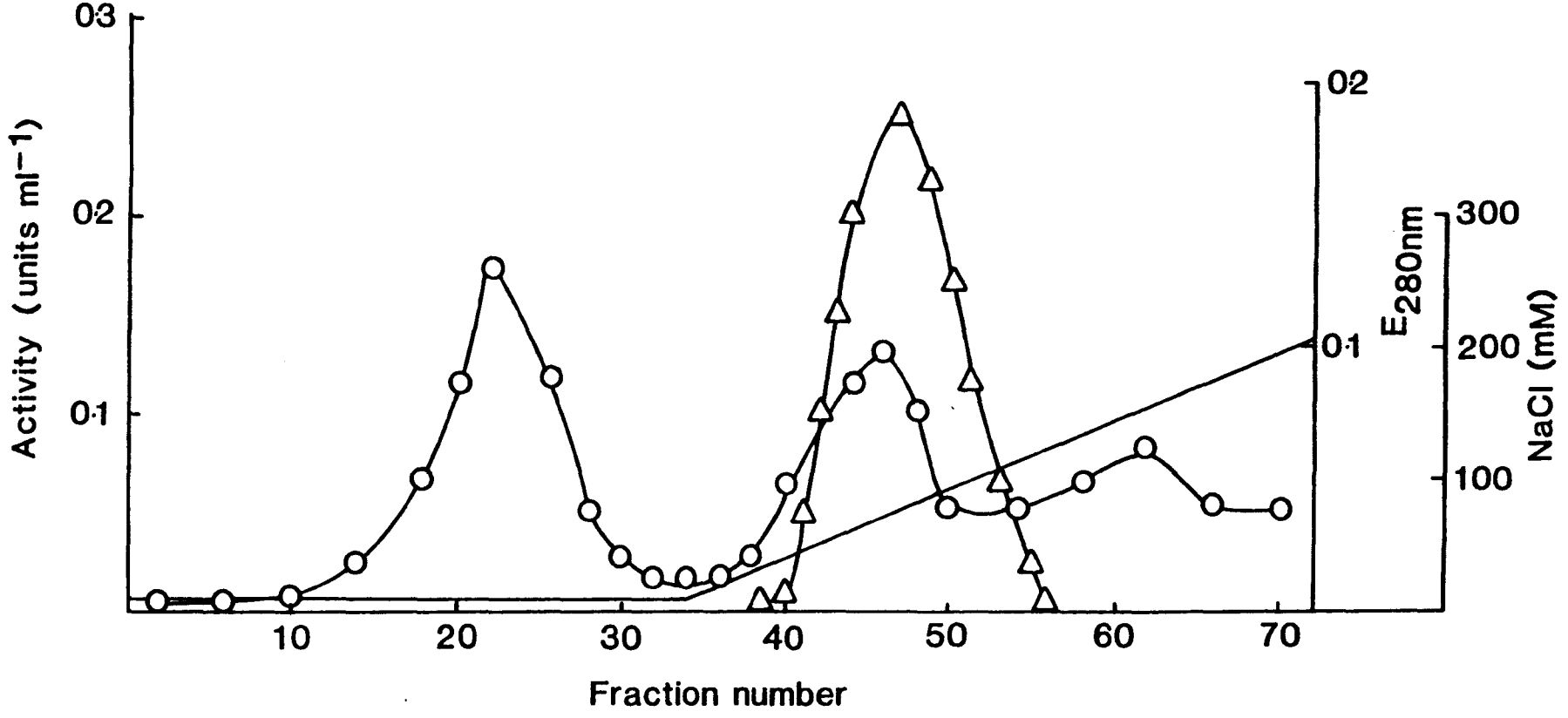
Key:

△ Phosphohexuloisomerase activity (units ml^{-1})

○ Extinction at 280 nm

——— NaCl gradient (mm)

Fraction volume = 4.9 ml.


0·3
0·2
0·1
Activity (units ml⁻¹)
0·2
0·1
E280nm
300
200
100
NaCl (mM)
10
20
30
40
50
60
70
Fraction number

The stability of phosphohexuloisomerase

Mention of the stability of the enzyme has been made throughout the purification procedure. The most striking point is the extreme instability of the enzyme when it is in an environment containing low protein, this being best visualised during the continuous assay of the enzyme when the activity decreases while the enzyme assay is proceeding. As noted previously the instability was overcome by keeping the protein concentration as high as possible during the purification process, by ultrafiltering column eluates to a small volume, and by storage of the purified enzyme in buffers containing 10 mg ml^{-1} of bovine serum albumin.

The purified enzyme in an environment of high protein concentration at $0^{o}C$ loses all activity within three days. At $-15^{o}C$ the enzyme is stable for up to two months. However, repeated cycles of freezing and thawing do reduce enzyme activity considerably. The enzyme is not stable at raised temperatures, losing all activity within 10 minutes at $50^{o}C$.

The effect of the pH on stability during storage was tested by placing a sample of purified enzyme in a buffer of an appropriate pH and storing for one hour at $0^{o}C$, then assaying for activity in the spectrophotometric assay described in the Materials and Methods section, at pH 8.6. Figure 30 shows the results obtained. The stability peak was broad, the enzyme being stable over a pH range from 7.0 to 8.5. However, the stability does drop considerably outside those boundaries. The partially purified enzyme was in fact always stored within the stability boundary in sodium potassium phosphate buffer at pH 7.0.

FIGURE 30 pH stability of purified phosphohexuloisomerase of methylamine grown Arthrobacter 2b2

The purified enzyme was stored at 0°C for one hour in an appropriate buffer before being assayed at pH 8.6 in the continuous assay described in the Materials and Methods section.

Key:

○ Sodium potassium phosphate buffer.

△ Tris-HCl buffer.

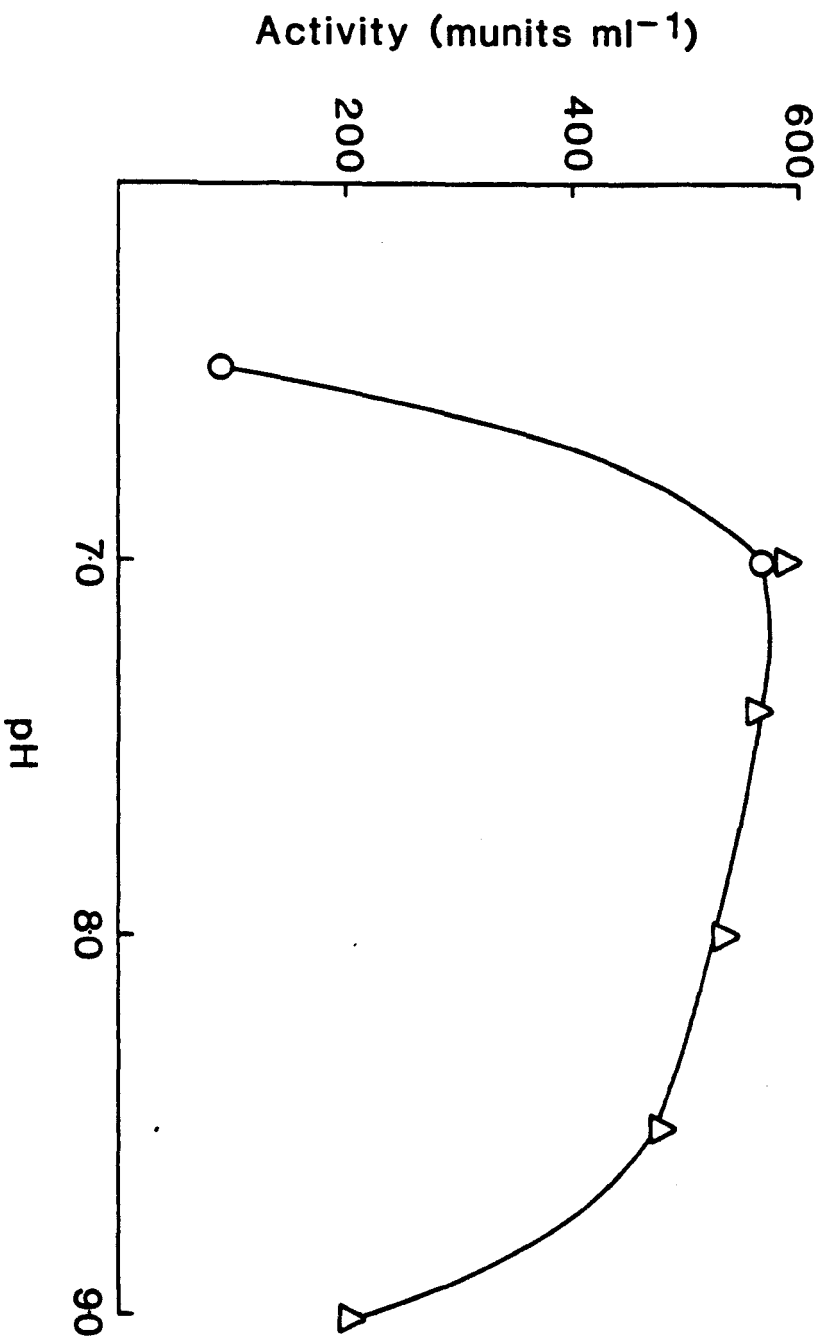

Activity (munits ml^{-1})
200
400
600
pH
7.0
8.0
9.0

TABLE 11 The effect of divalent metal ions on phosphohexuloisomerase activity of methylamine grown Arthrobacter 2B2

The results are expressed as a percentage of the activity obtained in the continuous assay procedure described in the Materials and Methods section.

Metal ion	Concentration (mM)	Activity (%)
Mg	1	100
Ca	0.5	100
Mn	1	100
Co	1	100
Cu	1	19
Zn	0.5	72
Hg	0.5	0
None + EDTA	-	100

pH optimum

The buffer used for the investigation were 20 mM sodium potassium phosphate over the pH range 7.2 to 7.9, and 20 mM tris-HCl over the pH range 8.0 to 8.9. Assays were carried out in the continuous spectrophotometric assay system, the pH of the buffer being checked before each assay and the pH of the complete assay system being checked afterwards to verify that the pH had remained the same throughout. The coupling enzymes were always present in excess quantities such that the assay was always phosphohexuloisomerase limiting.

The results shown in Figure 31 demonstrate that the enzyme shows slightly higher activity in tris-HCl buffer than in sodium potassium phosphate buffer of a similar pH. The pH optimum of the enzyme was determined to be 8.5.

Divalent metal ions

The activity of the enzyme was tested for the effect of divalent metal ions using the assay procedure described in the Materials and Methods section. The assay system was set up without the presence of EDTA and with the addition of a suitable metal ion. At higher concentrations certain of the metals used formed a precipitate during the assay. Their concentration was therefore reduced to a level at which precipitation did not occur.

Table 11 shows the results obtained in the investigation. $MgCl_2$, $CaCl_2$, $MnCl_2$ and $CoCl_2$ all gave 100% activity when compared to that obtained in an assay with no metal ions but containing EDTA. When $ZnSO_4$ was added to the assay, the activity dropped to 72% and it was reduced still further to 19% when $CuCl_2$ was included in the assay. $HgCl_2$ totally

FIGURE 31 The pH optimum of Phosphohexuloisomerase of methylamine grown Arthrobacter 2B2

The enzyme was assayed over the pH range 7.2 to 8.5 using the continuous spectrophotometric assay described in the Materials and Methods except that the buffers used were 20 mM sodium potassium phosphate and 20 mM Tris-HCl.

Key:

○ sodium potassium phosphate.

△ tris-HCl.

Activity (munits ml^{-1})
20
40
60
80
100
pH
7·5
8·0
8·5
9·0

inhibited the enzyme, no activity being recorded when it was included in the assay.

Substrate specificity

Extensive tests by Ferenci et al., (1974), using the phosphohexuloisomerase from Methylococcus capsulatus showed it to be specific for hexulose 6-phosphate as a substrate. With this in mind the enzyme was tested only for any phosphoglucoisomerase activity. The assay was carried out using the continuous procedure described for phosphohexuloisomerase in the Materials and Methods section, but replacing hexulose 6-phosphate with fructose 6-phosphate and omitting phosphoglucoisomerase from the assay. No conversion to glucose 6-phosphate and hence reduction of NADP was found using this assay procedure, therefore the purified phosphohexuloisomerase shows no detectable phosphoglucoisomerase activity.

The molecular weight determination of phosphohexuloisomerase

The molecular weight of the native enzyme was determined by Sephadex G200 gel filtration chromatography using the method of Andrews (1964). Purified phosphohexuloisomerase (5 units) was applied to a Sephadex G200 column (75 x 2.5 cm) equilibrated with 20 mM sodium potassium phosphate buffer pH 7.0, and eluted with the equilibration buffer at a flow rate of 10 ml h^{-1}. The enzyme activity was detected by assaying the fractions collected in the continuous assay described in the Materials and Methods section. The column was calibrated with standard proteins of known molecular weight. The standards used were cytochrome c (M. Wt. 12400),

bovine serum albumin (M. Wt. 65000-70000), lactate dehydrogenase (M. Wt. 130000-140000), catalase (M. Wt. 230000-250000), β galactosidase (M. Wt. 510000-530000). The calibration proteins were detected either by their absorbance cytochrome c at 412 nm, bovine serum albumin at 280 nm), or by their enzymic assay as described in the Materials and Methods section. The results are shown in Figure 32. Phosphohexuloisomerase activity peaked at an elution volume of 277 ml, corresponding to a molecular weight of 108000. Phosphohexuloisomerase activity in crude cell free extract applied to the calibrated Sephadex G200 column was detected at a similar elution volume to the purified enzyme, suggesting that the enzyme had the same molecular weight before and after purification.

SDS polyacrylamide gel electrophoresis of purified phosphohexuloisomerase

The subunit structure of the enzyme was examined using the denaturing polyacrylamide gel electrophoresis technique previously described in the Materials and Methods section. The calibration proteins used are shown in Figure 33. They were run on separate gels as were the samples of purified phosphohexuloisomerase. The sample gels contained a single protein band when stained with Coomassie Blue, and when the mobility of this band was compared with those of the standards (Figure 33), a subunit molecular weight of 35500 was determined.

The total molecular weight was estimated to be 108000 by gel filtration chromatography. The results from SDS polyacrylamide gel electrophoresis suggest that the enzyme is a trimer, constructed of three identical subunits.

FIGURE 32 Determination of the molecular weight of the phosphohexuloisomerase of methylamine grown Arthrobacter 2B2 by use of a Sephadex G200 column

A Sephadex G200 column (75 x 2.5 cm) was equilibrated with 20 mM sodium potassium phosphate buffer pH 7.0. The column was calibrated with cytochrome c (A), bovine serum albumin (B), lactate dehydrogenase (C), catalase (D) and β galactosidase (E). Purified phosphohexuloisomerase (O) was eluted from the column at a fraction volume corresponding to a molecular weight of 108000. The volume of each fraction was 4.5 ml and the flow rate was 10 ml h^{-1}.

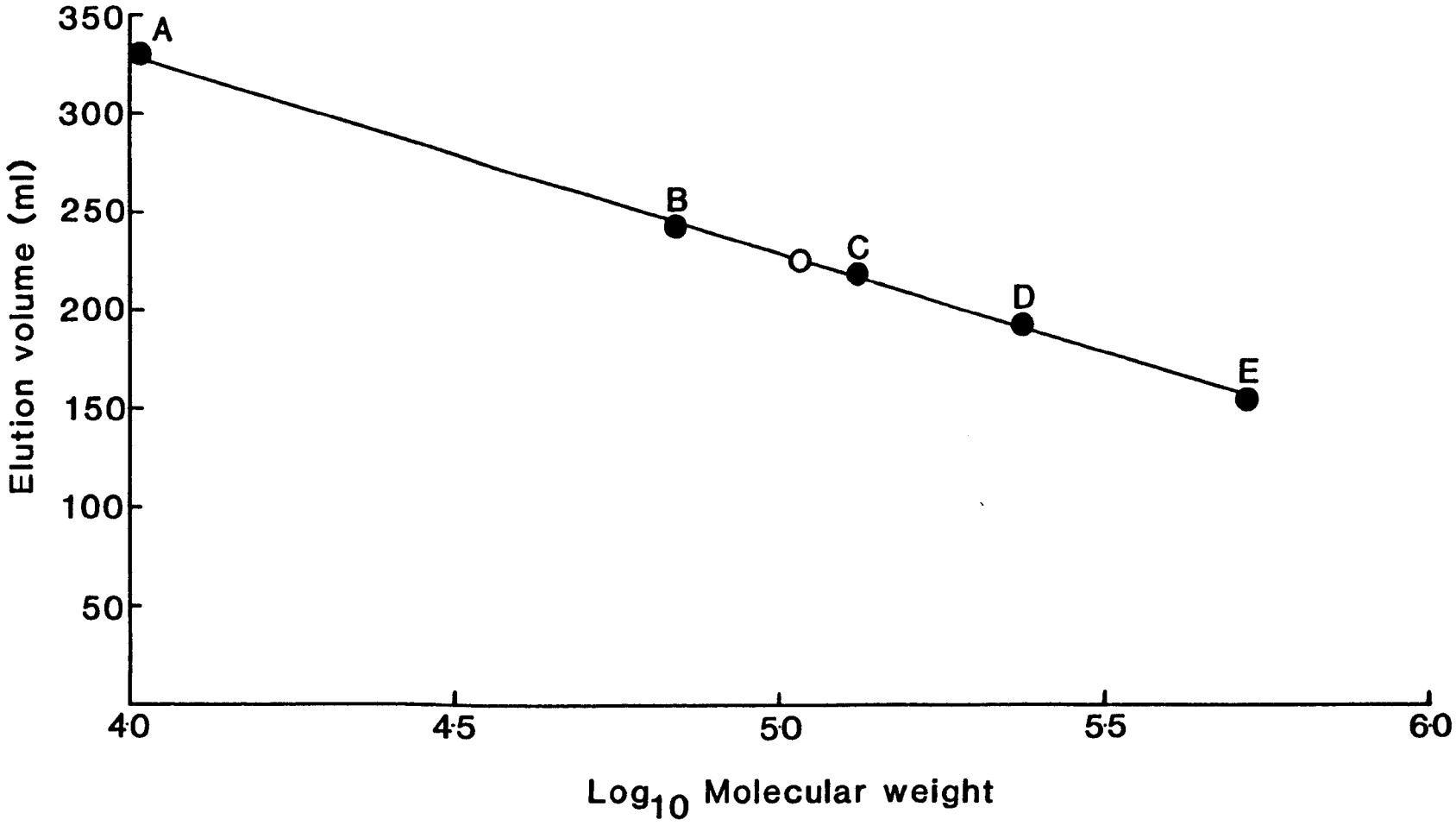
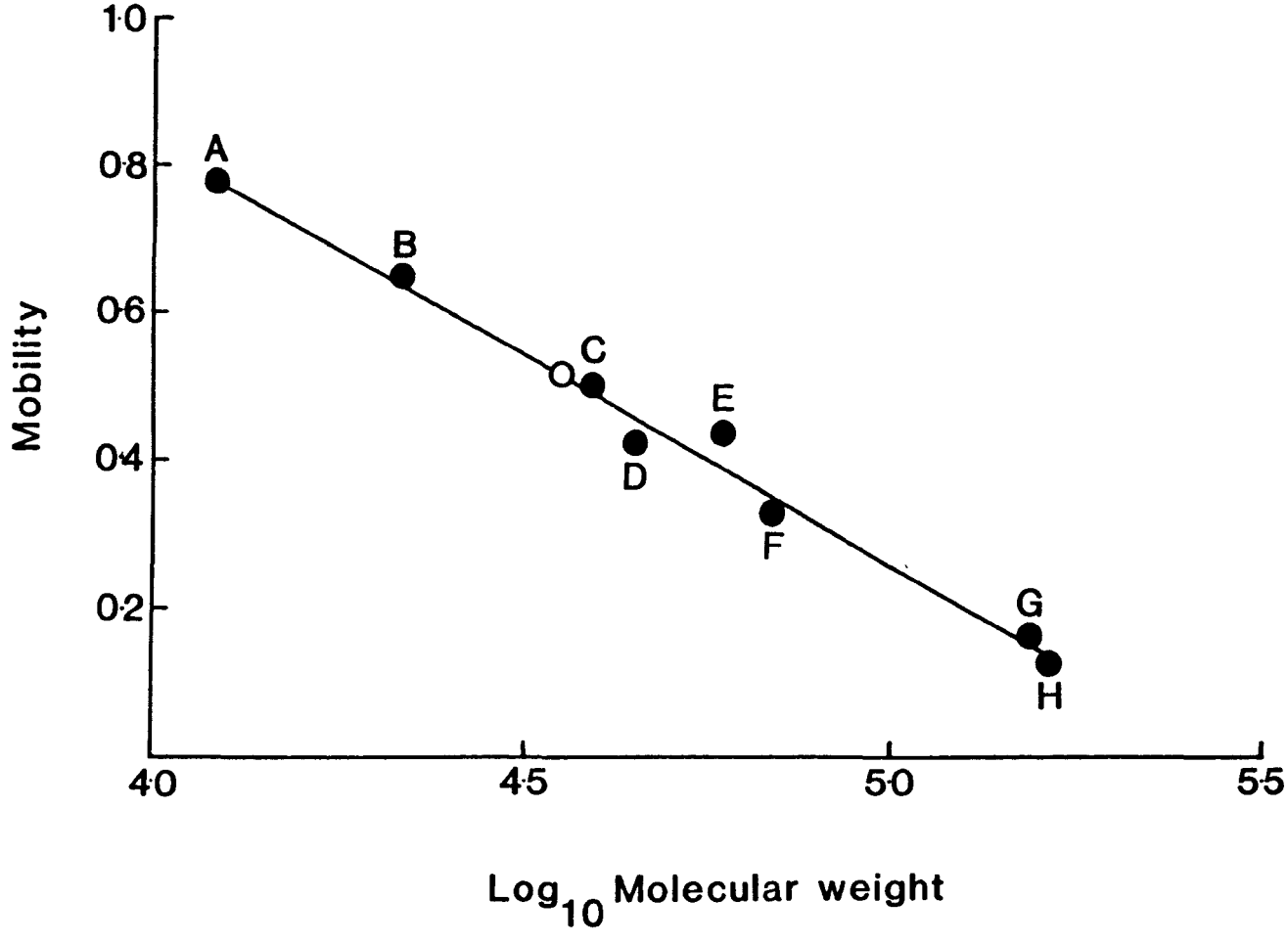

350
300
250
200
150
100
50
Elution volume (ml)
A
B
C
D
E
4·0
4·5
5·0
5·5
6·0
Log10 Molecular weight

FIGURE 33 SDS polyacrylamide gel electrophoresis of the phosphohexuloisomerase of methylamine grown Arthrobacter 2B2

The procedure used was that described in the Materials and Methods section.

The calibration proteins used were:

A	Cytochrome c	(M.W. 12500)
B	Trypsin inhibitor	(M.W. 21500)
C	RNA polymerase α chain	(M.W. 39000)
D	Ovalbumin	(M.W. 45000)
E	Catalase	(M.W. 60000)
F	Bovine serum albumin	(M.W. 68000)
G	RNA polymerase β chain	(M.W. 155000)
H	RNA polymerase β' chain	(M.W. 165000)
O	Phosphohexuloisomerase	


1·0
0·8
0·6
0·4
0·2
Mobility
A
B
C
D
E
F
G
H
4·0
4·5
5·0
5·5
Log10 Molecular weight

The determination of the Michaelis constants of phosphohexuloisomerase

The procedure of Eisenthal and Cornish-Bowden (1974) was used to determine the constants. It should be noted that the hexulose 6-phosphate that has been used in the purification and determination of properties of the enzyme has up to now been slightly impure. It was manufactured by the action of phosphoriboisomerase and hexulose phosphate synthase on ribose 5-phosphate and formaldehyde, as described in the Materials and Methods section. It was therefore a mixture of ribose 5-phosphate, ribulose 5-phosphate and hexulose 6-phosphate. For the determination of the kinetic constants a pure form of hexulose 6-phosphate was used, this being prepared by the method of Strøm et al., (1974), by Dr. A. J. Beardsmore.

The concentration of the hexulose 6-phosphate in solution was determined by adding a known volume of it to the continuous spectrophotometric assay, and adding an excess amount of phosphohexuloisomerase. The concentration was calculated from the total change in optical density obtained. The Km was determined by the addition of varying quantities of the pure hexulose 6-phosphate to the assay mixture, and adding a constant amount of the purified phosphohexuloisomerase.

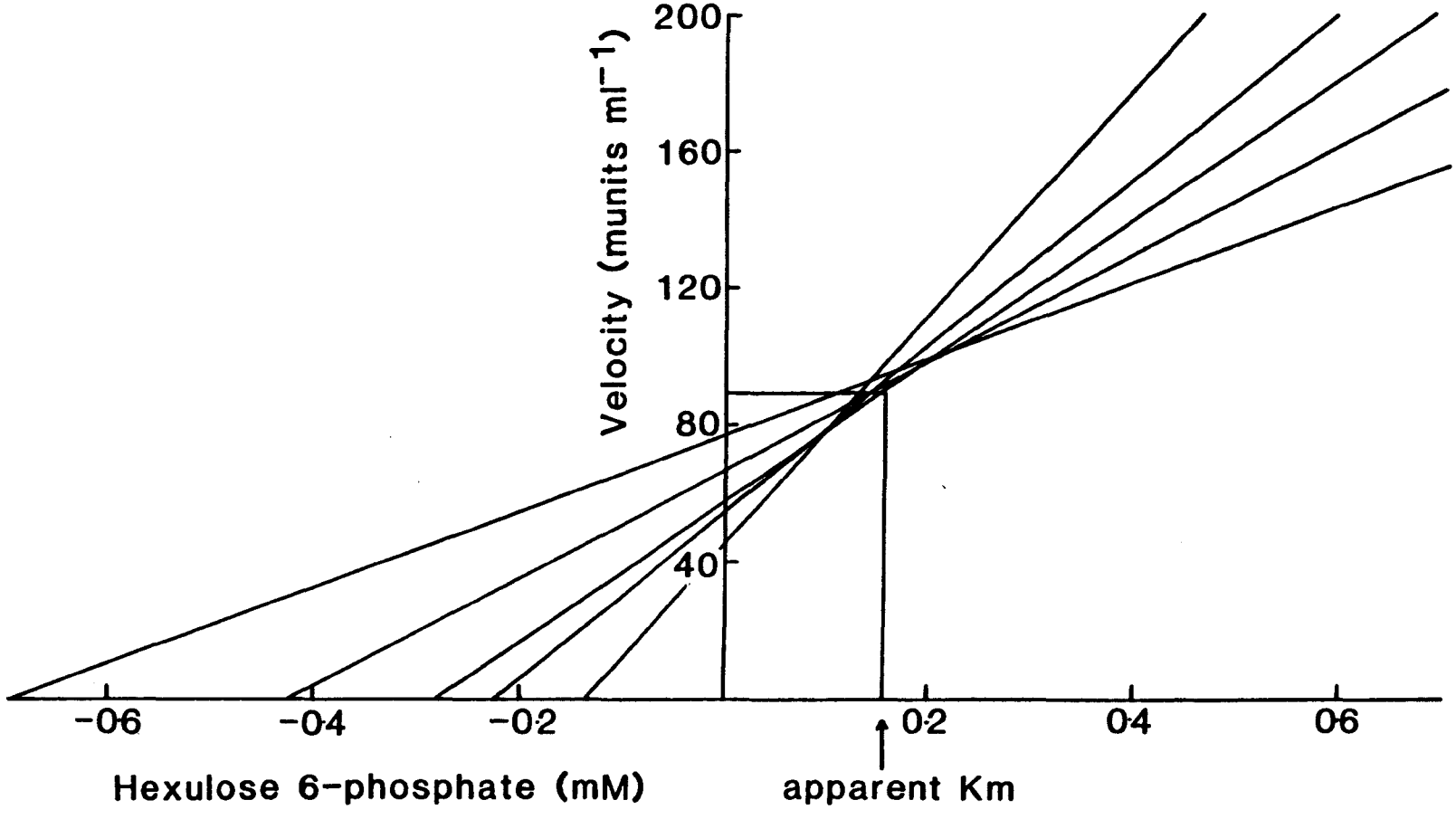
The results are shown in Figure 34. The apparent Km for hexulose 6-phosphate was calculated to be 1.3×10^{-4} M and the Vmax as 10.9 units $(\text{mg protein})^{-1}$.

Summary of results

Phosphohexuloisomerase from Arthrobacter 2B2 was purified 35 fold with a recovery of 22%. It was shown to be

FIGURE 34 The determination of the apparent Km for hexulose 6-phosphate of phosphohexuloisomerase of methylamine grown Arthrobacter 2B2

The Km was determined using the method of Eisenthal and Cornish Bowden (1974).


200
160
120
80
40
Velocity (munits ml^{-1})
-06
-04
-02
02
04
06
Hexulose 6-phosphate (mM)
apparent Km

a soluble enzyme which was extremely unstable in solutions of low protein concentration. The stability was however improved when the enzyme was stored in solutions containing high levels of protein. The behaviour of the enzyme in solutions containing low protein concentrations explains why it fails to work satisfactorily as a linkage enzyme in the hexulose phosphate synthase assay, the enzyme denaturing in the low protein concentration present in the assay mixture before initiation by the substrate. The hexulose phosphate synthase assay would consequently give little or no rate, and the extract tested would appear to be lacking hexulose phosphate synthase. This may be the explanation for the apparent lack of hexulose phosphate synthase in the organism Arthrobacter P1 (Levering et al., 1981a), when the hexulose phosphate synthase assay was used without the addition of a stable phosphohexuloisomerase (for example, that from Methylophilus methylotrophus) as a linkage enzyme.

The purified enzyme from Arthrobacter 2B2 is stable on freezing, but is inactivated quickly at raised temperatures. It appeared to have no requirement for divalent metal ions and was indeed inhibited by certain metal ions. The pH optimum of the enzyme was determined to be 8.5 and it showed no detectable phosphoglucoisomerase activity. The molecular weight was calculated to be 108000, and it appeared to be made up of three identical subunits, each of molecular weight 35500.

Discussion

When compared to the phosphohexuloisomerases which have been purified from Methylococcus capsulatus (Ferenci et al., 1974), and Methylophilus methylotrophus (Beardsmore et al., 1982), the enzyme from Arthrobacter 2B2 appears dissimilar.

The molecular weights of the enzymes from the other organisms were 67000 and 70000 respectively and that from Methylophilus methylotrophus seemed to associate into polymeric forms when purified, with dimers, trimers and other higher molecular weight variants being detected on application to a gel filtration column. Such polymers were not detected with the enzyme from Arthrobacter 2B2. The Km values for all three enzymes are very similar. However, the Vmax value for the enzyme from Arthrobacter 2B2 is considerably lower than those from the other two enzymes.

CHAPTER 7

The Utilisation of Choline as Sole Source of Carbon

In Chapter 4 when the growth of Arthrobacter 2B2 on methylamine was considered, it was noted that methylamine grown cells contained high activities of hexulose phosphate synthase and phosphohexuloisomerase, key enzymes of ribulose monophosphate cycle of formaldehyde fixation, and good indicators that the cycle was operative in the organism. However, the methylamine grown cells also contained a high activity of NADPH linked hydroxypyruvate reductase, an enzyme which is involved in the serine pathway of C_1 fixation. When further assays of other serine pathway enzymes were carried out, none were found, eliminating the possibility of the use of this pathway during growth on methylamine.

The presence of high levels of NADPH-linked hydroxypyruvate reductase in methylamine grown Arthorbacter 2B2 contrasted with the absence of this enzyme in methylamine grown Arthrobacter P1 (Levering et al.,1981b). Other enzymes of the serine pathway were absent in both methylamine grown Arthrobacter 2B2 and Arthrobacter P1. Both organisms when grown on methylamine possessed high activities of hexulose phosphate synthase and phosphohexuloisomerase, suggesting the operation of the ribulose monophosphate cycle during growth on this substrate. However, choline grown Arthrobacter P1 contained enzymes of the serine pathway, including very high levels of NADPH and NADH - linked hydroxypyruvate reductases. It was reported (Levering et al., 1981b) that the serine pathway enzymes were involved in the metabolism of glycine (produced by choline oxidation) to yield pyruvate (see Figure 11). These findings led to an investigation of the enzymes present in Arthrobacter 2B2 when grown on choline, the results being compared to those obtained when the organism was grown on methylamine or acetate.

The organism was grown on the inorganic salts medium described on page 39, supplemented with 0.2ml l^{-1} of a trace element solution (Vishniac and Santer, 1957), and 1 μg l^{-1} biotin. The carbon source was added at a

TABLE 12 Assay of serine pathway enzymes during the growth of Arthrobacter 2B2 on choline, methylamine and acetate

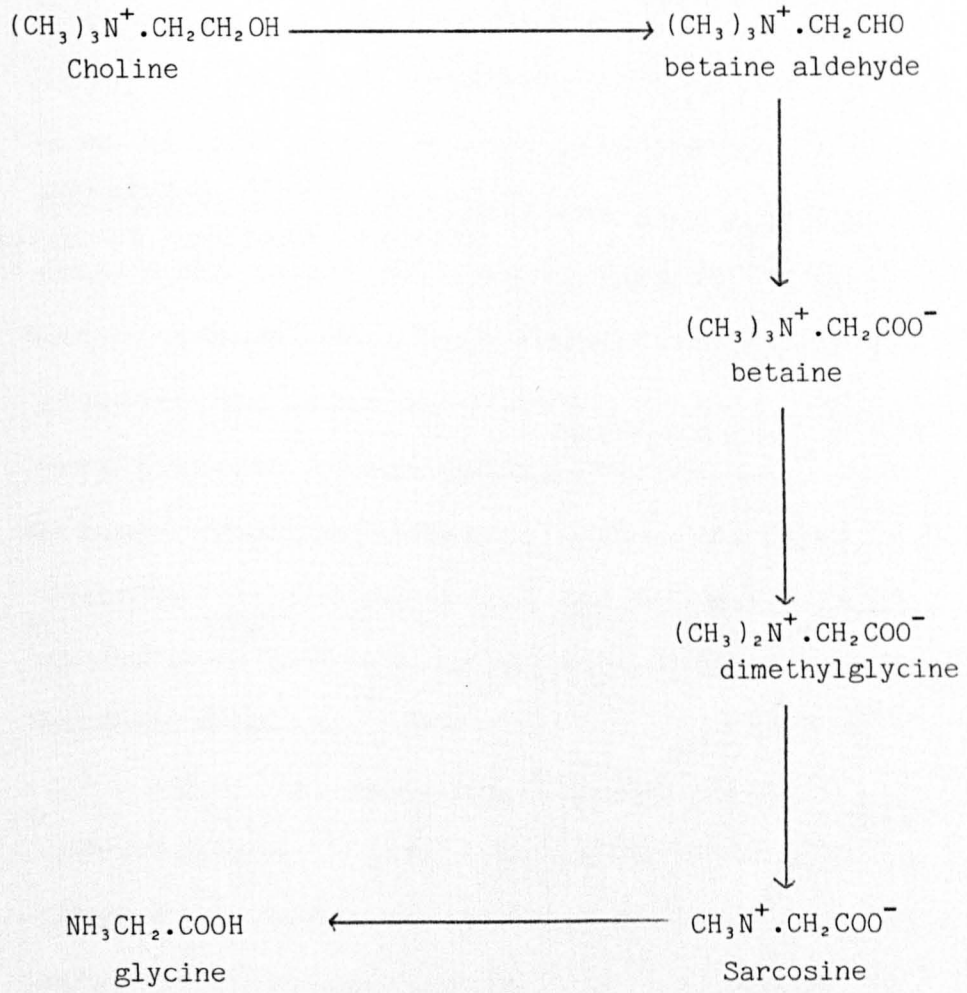
The preparation of crude cell free extracts and the assay procedures used are described in the Materials and Methods section. The assays for glycerate kinase, and serine dehydratase were carried out on benzene treated cells as little activity was detected using cell free extracts prepared by sonication. All activities are expressed as m units $(mg\ protein)^{-1}$.

Enzyme	Growth substrate		
	Choline	Methylamine	Acetate
Hydroxypyruvate reductase NADPH	1255	603	211
Hydroxypyruvate reductase NADH	1015	0	6.0
Serine-glyoxylate aminotransferase	0	0	0
Serine- α ketoglutarate aminotransferase	5.1	0	0
Glycerate kinase	79	0	0
Serine transhydroxymethylase	2	0.4	0.8
Malyl CoA lyase	0.2	0.3	0.7
Isocitrate lyase	4.4	8.4	222
Serine dehydratase	67	0	0
Hexulose phosphate synthase	9	342	2.1
Phosphohexuloisomerase	10	240	1.3

concentration of 50 mM. The doubling times of the organism on choline, methylamine and acetate were 7.6 hours, 6.9 hours and 7.0 hours respectively. In all cases cells were harvested in the exponential phase of growth, and cell free extracts were produced by sonicating the cell paste in 4 volumes of sodium potassium phosphate buffer pH 7.0, for 5 one minute periods interspersed with periods of cooling in ice. Enzyme assays were carried out using the procedure described in the Materials and Methods section. Table 12 shows the results obtained, and includes the specific activities of hexulose phosphate synthase and phosphohexuloisomerase for reference. Cell free extract of choline grown cells had a two-fold higher level of NADPH-linked hydroxypyruvate reductase than extracts of cells grown on methylamine. It also had higher levels of an NADH-linked hydroxypyruvate reductase; the presence of such an enzyme could not be detected in extracts of methylamine grown cells. Crude extracts of choline grown cells did show activities of all the enzymes necessary to operate the serine pathway, in a similar manner to that reported in Arthrobacter P1 (Levering et al., 1981b). However, when the specific activities of the enzymes were considered together with the doubling time of the organism, the activities with one exception, did not appear to be of physiological significance. The one exception being the hydroxypyruvate reductase levels.

Rather than using enzymes of the serine pathway, an alternative route of glycine metabolism yielding pyruvate, would be the use of serine dehydratase. As the results on Table 12 indicate, serine dehydratase is induced during the growth of Arthorbacter 2B2 on choline, and its activity is much higher than some of the serine pathway enzymes (Serine α-ketoglutarate aminotransferase, serine transhydroxymethylase, malyl CoA lyase, and isocitrate lyase). This indicates that serine dehydratase could play a role in the metabolism of glycine, to yield pyruvate, in choline grown Arthrobacter 2B2.

An examination of the pathway involved in the initial breakdown of choline was considered next. As mentioned in Chapter 1 the method of choline oxidation adopted by mammalian cells and proposed to occur in Arthrobacter P1 (Levering et al., 1981b), is as follows:



Initially an attempt was made to grow Arthrobacter 2B2 using the intermediates betaine, dimethylglycine, sarcosine and glycine as carbon sources.

It was determined that Arthrobacter 2B2 although capable of growth on choline, sarcosine and glycine, was unable to grow using betaine or dimethylglycine as a carbon source. This suggested either that the organism could not

TABLE 13 Assay of enzymes involved in choline metabolism in Arthrobacter 2B2

The preparation of crude cell free extracts and the assay procedures used are described in the Materials and Methods section. All activities are expressed as units $(mg\ protein)^{-1}$.

Enzyme		Growth substrate		
		Glucose	Choline	Methylamine
Choline oxidase		0	0.15	0
Betaine aldehyde dehydrogenase				
	NAD	0	0.14	0
	NADP	0	0.03	0
Dimethylglycine oxidase		0	0.03	0
Sarcosine oxidase		0	0.07	0

metabolise choline via this route as it would be expected that the intermediates of the breakdown pathway would support growth, or that it was metabolised in this way, but the organism had no mechanism to transport betaine and dimethylglycine into the cell and therefore could not use them as a carbon source.

In order to determine whether choline was being broken down by this route, assays were carried out using crude cell free extracts of choline-grown Arthrobacter 2B2 in order to detect the levels of enzymes involved with the pathway. The activities recorded were compared to those observed using glucose- and methylamine-grown cells. Crude cell free extracts were prepared by sonicating choline-grown cell paste in four volumes of 10 mM sodium potassium phosphate buffer pH 7.0 as described in the Materials and Methods section. Enzyme assays were carried out as described in the Materials and Methods section.

Table 13 shows the results obtained during the investigation. Cell free extracts of glucose and methylamine-grown cells had undetectable levels of the enzymes. However, extracts of choline-grown cells had reasonable levels of all of the enzymes involved in this pathway of choline metabolism.

One of the products of the metabolism of choline via this pathway is formaldehyde. This is a highly toxic substance and would severely damage a cell if it was allowed to build up to even low levels within it. Any cell which produces formaldehyde during metabolism must therefore have a method by which it can be removed in some way.

Since oxidation of choline by choline oxidase results in the production of formaldehyde, the latter must be removed in

some way during growth on choline by Arthrobacter 2B2.

A method of formaldehyde oxidation which is widespread amongst organisms using the ribulose monophosphate cycle for C_1 assimilation is the dissimilatory ribulose monophosphate cycle (Figure 10). During the operation of this cycle hexulose 6-phosphate is synthesized from ribulose 5-phosphate and formaldehyde. This is isomerised first to fructose 6-phosphate then glucose 6-phosphate which is acted on by glucose 6-phosphate dehydrogenase to give 6-phosphogluconate. This is converted to ribulose 5-phosphate and carbon dioxide by 6-phosphogluconate dehydrogenase. In extracts of methylamine-grown Arthrobacter 2B2 there are high levels of hexulose phosphate synthase and phosphohexuloisomerase. The organism also contains glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, making a cyclic oxidation pathway operable. However, extracts of choline-grown cells contain very low levels of hexulose phosphate synthase and phosphohexuloisomerase which makes the use of the dissimilatory ribulose monophosphate cycle look improbable. Another route which could be used for formaldehyde removal is a linear sequence involving the use of formaldehyde and formate dehydrogenases. When extracts of Arthrobacter 2B2 were assayed to test for the presence of formaldehyde dehydrogenase, activities of 176 m units $(\text{mg protein})^{-1}$ were recorded in choline-grown cells. In glucose- and methylamine-grown cells however, no activity of this enzyme could be detected. It is therefore possible that during the growth of Arthrobacter 2B2 on a medium containing choline as a carbon source, the formaldehyde produced is oxidised via a linear sequence by the enzymes formaldehyde dehydrogenase and formate dehydrogenase to carbon dioxide.

Summary of results

It was noted previously in Chapter 4 that carbon assimilation in methylamine-grown Arthrobacter 2B2 is via the ribulose monophosphate cycle of formaldehyde fixation. However, an enzyme profile of the organism showed the presence of high levels of NADPH-linked hydroxypyruvate reductase in the cell free extracts of such cells. Hydroxypyruvate reductase is a key enzyme of the serine pathway but assays of other serine pathway enzymes proved negative in extracts of the organism. This rather unexpected finding in Arthrobacter 2B2 led to the consideration of the results obtained in a similar organism, Arthrobacter P1 (Levering et al.,1981a,b), where the organism used the ribulose monophosphate cycle when growing on methylamine, but it contained no hydroxypyruvate reductase activity. When Arthrobacter P1 was grown on a medium containing choline as a carbon source, enzymes of the ribulose monophosphate cycle were again present, together with NADH and NADPH-linked hydroxypyruvate reductase activity. The presence of high levels of other enzymes involved in the serine pathway indicated that the concurrent operation of this pathway was likely.

When Arthrobacter 2B2 was grown on a medium containing choline as a carbon source, very low activities of the two key ribulose monophosphate cycle enzymes hexulose phosphate synthase and phosphohexuloisomerase were recorded, indicating that the cycle was not a major metabolic pathway during growth on choline. Assays of serine pathway enzymes proved positive although, with the exception of hydroxypyruvate reductase, they were at lower levels than the corresponding enzymes in Arthrobacter P1. NADPH-linked hydroxypyruvate reductase activity was present at a level of two times greater than that recorded in methylamine-grown cells. There was also a high level of NADH-linked hydroxypyruvate reductase activity, none being recorded in methylamine-grown cells.

In order to determine the choline breakdown pathway in Arthrobacter 2B2, enzyme assays were carried out. These showed the presence of the enzymes choline oxidase, betaine aldehyde dehydrogenase, dimethylglycine oxidase, and sarcosine oxidase. The results indicated that choline metabolism followed a similar pathway to that hypothosized to occur in Arthrobacter P1, and in mammalian cells. However, it was also noted that Arthrobacter 2B2 would not grow on a medium containing betaine or dimethylglycine as a carbon source, indicating that no transport mechanism for these compounds exists in the organism.

The breakdown of choline via this pathway leads to the production of glycine and formaldehyde. The glycine can be dealt with in essentially the same way as that put forward by Levering et al., (1981b), for Arthrobacter P1. Glycine can be condensed with formaldehyde by serine transhydroxymethylase to yield serine. This can be acted on sequentially by serine -α ketoglutarate aminotransferase, hydroxypyruvate reductase, and glycerate kinase, all of which are present in choline grown Arthrobacter 2B2, to yield phosphoglycerate. Phosphoglycerate can be converted to pyruvate via the glycolytic enzymes, phosphoglyceromutase enolase and pyruvate kinase. Alternatively, serine can be converted directly to pyruvate by serine dehydratase, activity of which has been found in choline-grown Arthrobacter 2B2.

As mentioned previously, the second product of choline oxidation is formaldehyde, a highly toxic substance which must be removed from the cell. Some of the formaldehyde can be removed during the conversion of glycine to pyruvate where it can be condensed with glycine, forming serine. However, other methods of formaldehyde removal must be available to the organism. It has been reported that Arthrobacter P1 contains no formaldehyde dehydrogenase during growth on either methylamine or choline (Levering et al., 1981a). Therefore, it has been suggested that the

formaldehyde produced during choline metabolism is removed by entering the ribulose monophosphate cycle, where it could be assimilated or dissimilated. However, in extracts of Arthrobacter 2B2 grown in a medium containing choline as a carbon source, the levels of key ribulose monophosphate cycle enzymes appear too low to suggest a major metabolic route for the sequence. The organism grown on choline does, however, contain quite high activities of a formaldehyde dehydrogenase, indicating that formaldehyde oxidation could proceed via a linear pathway.

The methods of formaldehyde oxidation in Arthrobacter 2B2, therefore, appear very complicated. When growing on methylamine there is no formaldehyde dehydrogenase present, and formaldehyde oxidation proceeds via the dissimilatory ribulose monophospate cycle. However, when growing on choline the levels of hexulose phosphate synthase and phosphohexuloisomerase are too low to suggest that the dissimilatory ribulose monophosphate cycle is the method of formaldehyde oxidation, and this, together with higher levels of formaldehyde dehydrogenase, indicate that a linear formaldehyde oxidation pathway operates.

CHAPTER 8

The purification and properties of the NADPH linked hydroxypyruvate reductase from methylamine-grown Arthrobacter 2B2

Results in Chapter 4 demonstrated that Arthrobacter 2B2 when growing on methylamine contained high activities of the enzymes involved in the ribulose monophosphate cycle and, with the exception of hydroxypyruvate reductase, enzymes of the serine pathway were absent. As carbon assimilation in methylamine-grown cells must be via the ribulose monophosphate cycle, the relatively high activities of NADPH-linked hydroxypyruvate reductase in extracts of such cells appears anomalous. When enzyme profiles of Arthrobacter P1 were considered (Levering et al., 1981a and b), it was noted that extracts of the organism grown on methylamine show high activities of ribulose monophosphate cycle enzymes indicating the operation of this pathway. However, no hydroxypyruvate reductase activity had been recorded. Therefore a problem remains as to the reason for the production of an NADPH-linked hydroxypyruvate reductase in Arthrobacter 2B2 grown in a medium containing methylamine as a carbon source. It was thought that the hydroxypyruvate reductase activity might be due to a relatively unspecific enzyme. In order to investigate this possibility, and to compare its properties with those of enzymes previously purified from other organisms, a partial purification of the NADPH-linked hydroxypyruvate reductase from Arthrobacter 2B2 was carried out.

Initial purification procedure

Crude cell free extracts were produced from cell pastes of Arthrobacter 2B2 grown on a medium containing methylamine as sole carbon source by sonication in four volumes of 20 mM sodium potassium phosphate buffer pH 7.0. The cell free extract was primarily subjected to ammonium sulphate fractionation. Initially the extract was raised to a level

TABLE 14 Ammonium sulphate fractionation of hydroxypyruvate reductase of methylamine grown Arthrobacter 282

Crude cell free extract was subjected to $(NH_4)_2SO_4$ fractionation using the procedure outlined in the Materials and Methods section.

Fraction	Total Activity (m units)	Specific Activity (m units mg protein^{-1})
Crude extract	27000	770
0-30% $(NH_4)_2SO_4$	656	164
30-40% $(NH_4)_2SO_4$	776	107
40-50% $(NH_4)_2SO_4$	2408	231
50-60% $(NH_4)_2SO_4$	17536	1992
60-70% $(NH_4)_2SO_4$	536	335

of 30% saturation by the addition of solid ammonium sulphate and, after removal of the precipitated protein, sequential 10% fractions were made up to a level of 70% ammonium sulphate. The results obtained are given in Table 14 and show that 64% of the total activity contained within the crude cell free extract precipitates out in the 50% to 60% ammonium sulphate fraction, giving a 2.6 fold purification. This was considered a good purification step and was included in further purification attempts.

As the next stage in purification the 50% to 60% ammonium sulphate fraction was applied to a Sephadex G200 gel filtration column (28 x 2.6 cm). The column was equilibrated with 20 mM sodium potassium phosphate buffer pH 7.0, and after the extract was loaded, it was eluted with a similar buffer, active fractions being detected using the continuous NADPH linked assay procedure described in the Materials and Methods section. The active fractions were pooled and assayed for activity, the results indicating that a 15% loss in total activity and a two fold purification had occurred when compared to the ammonium sulphate fraction.

The two steps described were considered to constitute a good partial purification procedure. However, for the final procedure a protamine sulphate precipitation was carried out before the ammonium sulphate fractionation in order to remove basic proteins.

Final purification procedure

(i) Preparation of the crude cell free extract

The crude cell free extract was prepared by resuspending 2 g of methylamine-grown Arthrobacter 2B2 cell paste in 8 ml

of 20 mM sodium potassium phosphate buffer pH 7.0, and sonicating as a single batch. The cell debris were removed by centrifugation at 38000 g for 20 minutes at $2^{o}C$. The pellet was discarded and the supernatant treated as the cell free extract.

(ii) Protamine sulphate precipitation

The cell free extract produced in step (i) was maintained at $0^{o}C$. A 20 mg ml^{-1} solution of cold protamine sulphate was then added slowly to the cell free extract to make a final concentration of 1 mg protamine sulphate per 10 mg protein in the crude extract. The mixture was stirred at $0^{o}C$ for 20 minutes before removing the precipitate by centrifugation at 38000 g for 20 minutes at $2^{o}C$. The supernatant was used in the next stage of purification.

(iii) Ammonium sulphate fractionation

The supernatant was raised to 50% saturation by the slow addition of solid ammonium sulphate and the precipitated protein was removed by centrifugation at 38000 g for 20 minutes at $2^{o}C$. The resulting supernatant was treated by the further addition of solid ammonium sulphate to a level of 60% saturation. After the precipitated protein had been collected by centrifugation it was redissolved in the sonication buffer.

(iv) Sephadex G200 chromatography

The redissolved protein from the ammonium sulphate fraction was loaded onto a Sephadex G200 column (28 x 2.6 cm) equilibrated with 20 mM sodium potassium phosphate buffer pH 7.0. The hydroxypyruvate reductase activity was eluted from the column with the equilibration buffer, active fractions being detected using the continuous NADPH-linked spectrophotometric assay described in the Materials and Methods section.

TABLE 15 The purification of NADPH-linked hydroxypyruvate reductase from methylamine-grown Arthrobacter 2B2

Fraction	Volume ml	Protein mg	Activity units	Specific Activity units mg^{-1}	Purification fold	Yield %
Crude cell free extract	8	42.4	29.7	0.7	1	100
Protamine sulphate precipitation	8	35.2	27.2	0.77	1.1	91
$(NH_4)_2SO_4$ fractionation	8	8.8	17.5	2.0	2.8	60
Sephadex G200 chromatography	58	3.2	16.0	5.0	7.1	54

Fractions 16 to 28 containing activity were pooled, then ultrafiltered to a small volume and stored until required at -15°C.

A table of the purification of NADPH linked hydroxypyruvate reductase is shown in Table 15. The results from the Sephadex G200 column are shown in Figure 35 . The enzyme was purified 7 fold with a yield of 54%. Samples of the partially purified enzyme were used in all of the following determinations.

Stability

The enzyme appeared relatively stable, no stabilisers being required either during the purification process or storage. At 0°C the enzyme lost 30% of its activity within a week; however when stored frozen at -15°C it proved more stable taking three months to lose 50% of its activity. Repeated cycles of freezing and thawing did not greatly affect the enzyme, 20% of its activity being lost during six cycles of freezing and thawing carried out over a two week period.

pH optimum

The buffers used for the investigation were 100 mM succinic acid - sodium hydroxide, over the pH range 4.0 to 6.0, and 100 mM sodium potassium phosphate over the range 6.0 to 8.0. Assays were carried out by including the appropriate buffer in the continuous assay mixture described in the Materials and Methods section. The pH of the buffer was checked before each assay, and the pH of the complete assay system was verified afterwards in order to confirm that

FIGURE 35 Sephadex G200 gel filtration chromatography of NADPH-linked hydroxypyruvate reductase from methylamine-grown Arthrobacter 2B2

Extract was applied to a Sephadex G200 column (28 x 2.6 cm) equilibrated with 20 mM sodium potassium phosphate buffer pH 7.0. The activity was eluted with equilibration buffer and the elution pattern followed as described in the Materials and Methods section.

Key:

○ = Extinction at 280 nm

△ = Hydroxypyruvate reductase activity

Fraction volume = 4.8 ml.

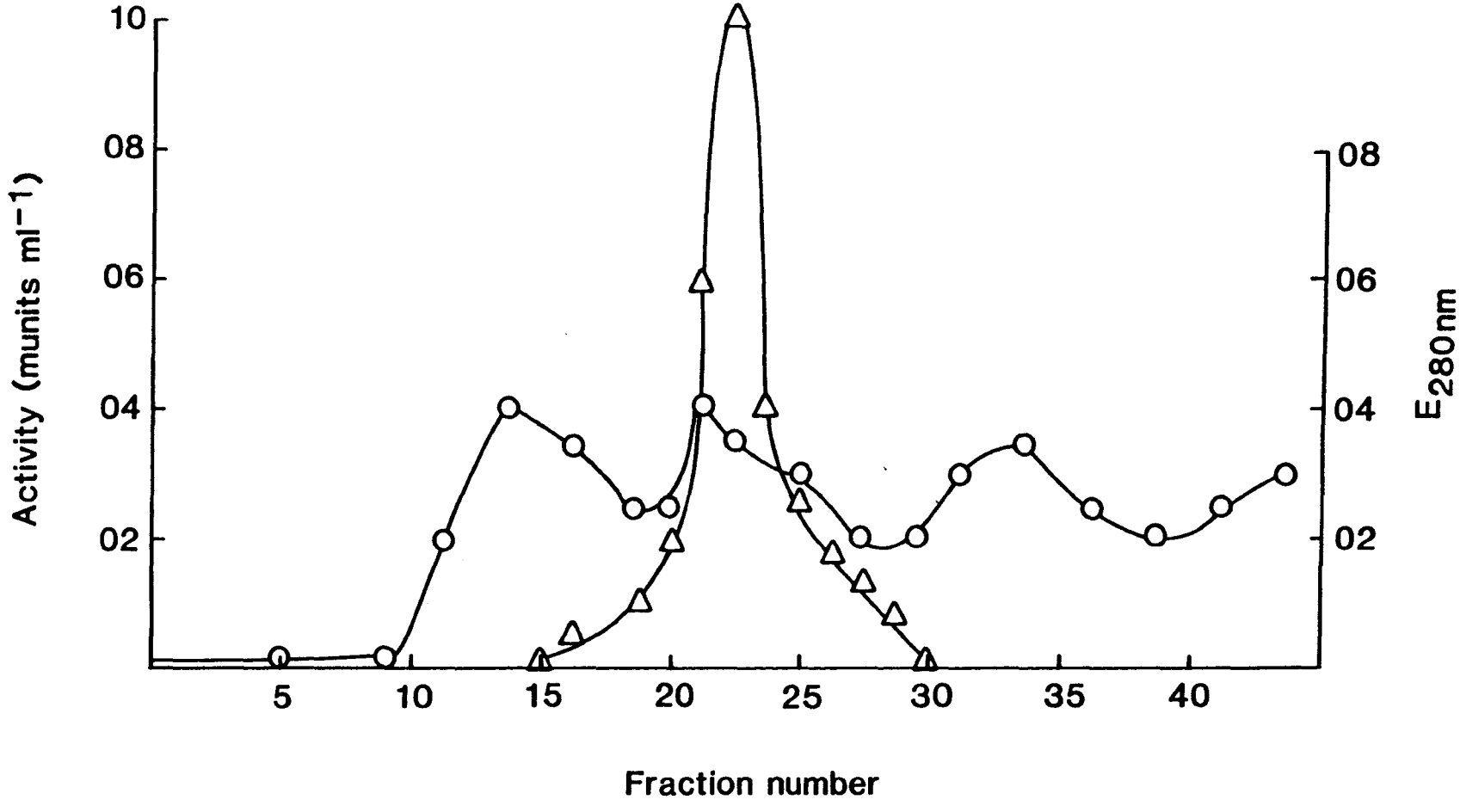

10
08
06
04
02
Activity (munits ml^{-1})
08
06
04
02
E_{280nm}
5
10
15
20
25
30
35
40
Fraction number

TABLE 16 Substrate specificity of hydroxypyruvate reductase of methylamine grown Arthrobacter 2B2

The substrate specificity was tested by the inclusion of the appropriate compound at a concentration of 2 mM to the continuous NADPH-linked assay which was described in the Materials and Methods section.

Substrate	Activity (%)
Hydroxypyruvate	100
Diacetyl	0
Glyoxylate	0
Methylglyoxal	14
Acetaldehyde	0
Glyoxal	0
Oxaloacetate	0
Pyruvate	0
Acetone	0
Glycolaldehyde	0
D-L-glyceraldehyde	0
Acetoacetate	0
Dihydroxyacetone	0
α -ketoglutarate	0
Formaldehyde	0

the pH had remained the same throughout.

The results are shown in Figure 36 and demonstrate that the pH optimum occurred at 6.0. It was also noted that the enzyme exhibited slightly higher activity when using a succinic acid - sodium hydroxide buffer system than when a sodium potassium phosphate buffer system at a similar pH was utilised.

Substrate specificity

The substrate specificity was tested by placing a sample of the partially purified enzyme in the continuous NADPH-linked assay mixture. Table 16 shows the results obtained from the investigation expressed as a percentage of the activity present when hydroxypyruvate was used as a substrate. It was noted that when NADPH was replaced by NADH in a continuous assay in which hydroxypyruvate was the substrate, no activity was detected.

The results show that the only compound with which the enzyme showed any activity was methylglyoxal which gave 14% of the activity obtained when hydroxypyruvate was used as a substrate.

Assays were also carried out in order to determine if the enzyme could work in reverse, catalysing the conversion of glycerate to hydroxypyruvate with the concomitant reduction of NADP. The enzyme was assayed in the reverse direction using a modified hydroxypyruvate reductase assay in which NADP replaced NADPH and D-L glycerate was used as the substrate. The assay was initially carried out at pH 7.0 and no activity could be detected. Repeat assays were performed at pH 6.0 and 8.0 in order to determine if the

FIGURE 36 The pH optimum of partially purified hydroxypyruvate reductase of methylamine grown Arthrobacter 2B2

The enzyme was assayed over the pH range 4.0 to 8.0 using the continuous NADPH-linked assay described in the Materials and Methods section, except that the buffers used were 100 mM succinic acid - sodium hydroxide and 100 mM sodium potassium phosphate.

Key:

○ succinic acid - sodium hydroxide

△ sodium potassium phosphate

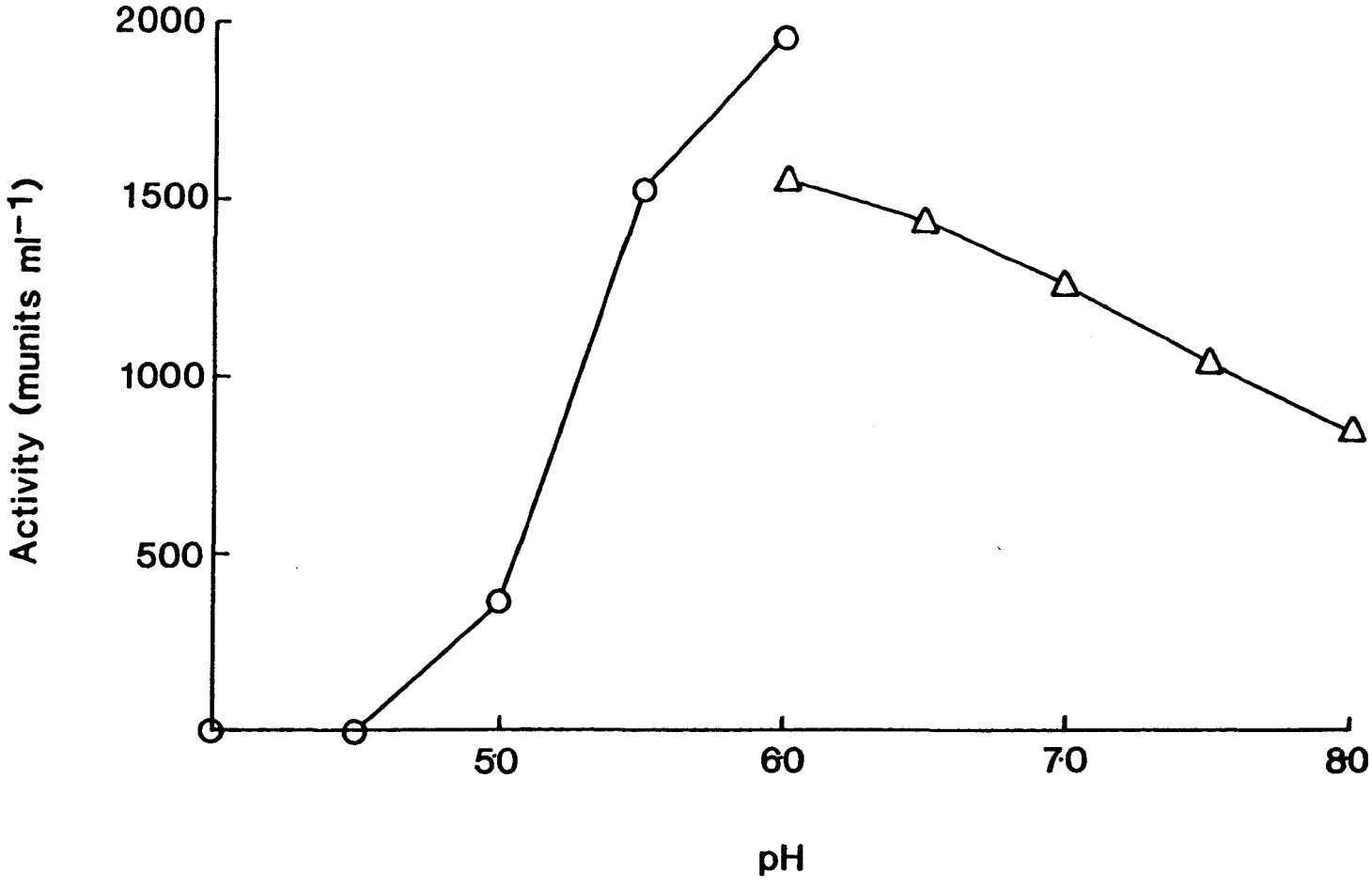

2000
1500
1000
500
Activity (munits ml⁻¹)
50
60
70
80
pH

TABLE 17 Effect of anions on hydroxypyruvate reductase of methylamine grown Arthrobacter 2B2

The enzyme was incubated in the continuous assay mixture supplemented by an addition of the appropriate compound. The results are expressed as the percentage inhibition, when the rates were compared to an assay omitting the anions.

Anion	Concentration mM	Inhibition %
None	-	0
Citrate	30	3
Bromide	33	26
Iodide	33	52

reverse reaction was catalysed at a higher or lower pH and again no activity was recorded. The assays were repeated using D(+) glycerate in order to assess whether the mixture of isomers present in D-L glycerate competitively inhibited the enzyme. Once more no activity could be detected.

Effects of anions

The effects of sodium iodide, sodium bromide and sodium citrate on the enzyme were tested by including the compound in the continuous NADPH-linked assay mixture described in the Materials and Methods. The results are shown in Table 17 and the inhibition is expressed as a percentage of the rate measured in an assay with no anion added.

The results indicate that citrate inhibits the enzyme by only 3% whilst bromide inhibits it by 26%, and iodide by 52%.

The molecular weight determination of hydroxypyruvate reductase

The native molecular weight of the partially purified enzyme was determined using Sephadex G200 gel filtration chromatography. The packing material was pre-swollen in 20 mM sodium potassium phosphate buffer pH 7.0 and a column (80 x 2.5 cm) packed and maintained at $4^{o}C$. Proteins of a known molecular weight were loaded onto the column and eluted with equilibration buffer which was pumped through the column at a flow rate of 10 ml h^{-1}. The standard proteins used were cytochrome c (M. Wt. 12500), bovine serum albumin (M. Wt. 65000-70000), lactate dehydrogenase (M. Wt. 13000-14000), catalase (M. Wt. 230000-250000), and β galactosidase

(M. Wt. 510000-530000). Partially purified hydroxypyruvate reductase (5 units), was then applied to the column and eluted with equilibration buffer pumped through at a flow rate of 10 ml h^{-1}. The standard proteins were detected either by their absorbance (cytochrome c at 412 nm, bovine serum albumin at 280 nm), or by their enzymic assay as described in the Materials and Methods section. Hydroxypyruvate reductase activity was determined using the continuous NADPH-linked assay procedure.

The determination of the molecular weight of hydroxypyruvate reductase is shown in Figure 37. The molecular weight of the purified enzyme was calculated to be 204000.

The determination of the Michaelis constants of hydroxypyruvate reductase

The constants were determined by the direct linear plot of Eisenthal and Cornish-Bowden (1974). The Km for NADPH was determined by adding differing quantities of NADPH to the continuous assay mixture containing 2 mM hydroxypyruvate. The Km for hydroxypyruvate was calculated by including various quantities of hydroxypyruvate to an assay mixture containing 0.4 mM NADPH.

The results are shown in Figures 38 and 39, the apparent Km and Vmax values for NADPH being determined as 1.6×10^{-5} M and 3.85 units (mg protein)$^{-1}$ respectively, and those for hydroxypyruvate as 3.35×10^{-4} M and 3.85 units (mg protein)$^{-1}$ respectively.

Summary of results

The enzyme was purified 7 fold with a yield of 54%.

FIGURE 37 Molecular weight determination of NADPH-linked hydroxypyruvate reductase from methylamine-grown Arthrobacter 2B2

The enzyme was eluted from a Sephadex G200 column (80 x 2.5 cm) equilibrated with 20 mM sodium potassium phosphate buffer pH 7.0. The column was calibrated with cytochrome c (A), bovine serum albumin (B), lactate dehydrogenase (C), catalase (D), and β galactosidase (E). Partially purified hydroxypyruvate reductase (O) was eluted from the column at a fraction volume corresponding to a molecular weight of 204000. The volume of each fraction was 3.3 ml and the flow rate 10 ml h^{-1}.

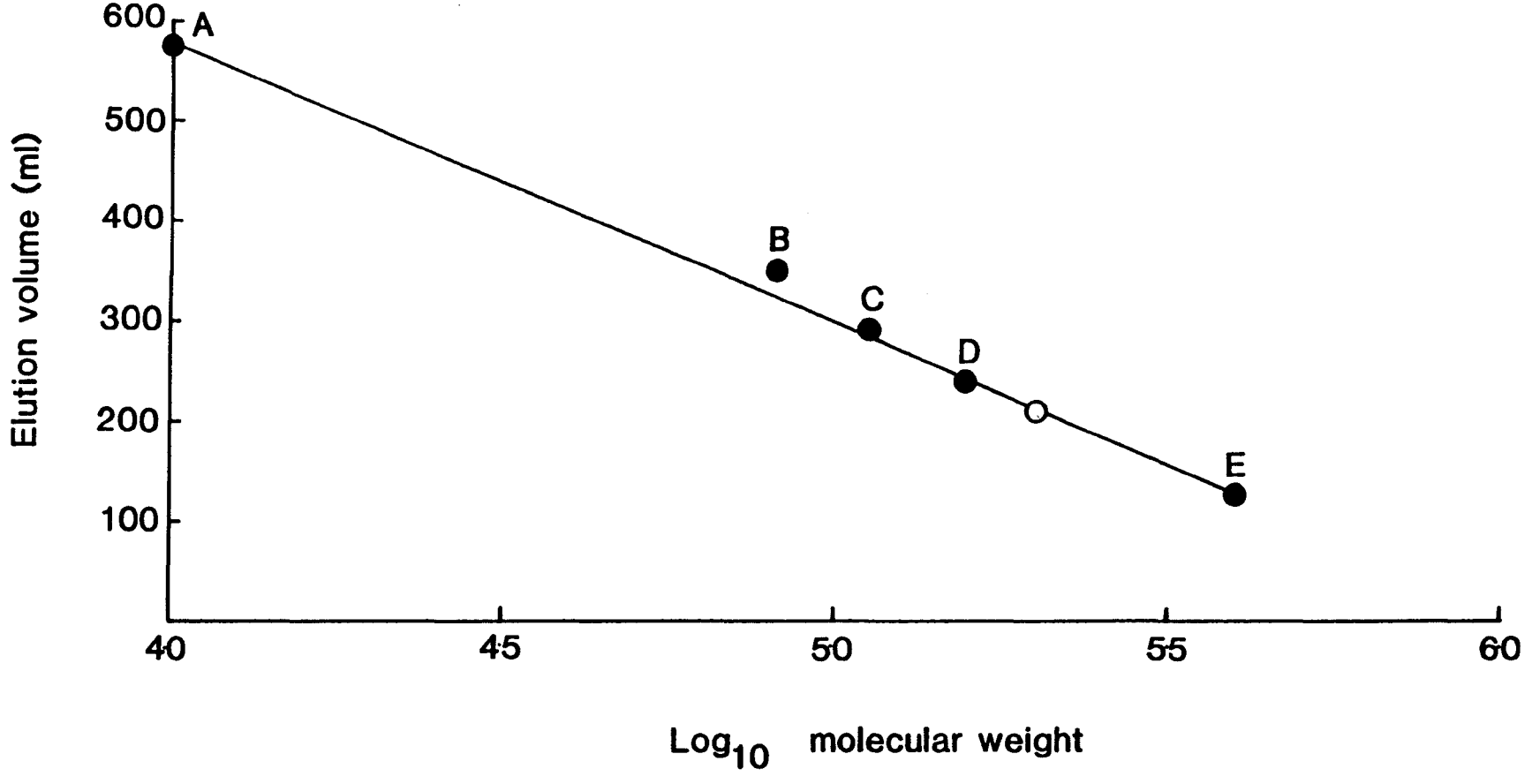

600
500
400
300
200
100
Elution volume (ml)
A
B
C
D
E
40
45
50
55
60
Log10 molecular weight

FIGURE 38 The determination of the apparent Km for hydroxypyruvate of NADPH-linked hydroxypyruvate reductase from methylamine-grown Arthrobacter 2B2

The Km was determined by a direct linear plot by adding different quantities of hydroxypyruvate to an assay mixture containing 0.4 mM NADPH.

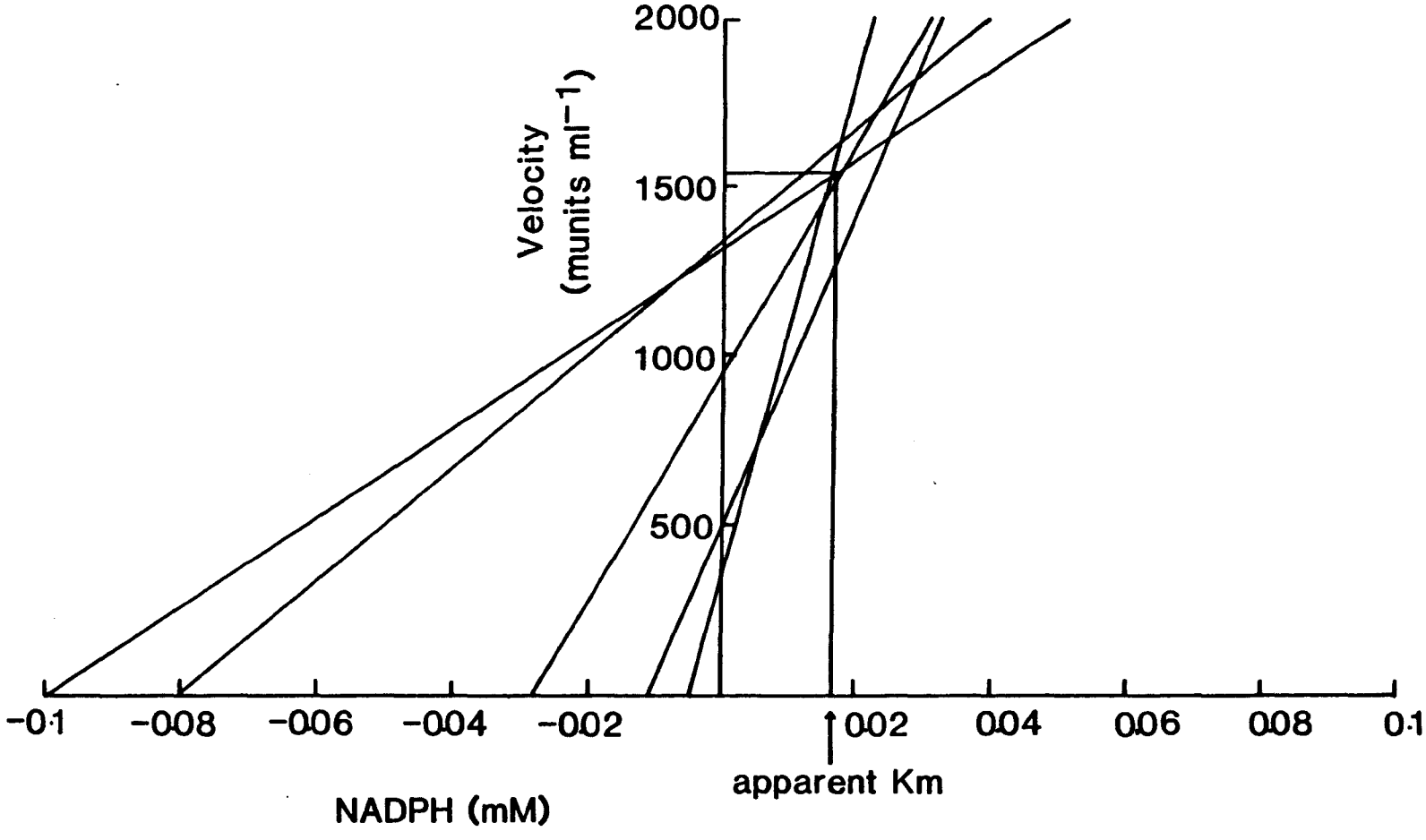
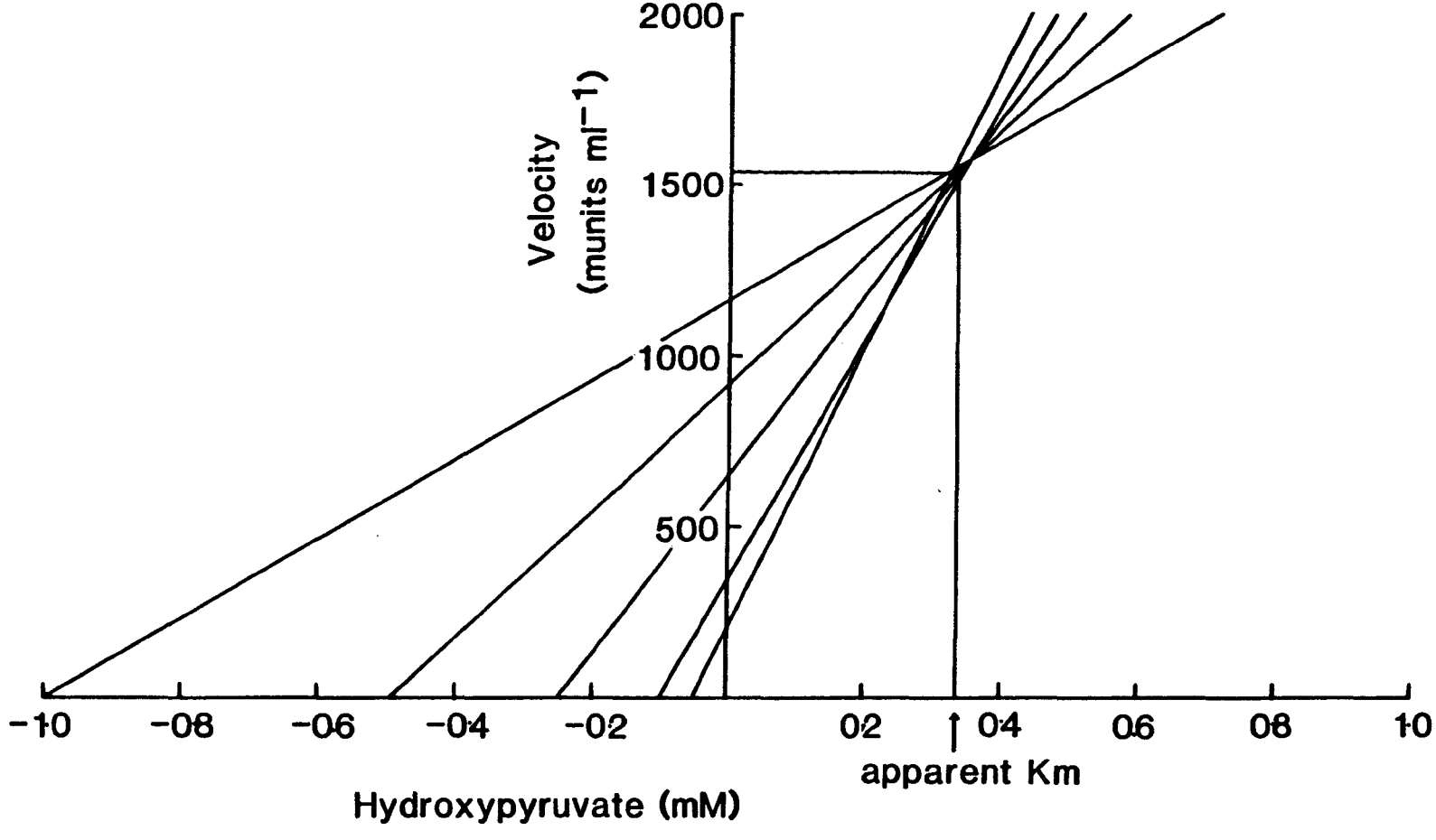

2000
1500
1000
500
Velocity
(munits ml^{-1})
-0·1
-0·08
-0·06
-0·04
-0·02
0·02
0·04
0·06
0·08
0·1
apparent Km
NADPH (mM)

FIGURE 39 The determination of the apparent Km for NADPH of NADPH-linked hydroxypyruvate reductase from methylamine-grown Arthrobacter 2B2

The Km was determined by a direct linear plot by adding different quantities of NADPH to an assay mixture containing 2 mM hydroxypyruvate.


2000
1500
1000
500
Velocity
(munits ml^{-1})
-10
-08
-06
-04
-02
02
04
06
08
10
apparent Km
Hydroxypyruvate (mM)

it appeared to be a soluble enzyme with a pH optimum of 6.0 and an apparent molecular weight of 204000. The partially purified enzyme was specific for NADPH and had a very narrow substrate range, methylglyoxal being its only other substrate besides hydroxypyruvate. This tends to imply that the enzyme is a hydroxypyruvate reductase and not some other relatively unspecific enzyme.

Discussion

When compared to similar enzymes from other organisms, the hydroxypyruvate reductase from methylamine grown *Arthrobacter 2B2* appears quite different. It is the only enzyme to be strictly NADP-linked, others being strictly NAD-linked e.g. *Pseudomonas AM1*, (Large and Quayle, 1963), or having a dual NAD/NADP-linkage e.g. *Paracoccus denitrificans*, (Bamforth and Quayle, 1977). The enzymes from *Pseudomonas AM1* and *Paracoccus denitrificans* were both competatively inhibited by citrate, whilst the enzyme from *Arthrobacter 2B2* retained almost full activity with citrate present. Further to this the enzyme from *Paracoccus denitrificans* was inhibited to a similar degree by bromide and iodide, while that from *Arthrobacter 2B2* was inhibited to a greater extent by iodide than by bromide.

The hydroxypyruvate reductase from methylamine grown *Arthrobacter 2B2* therefore seems to be a genuine hydroxypyruvate reductase. Its Km shows it to have a high affinity for hydroxypyruvate, but its characteristics show it to be dissimilar to hydroxypyruvate reductases studied previously in other organisms.

CHAPTER 9

The Purification and Properties of the Hydroxypyruvate Reductase present in Choline-grown Arthrobacter 2B2

TABLE 18 Ammonium sulphate fractionation of the NADH-linked hydroxypyruvate reductase from choline-grown Arthrobacter 2B2

Fraction	Total Activity (m units)	Specific Activity (m units mg protein^{-1})
Crude extract	17145	406
0-30% $(NH_4)_2SO_4$	1542	367
30-40% $(NH_4)_2SO_4$	1734	141
40-50% $(NH_4)_2SO_4$	3468	281
50-60% $(NH_4)_2SO_4$	6942	1068
60-70% $(NH_4)_2SO_4$	0	0

The results presented in Chapter 7 demonstrate that the cell free extracts of Arthrobacter 2B2 grown on a medium containing choline as the sole source of carbon had both NADH and NADPH-linked hydroxypyruvate reductase activity, unlike methylamine-grown cells which had only NADPH-linked activity. The NADH-linked activity therefore appeared to have been produced as a response to growth on choline. A study of the enzyme responsible was carried out in order to determine its properties.

Initial purification procedure

The initial purification procedure utilised similar techniques to those used for the purification of the NADPH-linked enzyme from methylamine-grown cells.

Crude cell free extracts were produced by sonicating choline-grown cell paste in four volumes of 20 mM sodium potassium phosphate buffer at pH 7.0. The cell debris was removed by centrifugation and the supernatant was used as the cell free extract.

The cell free extract was raised to a level of 30% saturation with solid ammonium sulphate. The precipitated protein was then removed by centrifugation before carrying out sequential 10% ammonium sulphate fractionations to a level of 70% saturation. The results are shown in Table 18 and demonstrate that the 50% to 60% ammonium sulphate fraction gives a 2.6 fold purification, together with a yield of 48%. This fraction was next applied to a Sephadex G200 gel filtration column (22 x 2.6 cm) equilibrated with 20 mM sodium potassium phosphate buffer at pH 7.0. The extract was loaded onto the column and eluted with the equilibration buffer, active

fractions being detected using the continuous NADH-linked assay described in the Materials and Methods section. The active fractions were pooled and assayed for activity, the results showing that the purification factor had dropped dramatically to 0.39 and that the pooled Sephadex G200 fractions still retained NADPH-linked hydroxypyruvate reductase activity.

The results indicated that the NADH-linked enzyme was difficult to separate from the NADPH-linked one, and also that it loses considerable activity on application to a Sephadex G200 column. The gel filtration chromatography step was therefore dropped from the purification procedure; the ammonium sulphate fractionation was, however, retained.

The next procedures used were aimed primarily at the separation of the NADH and NADPH-linked enzymes. Initially an affinity column was employed. The material used in the column was 5 AMP-Sepharose 4B. This permits group specific separation of enzymes which require cofactors, common to which is the adenylic moiety 5 AMP. Such enzymes include those with NADH as a cofactor. The 50% to 60% ammonium sulphate fraction was desalted by dialysis for two hours against 100 volumes of 0.1 M sodium potassium phosphate buffer pH 7.0, before being loaded onto a column packed with 5 AMP-Sepharose 4B (5 x 1 cm) equilibrated with 0.1 M sodium potassium phosphate buffer pH 7.0. The column was washed initially with equilibration buffer to remove unbound protein before being subjected to a gradient of 0 to 1 mM NADH in equilibration buffer. The fractions were assayed for activity using the continuous NADH-linked assay described in the Materials and Methods section, the NADH-linked activity being found along

with NADPH-linked activity in the fractions containing unbound protein.

As the technique of affinity chromatography had been unsuccessful in separating the NADH and NADPH-linked activities, the next separation attempt utilised a DEAE cellulose ion exchange column. The 50% to 60% ammonium sulphate fraction was desalted by dialysis for two hours against 100 volumes of 0.1 M sodium potassium phosphate buffer pH 7.0. The dialysate was then loaded onto a DEAE cellulose ion exchange column equilibrated with dialysing buffer. The column was initially washed with equilibration buffer to remove unbound protein and then as no hydroxypyruvate reductase activity was detected, a gradient of 0 to 500 mM sodium chloride in equilibration buffer was applied to the column. NADH-linked hydroxypyruvate reductase activity was detected in fractions 45 to 60. When assays were carried out for the presence of NADPH-linked hydroxypyruvate reductase, the activity was detected in fractions 28 to 65. However, it was clear that the overall activity was present in two separate peaks (Figure 40). The first peak covered fractions 28 to 40, the second coincided with the NADH-linked activity and covered fractions 45 to 60. These results indicated that of the two hydroxypyruvate reductases present in choline-grown cells, one was strictly NADPH-linked, the other was a dual specificity enzyme being active with either NADH or NADPH.

The successful separation of the two hydroxypyruvate reductases relied on the use of Whatman DE52 pre-swollen DEAE cellulose. In order to improve the separation of the enzymes, the use of DEAE Sephacel was tried, this material being claimed to have better separating properties.

FIGURE 40 Separation of NADH and NADPH-linked hydroxypyruvate reductase from choline-grown Arthrobacter 2B2

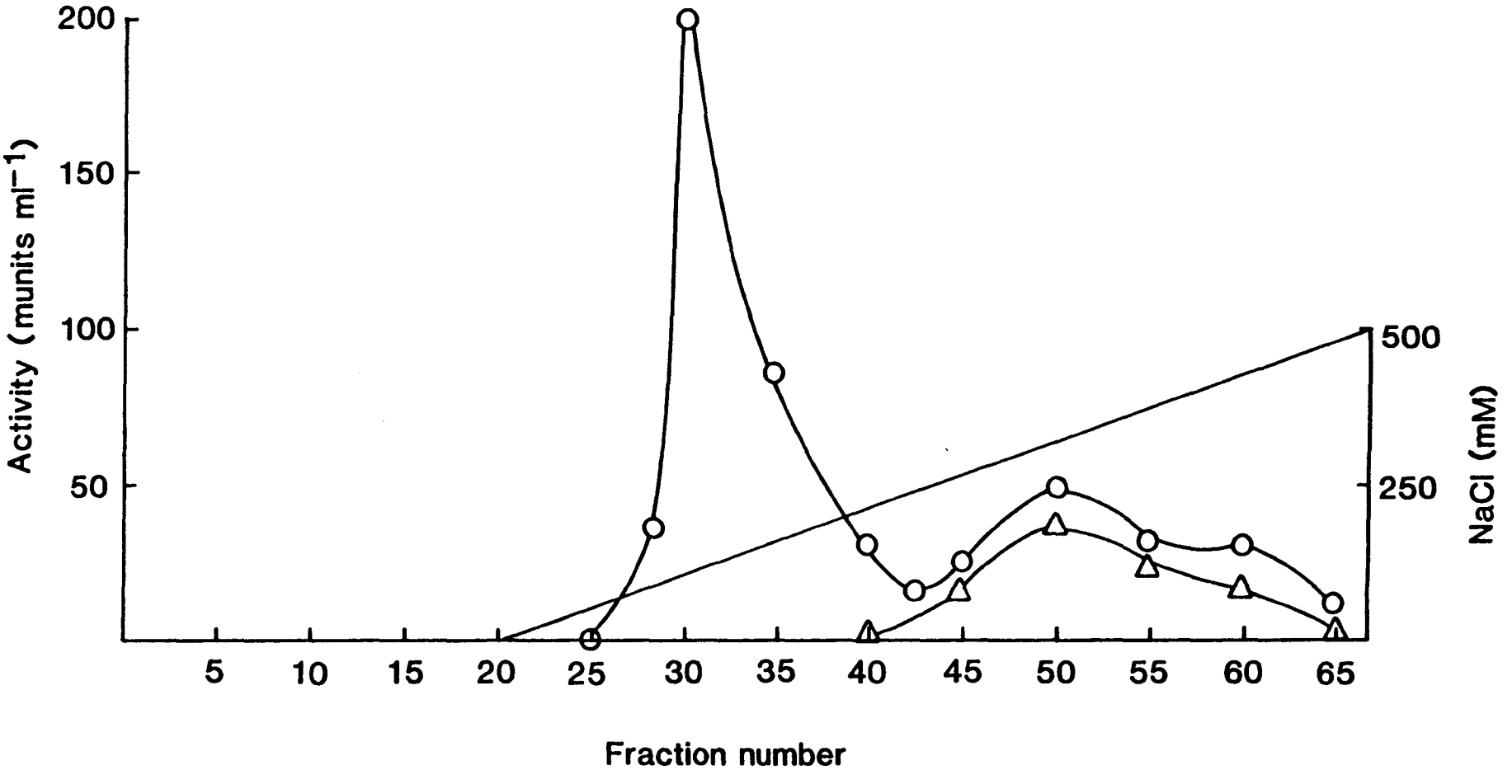
The separation was carried out by eluting a dialysed 50% to 60% ammonium sulphate fraction from a DEAE cellulose column using a 0 mM to 500 mM sodium chloride gradient.

Key:

○ NADPH-linked hydroxypyruvate reductase

△ NADH-linked hydroxypyruvate reductase

Fraction volume = 2.2 ml.


200
150
100
50
Activity (munits ml^{-1})
500
250
NaCl (mM)
5
10
15
20
25
30
35
40
45
50
55
60
65
Fraction number

Final separation procedure

(i) Production of crude cell free extract

The crude cell free extract was produced by resuspending 2 g of choline grown cell paste in 8 ml of 10 mM sodium potassium phosphate buffer pH 7.0 and sonicating as a single batch. The cell debris was removed by centrifugation at 38000 g for 20 minutes at 2°C. The pellet was discarded and the supernatant treated as the cell free extract.

(ii) Ammonium sulphate fractionation

The cell free extract was raised to a level of 50% saturation by the slow addition of solid ammonium sulphate and after equilibration for 30 minutes at 0°C, the precipitate was removed by centrifugation. The supernatant was then raised to 60% saturation with solid ammonium sulphate and the resulting precipitate was collected by centrifugation and resuspended in sonication buffer.

(iii) DEAE Sephacel ion exchange chromatography

The ammonium sulphate fraction produced in stage (ii) was applied to a DEAE Sephacel column equilibrated with 10 mM sodium potassium phosphate buffer pH 7.0. The unbound protein was removed from the column by washing with equilibration buffer. Following this a gradient of 0 to 500 mM sodium chloride in equilibration buffer was applied to the column to elute bound proteins.

Both NADH and NADPH-linked hydroxypyruvate reductase activities remained bound to the column after the initial wash, requiring a sodium chloride gradient in order to elute them. NADH-linked activity was detected in fractions 50 to 58; NADPH activity was found in fractions 41 to 58, two peaks being apparent, the first spanning fractions 41 to 48, and

the second fractions 50 to 58.

The results of the ion exchange separation are shown in Figure 41. They confirm the previous data that there appeared to be two hydroxypyruvate reductases present in cell free extracts of choline-grown cells, one being strictly NADPH-linked, the other being active with both NADH and NADPH.

Although there was a separation of two enzymes on DEAE cellulose columns, it was considered a possibility that the second peak of NADPH-linked activity could be due to a carry-over of activity from the first peak, thus indicating that the second peak was an NADH-linked enzyme and not a dual NADH/NADPH-linked enzyme. In order to determine whether this was correct the decay rates of the two peaks were considered. The two sets of pooled fractions collected from the DEAE cellulose column were stored at $4^{o}C$ and samples were withdrawn daily from each for hydroxypyruvate reductase assay. The pooled fractions from the first peak were assayed for NADPH-linked activity and those from the second peak were assayed for NADH and NADPH-linked activity, the results being shown in Figure 42. They show that both NADH and NADPH-linked activities from the second peak decay at a similar rate, whilst the NADPH-linked activity from the first peak decays far less rapidly. This indicates that the NADH and NADPH-linked activities in the second peak are situated in the same enzyme, which is quite unstable. The NADPH-linked activity in the first peak appears relatively stable compared to that in the second.

From the results of the separation, the ratio of NADPH : NADH-linked activities in the NADH/NADPH-linked hydroxypyruvate reductase was calculated to be 1.06 : 1. Using this figure it was possible to calculate the amount of

FIGURE 41 Final separation of hydroxypyruvate reductase from choline-grown Arthrobacter 2B2

The separation was carried out by eluting a dialysed 50% to 60% ammonium sulphate fraction from a DEAE Sephacel column using a 0 mM to 500 mM sodium chloride gradient.

Key:

○	E280 nm
△	NADPH-linked hydroxypyruvate reductase
●	NADH-linked hydroxypyruvate reductase

Fraction volume = 4.6 ml.

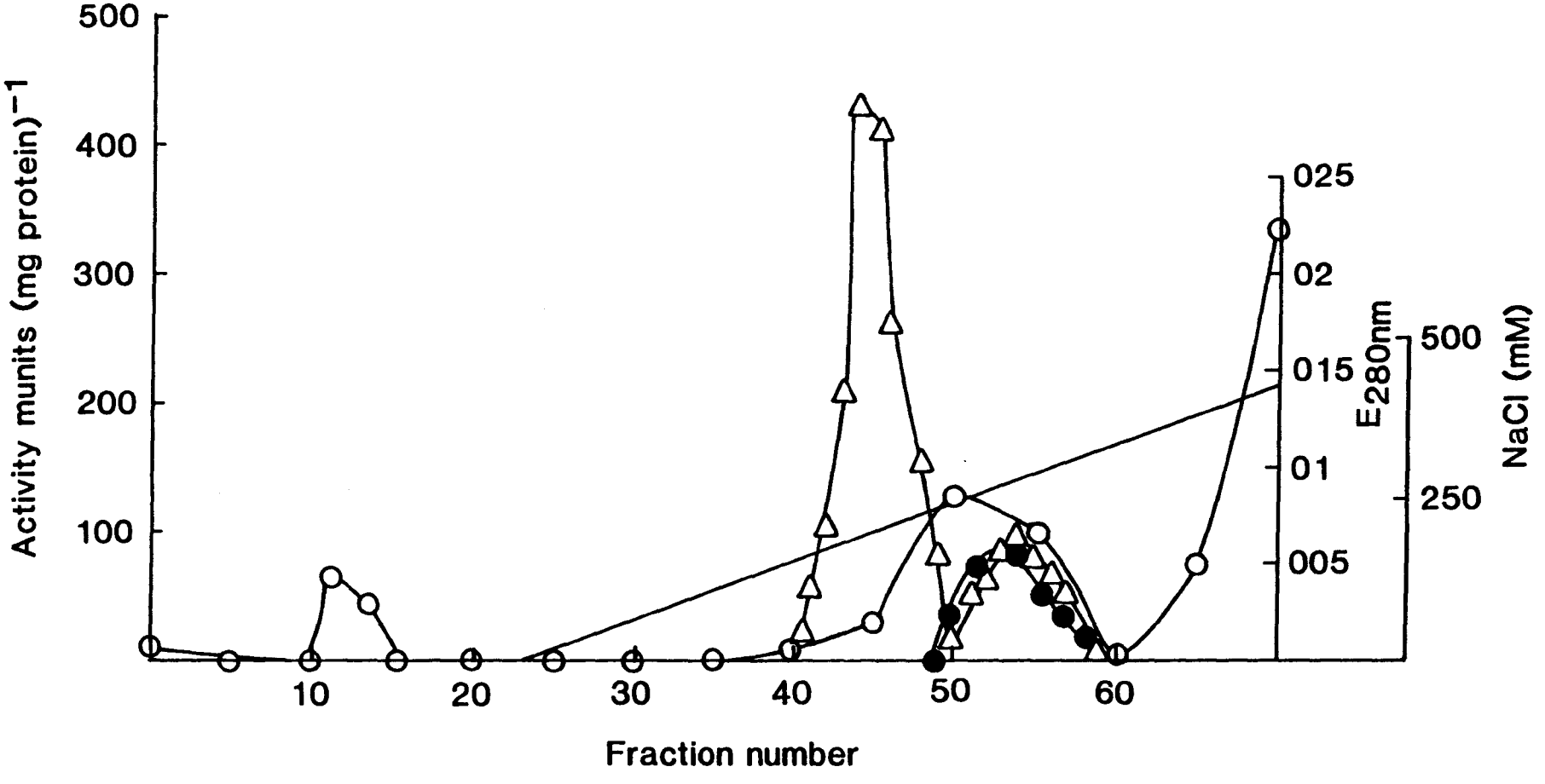

Activity munits (mg protein)$^{-1}$
500
400
300
200
100
10
20
30
40
50
60
Fraction number
025
02
015
01
005
E_{280nm}
500
250
NaCl (mM)

FIGURE 42 Decay curves of the separated NADPH and NADH/NADPH-linked hydroxypyruvate reductases from choline-grown Arthrobacter 2B2

The separated enzymes were stored at 4°C and assayed for activity each day.

Key:

○ NADPH-linked activity in the NADPH-linked enzyme

△ NADPH-linked activity in the NADH/NADPH-linked enzyme

□ NADH-linked activity in the NADH/NADPH-linked enzyme

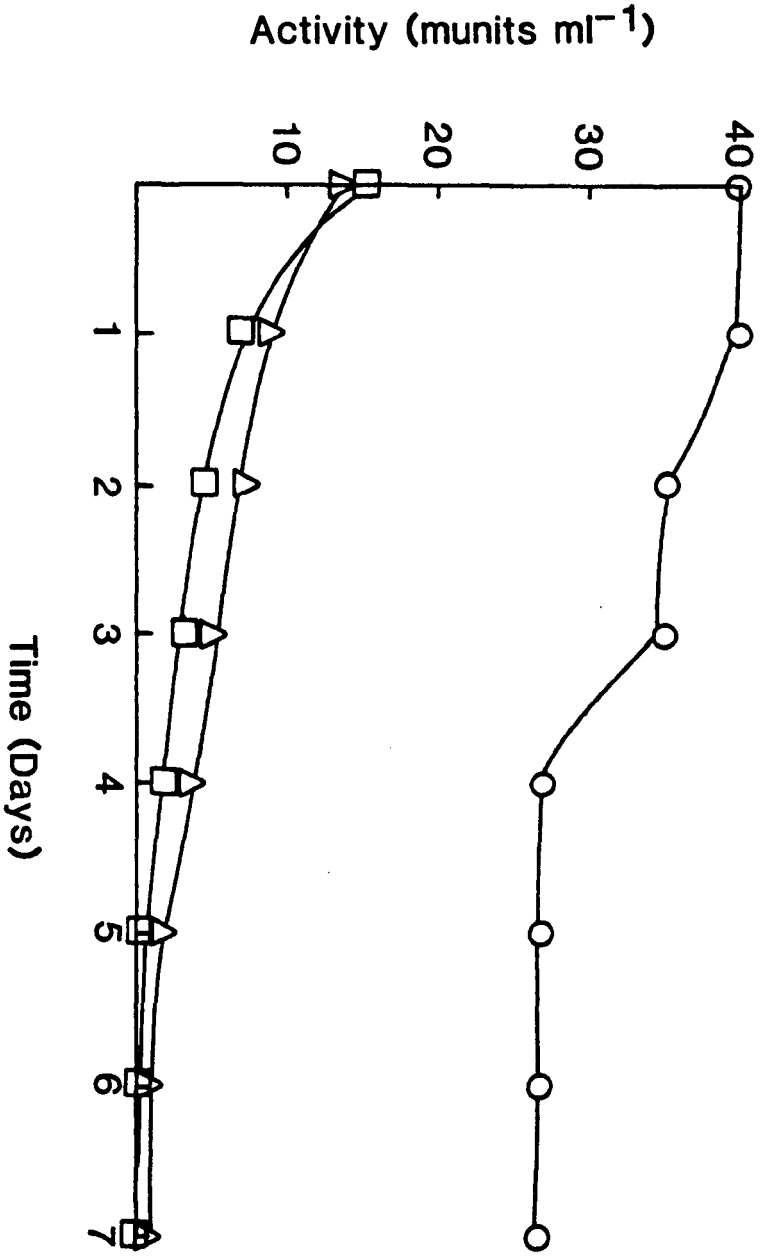

Activity (munits ml^{-1})
10
20
30
40
Time (Days)
1
2
3
4
5
6
7

TABLE 19 The purification of NADH/NADPH-linked hydroxypyruvate reductase from choline-grown Arthrobacter 2B2

Fraction	Volume ml	Protein mg	NADH Activity Units	NADH Sp Activity Units mg^{-1}	NADPH Activity Units	NADPH Sp Activity Units mg^{-1}	Purification fold	Yield %
Crude cell free extract	7.7	38.5	7.1	0.18	7.5	0.19	1	100
$(NH_4)_2SO_4$ fractionation	7.8	5.85	2.8	0.48	2.9	0.49	2.5	38
DEAE cellulose column	38.4	2.0	2.0	1.0	2.1	1.0	5.2	28

the total NADPH-linked activity recorded in any assay using unseparated enzymes which was due to the single NADPH-linked enzyme. The calculation was carried out by multiplying the activity recorded in the NADH-linked assay by 1.06. This gave the NADPH-linked activity which was due to the NADH/NADPH-linked enzyme. This figure could then be subtracted from the total NADPH-linked activity recorded in the continuous NADPH-linked assay, thus leaving the NADPH-linked activity which was due to the strict NADPH-linked enzyme. By carrying out this calculation for the crude cell free extract, and the 50% to 60% ammonium sulphate fractionation, it was possible to construct a full purification table for both enzymes subjected to the final separation procedure.

Properties of the NADH/NADPH-linked enzyme

Table 19 shows the results obtained for the NADH/NADPH-linked enzyme. It was purified 5.2 fold with a yield of 28%. There was no evidence for the presence of any NADH or NADPH-oxidase in the purified enzyme. Samples of the partially purified enzyme were used in all of the following determinations.

Stability

As Figure 42 demonstrates, the enzyme was relatively unstable when stored at $4^{o}C$, losing 50% of its activity in one day and all activity within five days. It was extremely unstable on passage through a Sephadex G200 column, making it impossible to use gel filtration as a purification step. Activity was retained when the enzyme was frozen at $-15^{o}C$, enzyme being stored in this way remaining active for up to two months.

TABLE 20 Effect of anions on the NADH/NADPH-linked hydroxypyruvate reductase from choline-grown Arthrobacter 2B2

A sample of the enzyme was placed in both the NADH and NADPH-linked hydroxypyruvate reductase assays, which had been supplemented by the addition of the appropriate anion. The results are expressed as the percentage inhibition of the rate recorded in an assay omitting the anions.

Anion	Concentration mM	Inhibition in NADH-linked assay (%)	Inhibition in NADPH-linked assay (%)
None	-	0	0
Citrate	30	87	60
Bromide	33	0	0
Iodide	33	0	0

Effects of anions

The effects of sodium iodide, sodium bromide and sodium citrate were tested by including the appropriate compound in: (i) the continuous NADH-linked hydroxypyruvate reductase assay, and (ii) the continuous NADPH-linked assay, both detailed in the Materials and Methods section. The results are shown in Table 20, the inhibition being expressed as a percentage of the rate measured in an assay which had no anion added.

The results show that both iodide and bromide had no effect on the activity shown in the assay, while citrate greatly inhibited it. An 87% inhibition was recorded in the NADH-linked assay, and a 60% inhibition in the NADPH-linked assay.

The molecular weight determination of NADH/NADPH-linked hydroxypyruvate reductase

The native molecular weight of the enzyme was determined by Sephadex G200 gel filtration chromatography. The column material was pre-swollen in 10 mM sodium potassium phosphate buffer pH 7.0, and a column (71 x 25 cm) was packed and maintained at $4^{o}C$. The column was calibrated by running proteins of a known molecular weight through it. The standard proteins used were β galactosidase (M. Wt. 510000-530000), catalase (M. Wt. 23000-25000), aldolase (M. Wt. 158000), lactate dehydrogenase (M. Wt. 130000-140000), bovine serum albumin (M. Wt. 65000-70000), ovalbumin (M. Wt. 43000), and cytochrome c (M. Wt. 12400). They were loaded onto the column and eluted with equilibration buffer which was pumped through at a flow rate of 10 ml h^{-1}. The standard proteins

were detected either by their enzymic assay as described in the Materials and Methods section, or by their absorbance (cytochrome c at 412 nm, bovine serum albumin and ovalbumin at 280 nm).

As noted previously the enzyme was very unstable on passage through Sephadex G200. However, the column used in this determination was not intended to be a purification step; only enough activity was required to be eluted from the column to allow the detection of the elution peak and thus the elution volume. The hydroxypyruvate reductase activity in the eluant was identified using the continuous NADH-linked and NADPH-linked assays described previously.

The elution profile of the Sephadex G200 column is shown in Figure 43 and clearly shows the co-elution of NADH- and NADPH-activities. The molecular weight determination of NADH/NADPH-linked hydroxypyruvate reductase is shown in Figure 44. The molecular weight of the separated enzyme was determined to be 214000.

The determination of the Michaelis constants of NADH/NADPH-linked hydroxypyruvate reductase

The Km and Vmax values were determined by the application of the direct linear plot of Eisenthal and Cornish-Bowden (1974). Constants were calculated for NADH and NADPH by including varying quantities of the pyridine nucleotides in the continuous assay containing a constant amount of the separated enzyme and 4 mM hydroxypyruvate. The Km for hydroxypyruvate was calculated once using the NADH-linked assay and again replacing the NADH with NADPH. Both NADH and NADPH were present in their respective assays at 0.4 mM

FIGURE 43 Elution of NADH/NADPH hydroxypyruvate reductase of choline grown Arthrobacter 2B2 on a Sephadex G200 gel filtration column.

A sample of separated NADH/NADPH-linked hydroxypyruvate reductase was loaded onto a Sephadex G200 column (71 x 2.5 cm) as a part of the determination of its molecular weight. The profile shows the co-elution of NADH and NADPH-linked activities.

Key:

○ NADH-linked hydroxypyruvate reductase

△ NADPH-linked hydroxypyruvate reductase

Fraction volume = 3 ml.

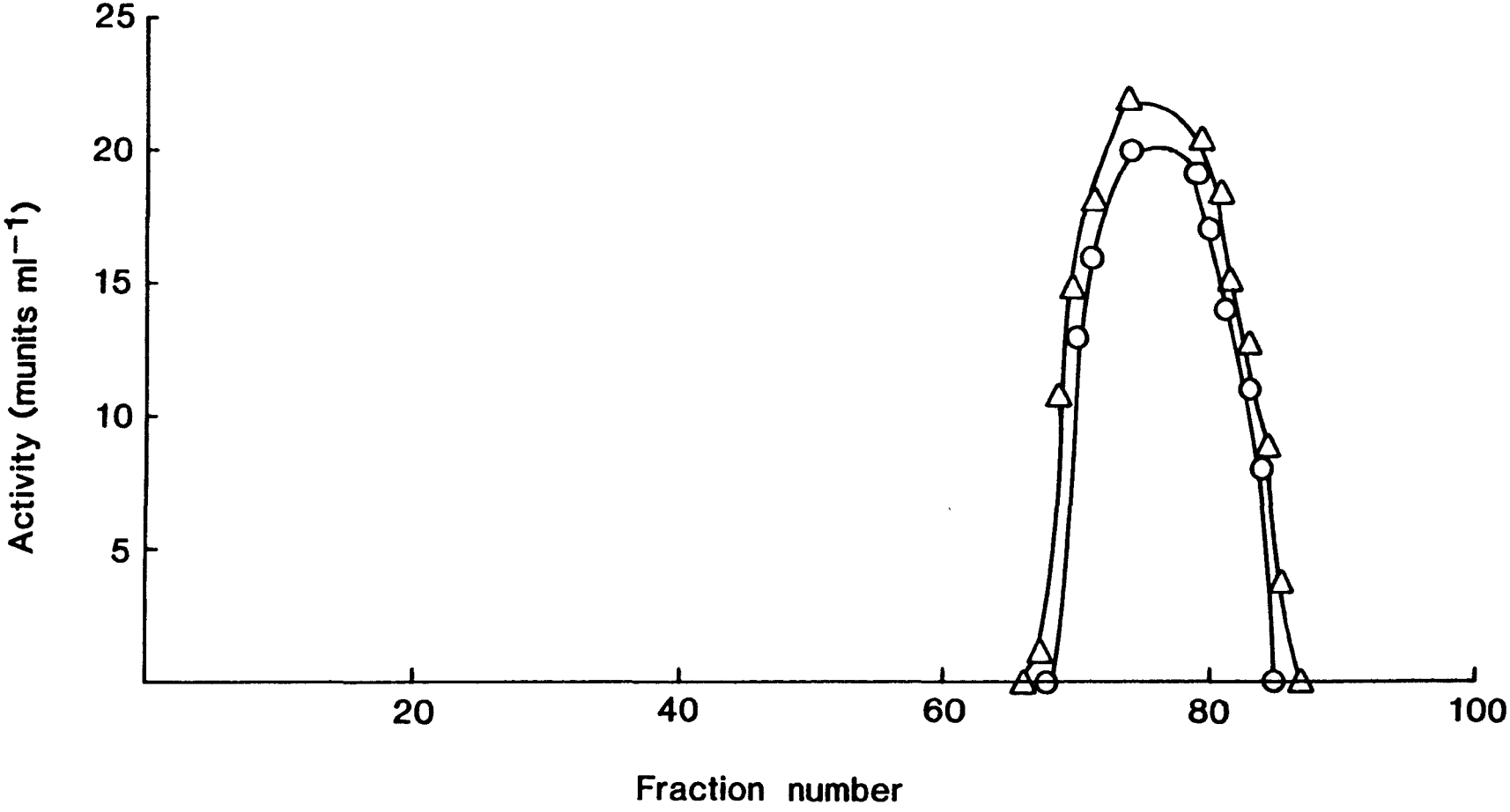

25
20
15
10
5
Activity (munits ml⁻¹)
20
40
60
80
100
Fraction number

FIGURE 44 Molecular weight determination of NADH/NADPH-linked hydroxypyruvate reductase from choline-grown Arthrobacter 2B2

The enzyme was eluted from a Sephadex G200 column (71 x 25 cm) equilibrated with 20 mM sodium potassium phosphate buffer pH 7.0. The column was calibrated with cytochrome c (A), ovalbumin (B), bovine serum albumin (C), lactate dehydrogenase (D), aldolase (E), catalase (F), and β galactosidase (G). NADH/NADPH-linked hydroxypyruvate reductase (O) was eluted from the column at a fraction volume corresponding to a molecular weight of 214000. The volume of each fraction was 3 ml and the flow rate 10 ml h^{-1}.

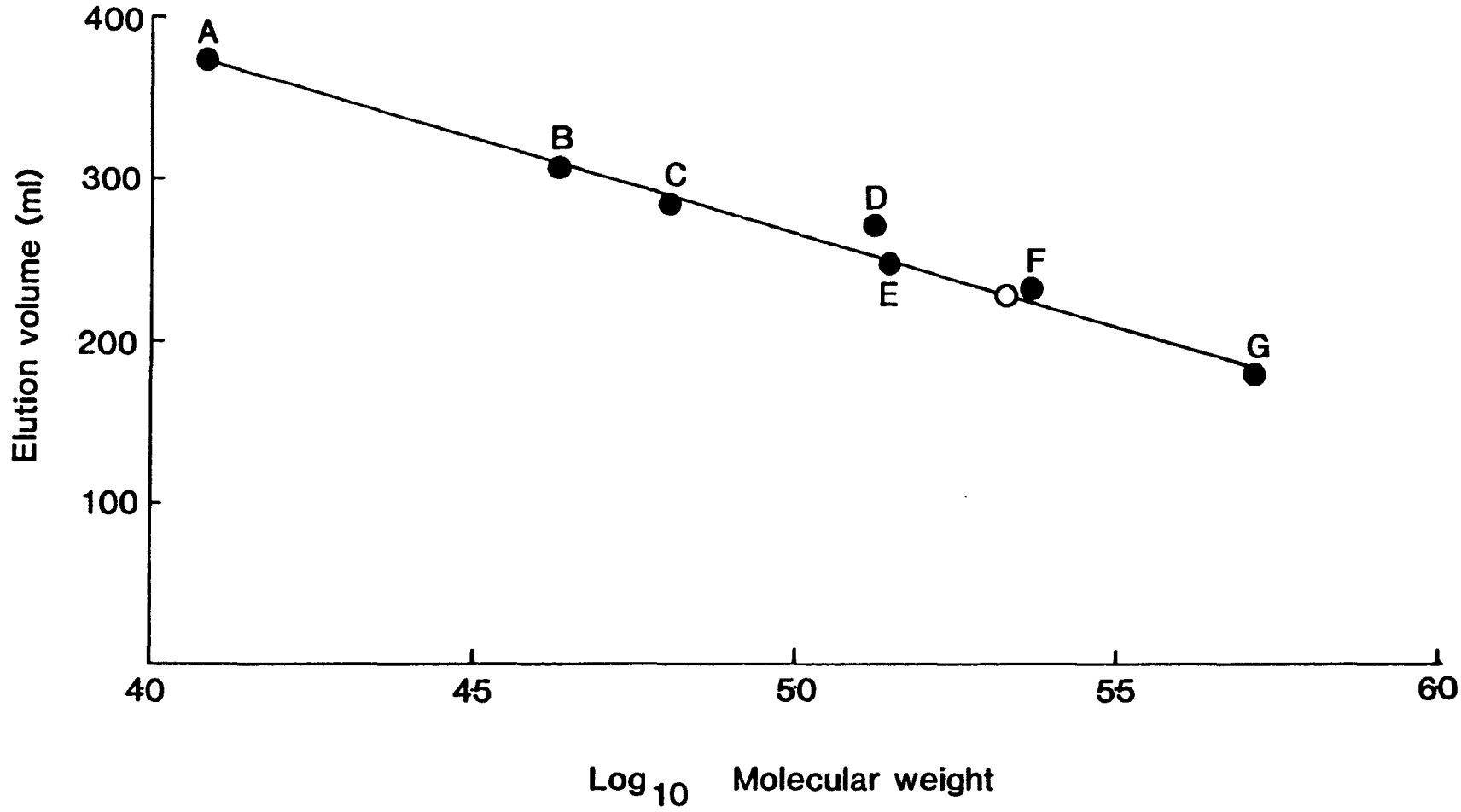

400
300
200
100
Elution volume (ml)
A
B
C
D
E
F
G
40
45
50
55
60
Log 10 Molecular weight

TABLE 2: The purification of NADPH-linked hydroxypyruvate reductase from choline-grown Arthrobacter 2B2

Fraction	Volume ml	Protein mg	Activity units	Specific Activity units mg^{-1}	Purification fold	Yield %
Crude cell free extract	7.7	38.5	10.0	0.26	1	100
$(NH_4)_2SO_4$ fractionation	7.8	5.85	8.8	1.51	5.8	88
DEAE cellulose column	31	0.84	7.0	8.45	32.5	70

concentrations, a constant amount of enzyme being added to each assay. By varying the concentration of hydroxypyruvate in each assay, the kinetic constants of the enzyme were established.

The results indicated that the apparent Km and Vmax values for NADH were 2.1 x 10^{-5} M and 2.7 units (mg protein)$^{-1}$ respectively, the values for NADPH being 1.1 x 10^{-5} M and 3.5 units (mg protein)$^{-1}$ respectively. The Km and Vmax for hydroxypyruvate were 5.7 x 10^{-5} M and 4.0 units (mg protein)$^{-1}$ using the NADH-linked assay, and 3.0 x 10^{-5} M and 7.5 units (mg protein)$^{-1}$ using the NADPH-linked assay.

Properties of the NADPH-linked enzyme

The purification and properties of the single NADPH-linked hydroxypyruvate reductase produced during growth on choline was next studied.

Table 21 shows the purification results obtained. It was purified 32.5 fold with a yield of 70%. Samples of the partially purified enzyme were used in all of the following determinations.

Stability

The enzyme was relatively stable at 4°C (Figure 42), losing only 33% of its activity after storage for seven days at this temperature. The enzyme was also stable when stored frozen at -15°C, and was not inactivated by passage through a Sephadex G200 column, unlike the NADH/NADPH-linked enzyme.

Effects of anions

The effects were tested by placing sodium iodide,

TABLE 22 Effect of anions on the NADPH-linked hydroxypyruvate reductase from choline-grown Arthrobacter 2B2

A quantity of the purified enzyme was placed in an NADPH-linked hydroxypyruvate reductase assay mixture which had been supplemented by the addition of an appropriate anion. The results are expressed as the percentage inhibition caused by the anion when compared to an assay lacking that anion.

Anion	Concentration mM	Inhibition %
None	-	0
Citrate	30	5
Bromide	33	26
Iodide	33	39

sodium bromide, or sodium citrate in the continuous assay mixture described in the Materials and Methods section. The activity in such assays was recorded and compared to that present in an assay which contained no anion, the results being expressed as the percentage inhibition of activity when compared to the latter assay.

The results are given in Table 22 and show that citrate inhibits the enzyme by only 3%, whilst the inhibition by bromide and iodide is greater at levels of 26% and 29% respectively.

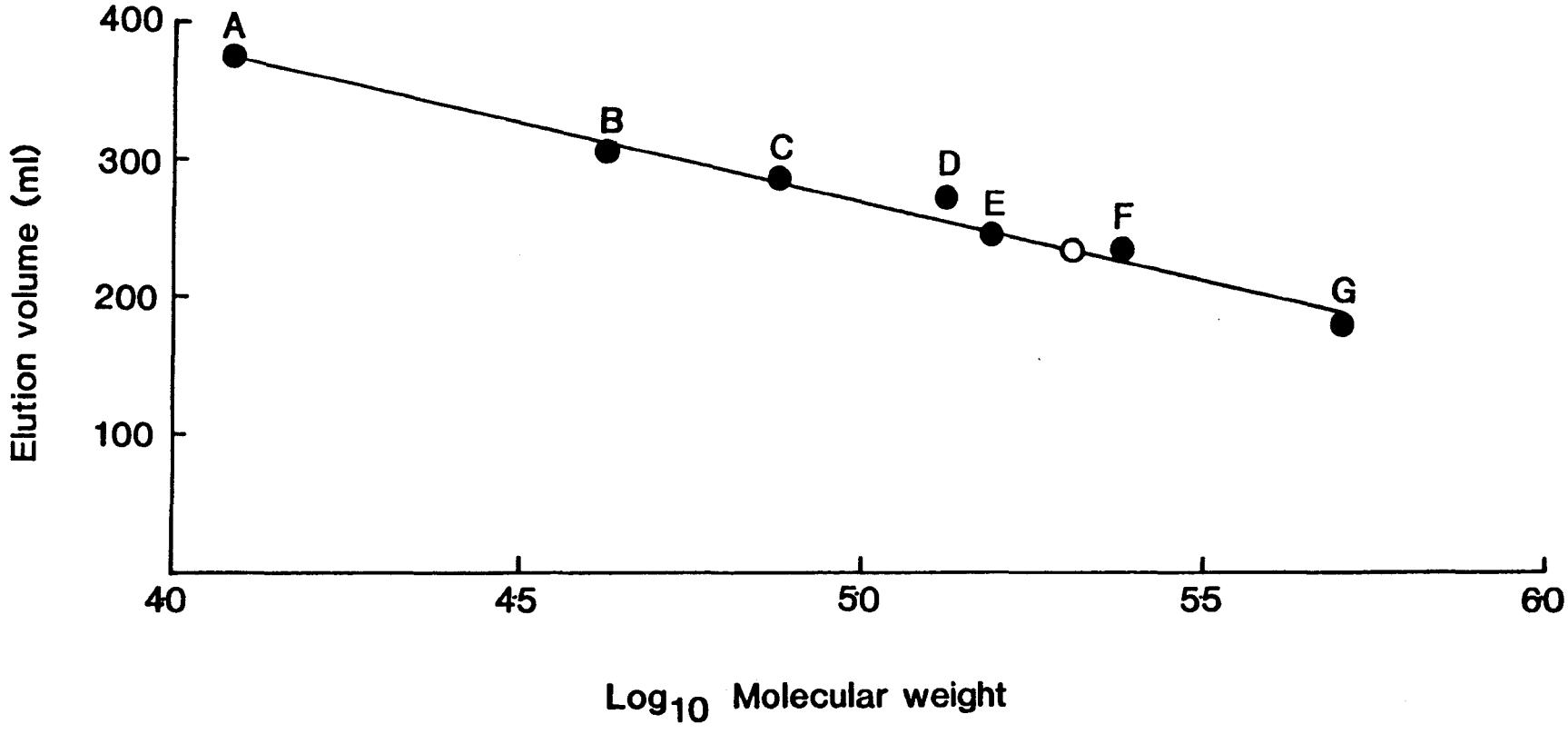
Determination of the molecular weight of the NADPH-linked hydroxypyruvate reductase

The molecular weight was determined by the application of Sephadex G200 gel filtration chromatography. The column (71 x 2.5 cm) was equilibrated with 20 mM sodium potassium phosphate buffer pH 7.0. It was maintained at $4^{o}C$ and eluted with equilibration buffer which was pumped through at a flow rate of 10 ml h^{-1}. The column was calibrated with the following proteins of known molecular weight: β galactosidase (M. Wt. 510000-530000), catalase (M. Wt. 230000-250000), aldolase (M. Wt. 158000), lactate dehydrogenase (M. Wt. 130000-140000), bovine serum albumin (M. Wt. 65000-70000), ovalbumin (M. Wt. 43000), and cytochrome c (M. Wt. 12400). After elution of the calibration proteins, 2.2 units of purified NADPH-linked hydroxypyruvate reductase were loaded onto the column and eluted with equilibration buffer, pumped through the column at a flow rate of 10 ml h^{-1}.

The results are shown in Figure 45. The molecular weight was determined to be 204200.

FIGURE 45 Molecular weight determination of NADPH-linked hydroxypyruvate reductase from choline-grown Arthrobacter 2B2

The enzyme was eluted from a Sephadex G200 column (71 x 2.5 cm) using 20 mM sodium potassium phosphate buffer pH 7.0. The column was calibrated with cytochrome c (A), ovalbumin (B), bovine serum albumin (C), lactate dehydrogenase (D), aldolase (E), catalase (F), and β galactosidase (G). NADPH-linked hydroxypyruvate reductase (O) was eluted from the column at a volume corresponding to a molecular weight of 204200. The volume of each fraction was 3 ml and the flow rate was 10 ml h^{-1}.


400
300
200
100
A
B
C
D
E
F
G
40
45
50
55
60
Elution volume (ml)
Log10 Molecular weight

The determination of the Michaelis constants of NADPH-linked hydroxypyruvate reductase

The apparent Km and Vmax values were calculated by the method of Eisenthal and Cornish-Bowden (1974). The constants for NADPH were established by placing various concentrations of NADPH into an assay mixture containing a constant amount of enzyme and 2 mM hydroxypyruvate. The values for hydroxypyruvate were determined in a similar way but varying the concentration of hydroxypyruvate in assays containing a constant amount of enzyme and 0.4 mM NADPH.

The apparent Km and Vmax for NADPH were 3.5×10^{-5} M and 6.2 units $(\text{mg protein})^{-1}$ respectively, and those for hydroxypyruvate were 2.3×10^{-4} M and 3.5 units $(\text{mg protein})^{-1}$ respectively.

Summary of results

By employing DEAE cellulose ion exchange chromatography a separation of two hydroxypyruvate reductase enzymes from crude cell free extracts of choline grown cells was successfully obtained. The separation procedure proved one enzyme to be strictly NADPH-linked and the second to have a dual specificity for NADPH or NADH, the ratio of activity between NADPH and NADH in the latter enzyme being 1.06 : 1.

The procedure adopted provided a purification as well as a separation for both enzymes. The NADPH-specific enzyme was purified 32.5 fold with a yield of 70%, the NADH/NADPH-linked enzyme being purified 5.2 fold with a yield of 28%. The low figures obtained for the NADH/NADPH hydroxypyruvate reductase possibly reflect the instability of the enzyme. This was previously noted when the enzyme was applied to gel

filtration columns and confirmed when studies of the rate of enzyme decay at $4^{o}C$ were carried out.

A consideration of the properties of the two enzymes shows them to be differing in many respects. As mentioned previously the NADH/NADPH-linked enzyme was fairly unstable while the strict NADPH-linked enzyme was not. The NADPH-linked enzyme had a Km value for hydroxypyruvate which was a factor of ten greater than the corresponding value for the dual specificity enzyme, using either NADH or NADPH. The two enzymes also behaved differently when anions were included in their respective assays. The NADPH-linked hydroxypyruvate reductase was only slightly inhibited by citrate, whilst bromide and iodide inhibited to a much greater extent. However, bromide and iodide had no effect on the dual specificity enzyme, but citrate greatly inhibited it.

A comparison of the results obtained using the strict NADPH-linked enzyme purified from choline-grown cells, with those established from the NADPH-linked enzyme purified previously from methylamine-grown cells, shows them to be very similar. Their response to anions was the same and the apparent Km and molecular weight values were very similar. These results lead to the conclusion that the NADPH-linked activities produced during growth on methylamine and choline were due to the same enzyme.

CHAPTER 10

The Independent Synthesis of Two Hydroxypyruvate Reductases in Arthrobacter 2B2 Growing on Choline

Whilst in the process of separating, purifying and studying the characteristics of the two hydroxypyruvate reductases produced in cells grown on a medium containing choline as the sole source of carbon, it was noted that the relative amounts of the strict NADPH-linked enzyme and the NADH/NADPH-linked enzyme differed depending on the cell paste used to produce the cell free extract. There were usually appreciable but variable levels of NADH/NADPH-linked hydroxypyruvate reductase activity; however, in the case of some cell free extracts, there was almost a complete lack of strict NADPH-linked activity. A possible reason for this was considered to be that the two enzymes were produced at different stages during the growth cycle of Arthrobacter 2B2 when growing on choline. The result of this would be that the relative amounts of the two hydroxypyruvate reductases present in cell free extracts of choline-grown cells would depend on the exact stage of growth reached by the parent culture before the cells were harvested.

In order to test the hypothesis it was necessary to examine the production of the two enzymes during batch culture of the organism, using a medium containing choline as the sole source of carbon. A ten litre batch culture was established in a fermenter using the medium described in the Materials and Methods section supplemented with 50 mM choline. It was inoculated with a 700 ml starter culture which had been grown over a 24 hour period. Sampling was initiated after 12 hours growth by the withdrawal of 500 ml of the culture. The cells were removed by centrifugation at 6300 g for 15 minutes at 2°C and a crude cell free extract was prepared by sonication of 1 g wet weight of cells in 4 ml of 10 mM sodium potassium phosphate buffer at pH 7.0. The cell debris was removed by

centrifugation at 38000 g for 20 minutes at 2°C and the supernatant was treated as the crude cell free extract and used for the assay of the two hydroxypyruvate reductases.

Further samples were taken from the fermenter at three hour intervals until the culture had reached the stationary phase of growth as determined by its optical density at 610 nm. Crude cell free extracts of each sample were prepared by treatment in a similar manner to that described for the first sample. The extracts were then assayed for hydroxypyruvate reductase activity by (1) the continuous NADH-linked assay, and (2) the continuous NADPH-linked assay, both described in the Materials and Methods section. As the ratio of NADPH : NADH-linked activity in the NADH/NADPH-linked enzyme was known to be 1.06 : 1 (see Chapter 9), then the NADPH-linked activity due to that enzyme could be calculated by multiplying the NADH-linked activity by 1.06. By subtracting that value from the total NADPH-linked activity recorded in the NADPH-linked assay, the NADPH-linked activity which was due to the strictly NADPH-specific enzyme could be determined. This calculation was carried out for all of the samples withdrawn from the culture and the results are shown in Figure 46.

The results clearly show that during the exponential phase of growth the cells contain high levels of the NADH/NADPH-linked enzyme, then in mid to late exponential phase and continuing on into stationary phase the level of this enzyme is decreased and the activity of the strict NADPH-linked enzyme increases.

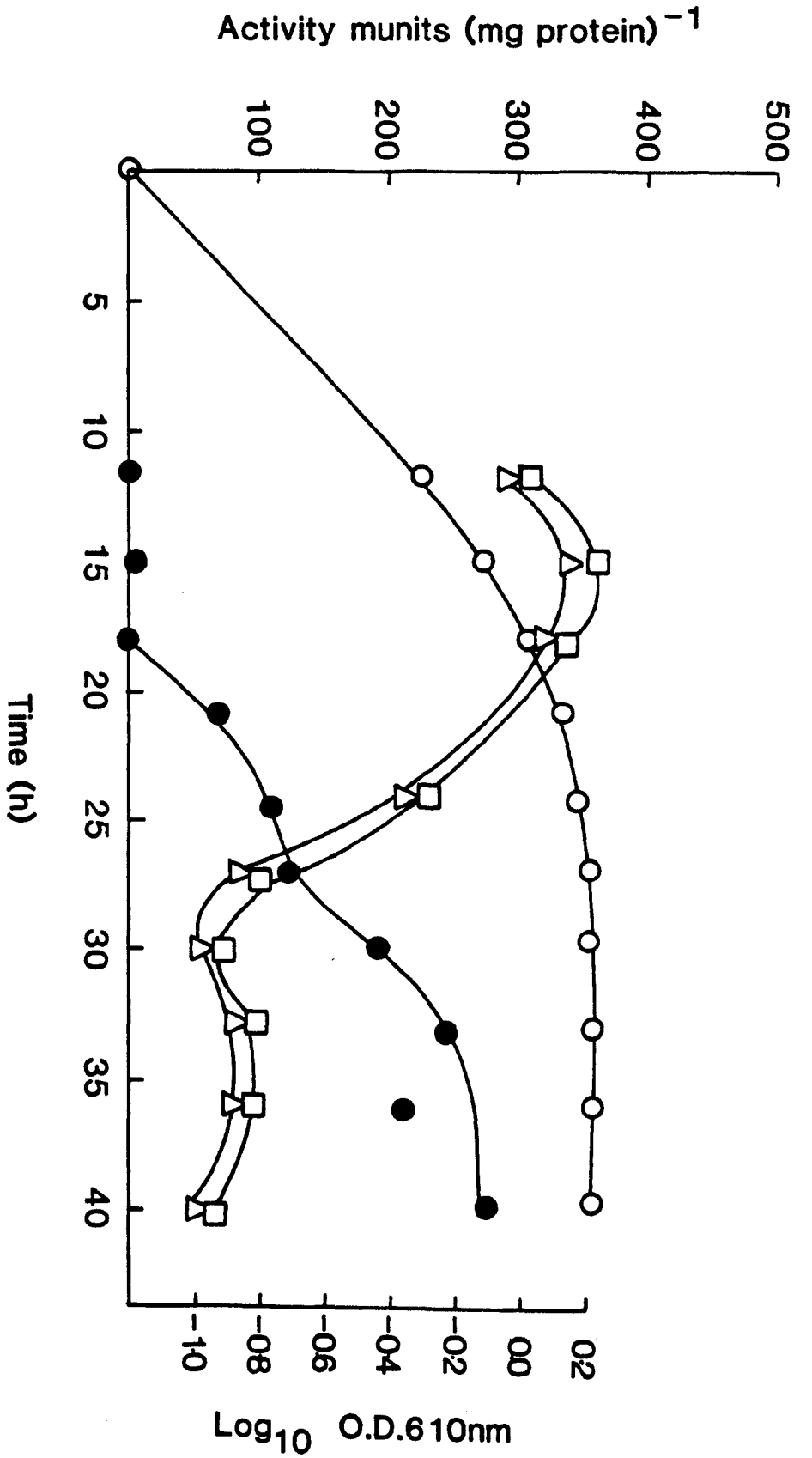
It was initially considered a possibility that this repression and induction of the two enzymes could have been

FIGURE 46 Synthesis of two hydroxypyruvate reductases during the growth of Arthrobacter 2B2 on choline

A ten litre batch culture was inoculated and samples withdrawn at timed intervals. Cell free extracts were produced from each sample and these were assayed for the presence of NADH and NADPH-linked hydroxypyruvate reductase activities.

Key:

O	Log_{10} O.D.$_{610nm}$	
Δ	NADH-linked hydroxypyruvate reductase	NADH/NADPH-linked enzyme
□	NADPH-linked hydroxypyruvate reductase	NADH/NADPH-linked enzyme
●	NADPH-linked hydroxypyruvate reductase	


Activity munits (mg protein)$^{-1}$
100
200
300
400
500
Time (h)
5
10
15
20
25
30
35
40
-10
-08
-06
-04
-02
00
02
Log$_{10}$ O.D.610nm

due to a build up of one of the breakdown products of choline metabolism. It was envisaged that choline was being oxidised to one of its breakdown intermediates and this was building up in the culture fluid until the choline had been consumed. The organism could then further oxidise the intermediate in order to continue growth. In order to test the theory it was necessary to be able to measure levels of choline and some of its oxidation products. Quantitative assays were therefore developed for choline, betaine and sarcosine. They relied on the use of thin layer chromatography and are described in the Materials and Methods section.

The next experiment was a repeat of the large scale batch culture carried out previously. A sample of 500 ml was withdrawn from the fermenter vessel 3 hours after inoculation. The cells were sedimented by centrifugation at 6300 g for 15 minutes at 2°C. The culture fluid was decanted and this together with the cell paste was stored frozen at -15°C in readiness for assay. Further samples were withdrawn from the culture vessel at three hour intervals until stationary phase had been established. The culture fluid samples were tested directly for choline, betaine and sarcosine using the methods described in the Materials and Methods section. Crude cell free extracts were produced from the cell pastes by sonicating 1 g of paste in 4 ml of 10 mM sodium potassium phosphate buffer at pH 7.0. After sonication the cell debris was removed by centrifugation at 38000 g for 20 minutes at 2°C, the supernatant being used as the cell free extract. Initially the extracts were assayed for the presence of NADH and NADPH-linked hydroxypyruvate reductase activities using the assay procedures described in the Materials and Methods section. From the results obtained the activities of NADH/

NADPH-linked enzyme, and the strictly NADPH-linked enzyme were determined using the calculation method described previously. After assaying for hydroxypyruvate reductase the cell free extracts were brought to a level of 10% (w/v) with respect to trichloroacetic acid by the addition of a known volume of 50% (w/v) trichloracetic acid. This treatment precipitated the protein in the cell free extract and this was removed by centrifugation, the resultant deproteinised cell free extract being assayed for the presence of choline, betaine and sarcosine.

The results of all the assays are displayed in Figure 47. The assays for betaine and sarcosine in the culture fluid were negative in all of the samples tested, as were assays for betaine in the deproteinised cell free extract. The results of the sarcosine assay on the deproteinised cell free extract were inconclusive. The developing reagent used in the assay was ninhydrin. This reacts with primary amines and thus amino acids show up. The developed thin layer chromatography plates exhibited a smear of colour along the running track with no identifiable spots, thus showing the samples to contain a wide variety of primary amines. The results of the choline assay on the culture fluid showed that there was an initial sharp decrease in choline within the first eight hours of growth. This then levelled out to give a more gradual decrease which continued into the stationary growth phase. The enzyme assay results are similar to those obtained previously. There was initially quite a high level of strictly NADPH-linked hydroxypyruvate reductase and reduced levels of the NADH/NADPH-linked enzyme. As the culture moved into exponential phase the levels of the NADH/NADPH-

FIGURE 47 Synthesis of hydroxypyruvate reductases and the oxidation of choline during the growth of Arthrobacter 2B2 in batch culture using choline as a carbon source

Samples were withdrawn from a ten litre batch culture at timed intervals. They were assayed for NADH and NADPH-linked hydroxypyruvate reductase activity and for the presence of choline.

Key:

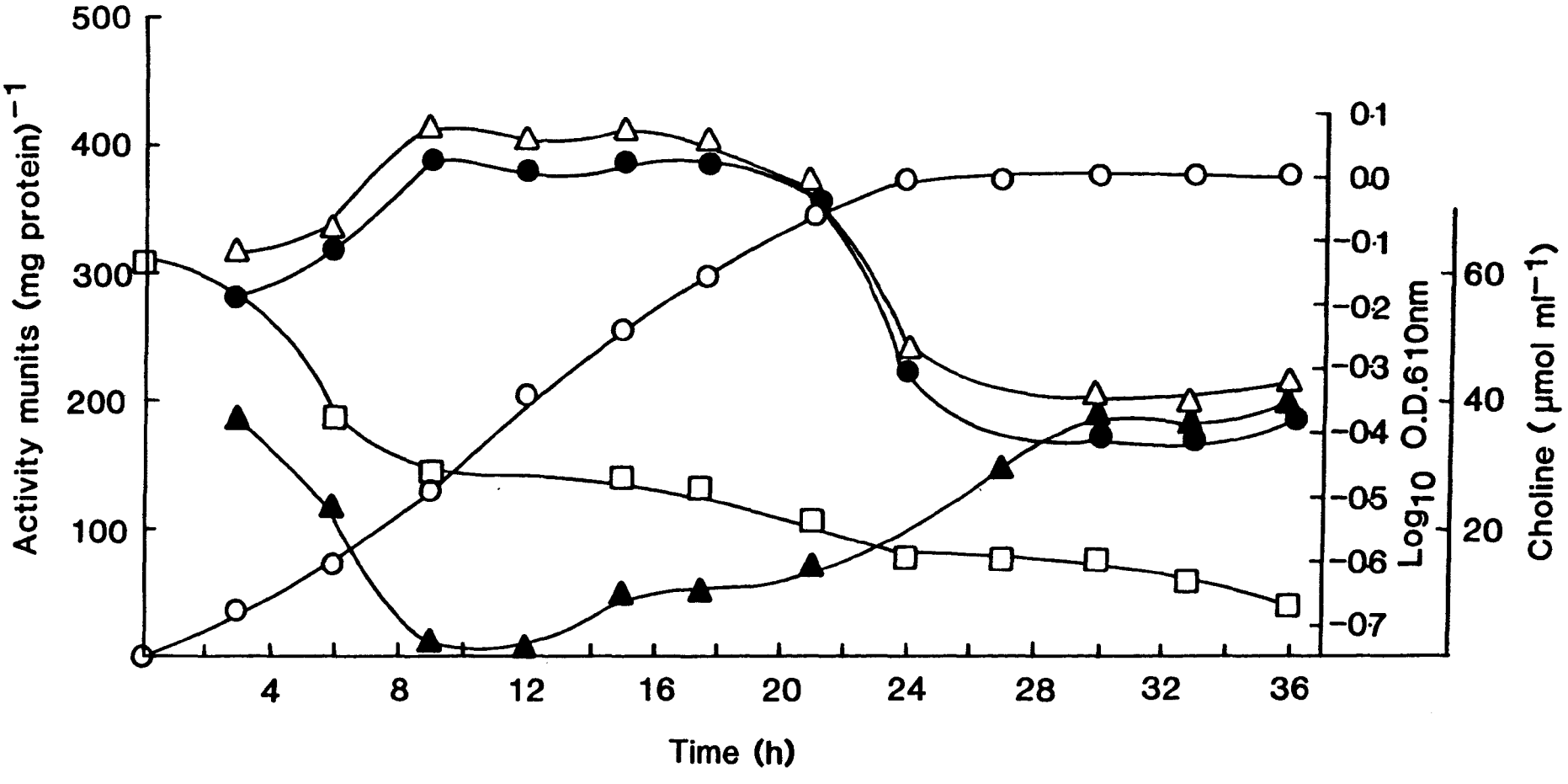
○ $\log_{10}$ O.D.$_{610nm}$

● NADH-linked hydroxypyruvate reductase) NADH/ NADPH-linked enzyme

△ NADPH-linked hydroxypyruvate reductase) NADH/ NADPH-linked enzyme

▲ NADPH-linked hydroxypyruvate reductase

□ Choline in culture filtrate


Activity munits (mg protein)$^{-1}$
500
400
300
200
100
0·1
0·0
-0·1
-0·2
-0·3
-0·4
-0·5
-0·6
-0·7
Log_{10} O.D.610nm
60
40
20
Choline (µmol ml^{-1})
4
8
12
16
20
24
28
32
36
Time (h)

linked enzyme increased and those of the strictly NADPH-linked enzyme reduced to zero. As late exponential phase was established the NADH/NADPH-linked activity began to decrease and the strict NADPH-linked activity increased, both of these trends continuing into stationary phase.

The results of the experiment confirmed those obtained previously but threw no light onto the reason for the sequential induction of the two enzymes. The analytical methods that were used failed to detect a build up of any oxidation intermediates of choline during the growth cycle.

The next experiments were designed to test the effects of the rate of growth on the production of the two hydroxypyruvate reductases. In order to do this it was necessary to grow the organism under conditions of chemostat continuous culture using carbon as the limiting nutrient and choline as the carbon source. The experimental procedure consisted of running the chemostat at three different growth rates and, after establishing steady state conditions at each rate, removing a sample of the culture and assaying for NADH and NADPH-linked hydroxypyruvate activity. Steady state conditions were assumed to be established after the passage of five culture volumes through the fermenter vessel. At each steady state one litre of culture was withdrawn from the fermenter and the cells were separated from the culture fluid by centrifugation at 6300 g for 15 minutes at 2°C. The supernatant culture fluid was discarded, and 1 g of cell paste was resuspended in 4 ml of 10 mM sodium potassium phosphate buffer pH 7.0. This was sonicated and the cell debris removed by centrifugation at 38000 g for 20 minutes at 2°C, the supernatant being used as the cell free extract

TABLE 23 Levels of hydroxypyruvate reductase during the growth of Arthrobacter 2B2 in chemostat continuous culture, using choline as a carbon source

Dilution rate	NADH/NADPH enzyme m units $(mg\ protein)^{-1}$		NADPH enzyme m units $(mg\ protein)^{-1}$
	NADH	NADPH	
$0.012\ h^{-1}$	131	138	1097
$0.03\ h^{-1}$	110	116	623
$0.079\ h^{-1}$	312	330	6

for the assay of the enzyme activities.

The maximum growth rate (μ max) of Arthrobacter 2B2 growing on choline as carbon source was estimated from its exponential rate of growth in batch culture to be 0.09 (Figure 48). The three dilution rates at which the chemostat was operated were 0.012 h^{-1}, 0.03 h^{-1} and 0.079 h^{-1}. The hydroxypyruvate reductase activity present in the cell free extracts made from each of the cultures was assayed using both NADH and NADPH. From the results the relative activities of the strict NADPH-linked enzyme and the NADH/NADPH-linked enzyme could be calculated by employing the previously described method involving the use of the 1.06 : 1 ratio of NADPH : NADH in the NADH/NADPH-linked enzyme.

The results in Table 23 show that at high dilution rates the activity of the strict NADPH-linked enzyme was extremely low whilst at low dilution rates the activity was almost two hundred times greater. The dual NADH/NADPH-linked enzyme had a lower activity at low dilution rates than at high dilution rates. However, the activity did not fluctuate as greatly as the NADPH-linked enzyme, with the highest dilution rate having three times the activity of the lowest.

The effects noted during the chemostat growth of Arthrobacter 2B2 on choline prompted a consideration of the growth of the organism on methylamine in a chemostat in order to see if there were any differences between the production of hydroxypyruvate reductase activity at high or low growth rates. The maximum growth rate (μ max) for the organism growing on methylamine was calculated from the growth curve of batch cultured cells as described previously for growth on choline. It was determined to be 0.1 . The two dilution

FIGURE 48 Estimation of the μ max of Arthrobacter 2B2 using choline as the sole source of carbon

From the rate of exponential growth of the organism the specific growth rate μ , can be calculated as being $\frac{\ln 2}{td}$, where td is the doubling time. Assuming that the growth conditions are not limiting, this value of μ is taken to be μ max.

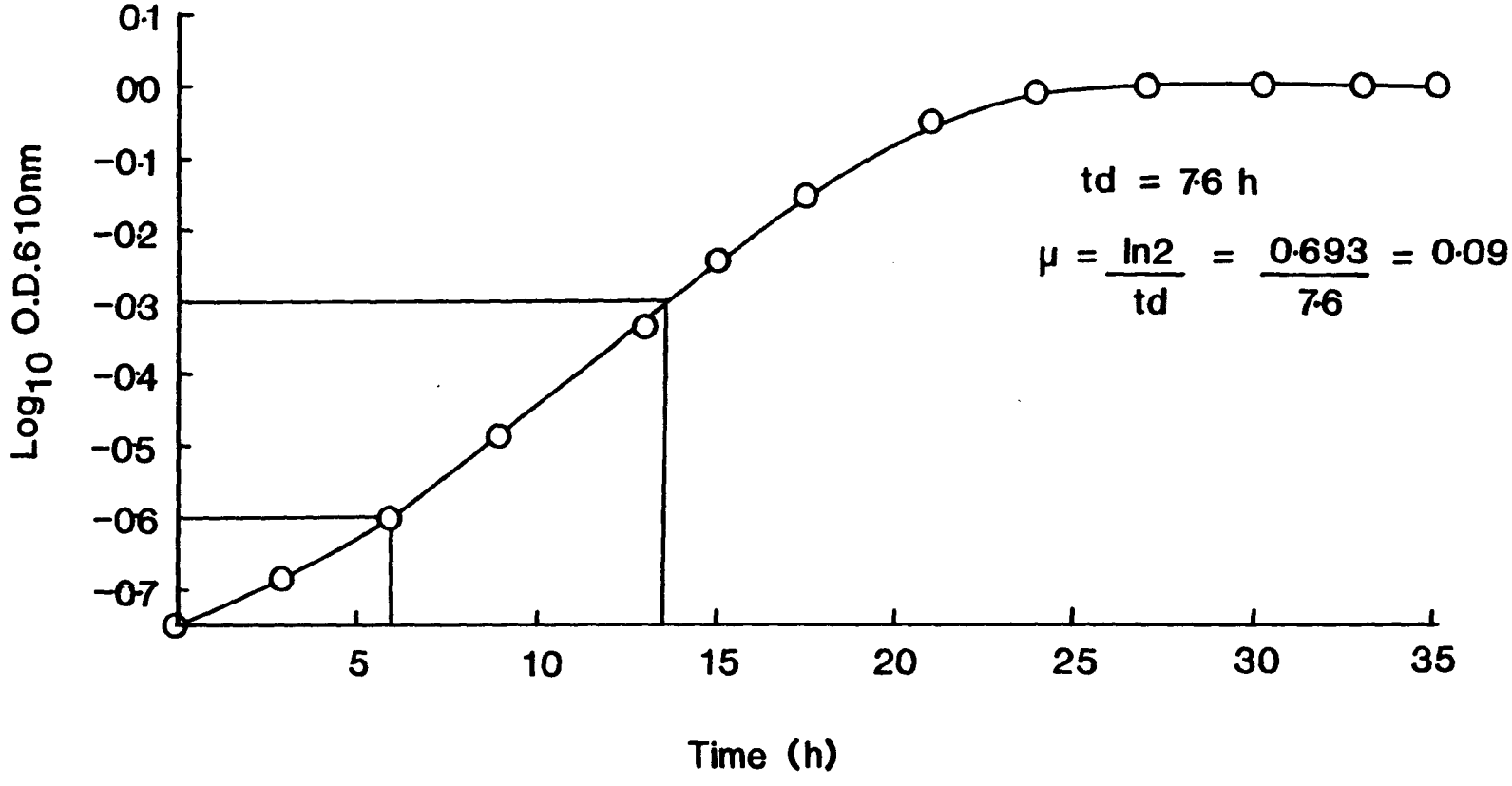

0·1
00
−0·1
−0·2
−0·3
−0·4
−0·5
−0·6
−0·7
Log10 O.D.610nm
5
10
15
20
25
30
35
Time (h)
td = 7·6 h
μ = ln2 / td = 0·693 / 7·6 = 0·09

TABLE 24 Levels of hydroxypyruvate reductase during the growth of Arthrobacter 2B2 in chemostat continuous culture using methylamine as a carbon source

Dilution rate	NADH/NADPH enzyme m units (mg protein)$^{-1}$		NADPH enzyme m units (mg protein)$^{-1}$
	NADH	NADPH	
0.098 h^{-1}	0	0	573
0.0084 h^{-1}	0	0	193

rates used in the chemostat were 0.098 h^{-1} and 0.0084 h^{-1}. Samples were withdrawn from the fermenter vessel after steady state conditions had been established. Cells were harvested from the culture medium and cell free extracts were prepared using the same procedure as that described for continuously cultured cells grown on choline. The cell free extracts thus produced were then examined for activity using the NADH and NADPH-linked hydroxypyruvate reductase assays described in the Materials and Methods section.

The results obtained from the investigation are shown in Table 24. It is clear that in carbon limited chemostat growth on methylamine, only the strict NADPH-linked hydroxypyruvate reductase is produced whether growth is at high or low dilution rates. The results do however indicate that more NADPH-linked enzyme is produced at higher growth rates than at lower ones.

Summary of results

Work carried out using large scale batch cultures of Arthrobacter 2B2 growing on choline as a sole source of carbon proved that the two hydroxypyruvate reductases that were present were produced at different times during the growth cycle of the organism. The NADH/NADPH-linked enzyme was prevalent during the exponential phase of growth. However, in late exponential phase the level of this enzyme began to decrease and this trend continued into stationary phase. The strict NADPH-linked enzyme, on the other hand, remained at relatively low levels until mid to late exponential phase was reached. The activity of the enzyme then began to increase

the upward trend continuing into stationary phase.

The pattern of enzyme synthesis was considered to be due to either an effect of the growth rate of the cells, or the build up of metabolite(s) of choline breakdown which would cause induction/repression of enzyme synthesis. Although the work on cells grown continuously under choline limitation show that at high growth rates the NADH/NADPH-linked enzyme is prevalent whilst at low growth rates the strict NADPH-linked enzyme is prevalent, growth rate alone is not thought to be the major factor controlling enzyme synthesis. The reason for this can be observed in the results of the batch culture experiments. These show that the reduction of NADH/NADPH-linked enzyme activity and the concurrent increase in NADPH-linked activity starts to occur whilst the culture is still in exponential phase and thus growing at μ max.

Although no build up of the choline oxidation intermediates betaine or sarcosine were detected, it is possible that some other oxidation product of choline causes the change in enzyme synthesis. It can be envisaged that during batch culture on choline the carbon source is only partially oxidised and there is therefore a build up of a choline metabolite in the medium, possibly repressing the strict NADPH-linked enzyme. As the choline is utilised, the organisms must further oxidise the intermediate in order to continue growth, and as the intermediate disappears the NADPH-linked enzyme is derepressed.

This theory fits the results obtained in the chemostat. At low dilution rates the cells must be under such great carbon limitation that no intermediate breakdown products of

choline are allowed to build up, and thus the NADPH-linked enzyme is derepressed and a very high activity is produced. However, at high dilution rates the organism is not under such rigorous carbon limitation, the choline oxidation intermediates can build up and the NADPH-linked enzyme is almost totally repressed.

The results obtained from the growth of Arthrobacter 2B2 in methylamine limited continuous culture show that no dual enzyme is produced whether growth is at a high or low dilution rate. This indicates that choline or some metabolite of choline induces the synthesis of this enzyme. The results also show that more of the strict NADPH-linked enzyme is produced at higher growth rates than at lower ones. In the absence of the NADH/NADPH-linked enzyme the strict NADPH-linked enzyme now becomes the only functional hydroxypyruvate reductase. However, operation of the ribulose monophosphate cycle alone during growth on methylamine could theoretically account for growth of the organism without recourse to the serine pathway, and this is borne out by the lack of other enzymes characteristic of the serine pathway. The metabolic role of the enzyme on this substrate is thus not clear at this stage, and hence interpretation of its regulation cannot be made. A simple explanation by way of a general derepression at very low rates of growth under carbon limitation is clearly not sufficient.

CHAPTER 11

The purification and properties of the formaldehyde dehydrogenase from *Methylophilus methylotrophus*

Introduction

Methylophilus methylotrophus is an obligate methylotroph using methanol or a methylated amine as a carbon source. A lot of work has been carried out using this organism and its enzyme profile has been intensively investigated. Nevertheless the method of formaldehyde oxidation adopted by the organism has not been clearly established. Taylor (1977b) could not detect any enzyme oxidising formaldehyde in Methylophilus methylotrophus, thus confining the oxidation to the dissimilatory ribulose monophosphate cycle. High activities of enzymes involved in the dissimilatory ribulose monophosphate cycle were found in the organism by Beardsmore et al.,(1982), these results seeming to confirm that the only formaldehyde oxidising pathway was the dissimilatory ribulose monophosphate cycle. However, Large and Haywood (1981), discovered the presence of a low level of NAD-linked formaldehyde dehydrogenase in the organism. Due to the uncertainty surrounding the formaldehyde dehydrogenase in Methylophilus methylotrophus, a study of this enzyme in the organism was made in order to establish its characteristics, and its possible role.

Crude cell free extracts of methanol-grown batch cultures were produced by resuspending cell paste in four volumes of 20 mM sodium potassium phosphate buffer pH 7.2 and sonicating using the procedure described in the Material and Methods section. Assay of the crude cell free extract showed the presence of very low levels of formaldehyde dehydrogenase, with activities of about 5 m units (mg protein)$^{-1}$. The levels of the enzyme in continuously cultured cells was next tested. Cells were grown in a chemostat at a dilution rate of 0.1 h^{-1} as described in the Materials and Methods section Cell free extracts were prepared as described before and assays for formaldehyde dehydrogenase were carried out. The results were similar to those obtained using batch grown cells, with very low activities of formaldehyde dehydrogenase being obtained.

Partial purification of formaldehyde dehydrogenase

(i) Preparation of crude cell free extract.

Crude cell free extract was prepared by the resuspension of 12 g of cell paste obtained from a methanol limited chemostat culture (dilution rate 0.1 h^{-1}), in 48 ml of 20 mM sodium potassium phosphate buffer pH 7.2. The suspension was sonicated in two batches and the cell debris was removed by centrifugation at 38000 g for 20 minutes at 2°C. The pellet was discarded and the supernatant treated as the crude cell free extract.

(ii) Ammonium sulphate fractionation.

The crude cell free extract from step (i) was raised to 40% saturation with $(NH_4)_2SO_4$ and the precipitated protein was removed by centrifugation. The resulting supernatant was treated by further fractionation with $(NH_4)_2SO_4$ to 50%

TABLE 25 The purification of formaldehyde dehydrogenase from Methylophilus methylotrophus

Fraction	Volume ml	Protein mg	Activity m units	Specific Activity m units mg protein^{-1}	Purification fold	Yield %
Crude cell free extract	48	921	3963	4.3	1	100
$(NH_4)_2SO_4$ fractionation	10	100	840	8.4	1.9	21
Heat step	8.5	35	878	24.5	5.6	22
Hydroxylapatite chromatography	16	1.4	105	73.3	17	3

saturation. After the precipitated protein had been collected by centrifugation, the pellet was resuspended in the sonication buffer.

(iii) Heat step

The 40% to 50% $(NH_4)_2SO_4$ fraction was subjected to heat treatment. This involved heating the sample to a temperature of 50°C for a ten minute period. The precipitate formed was removed by centrifugation at 38000 g for 20 minutes at 2°C, and discarded.

(iv) Hydroxylapatite chromatography

The supernatant from step (iii) was desalted by passage through a Sephadex G25 column (31 x 1.5 cm) equilibrated with sonication buffer, and then eluted with a similar buffer. Active fractions from the column were pooled and applied to a hydroxylapatite column (25 x 1.5 cm) equilibrated with sonication buffer. Unbound protein was washed off the column with equilibration buffer. Then a linear gradient of 20 mM to 300 mM sodium potassium phosphate made up in 400 ml of equilibration buffer was applied to the column and used to elute formaldehyde dehydrogenase activity. Fractions containing formaldehyde dehydrogenase activity were detected using the continuous NAD-linked assay described in the Materials and Methods section. Fractions 26 to 29 containing activity were pooled, concentrated to a small volume by ultrafiltration and stored at -15°C.

A table showing the purification of formaldehyde dehydrogenase is shown in Table 25. Figure 49 represents the hydroxylapatite step used in the purification of the enzyme.

As shown, the enzyme was purified 17 fold with a yield

FIGURE 49 Hydroxylapatite chromatography of formaldehyde dehydrogenase from Methylophilus methylotrophus

Extract was applied to a hydroxylapatite column (25 x 1.5 cm), equilibrated with 20 mM sodium potassium phosphate buffer pH 7.2. A gradient of 20 mM to 300 mM sodium potassium phosphate was initiated at fraction 16, and the elution pattern followed as described in the Materials and Methods section.

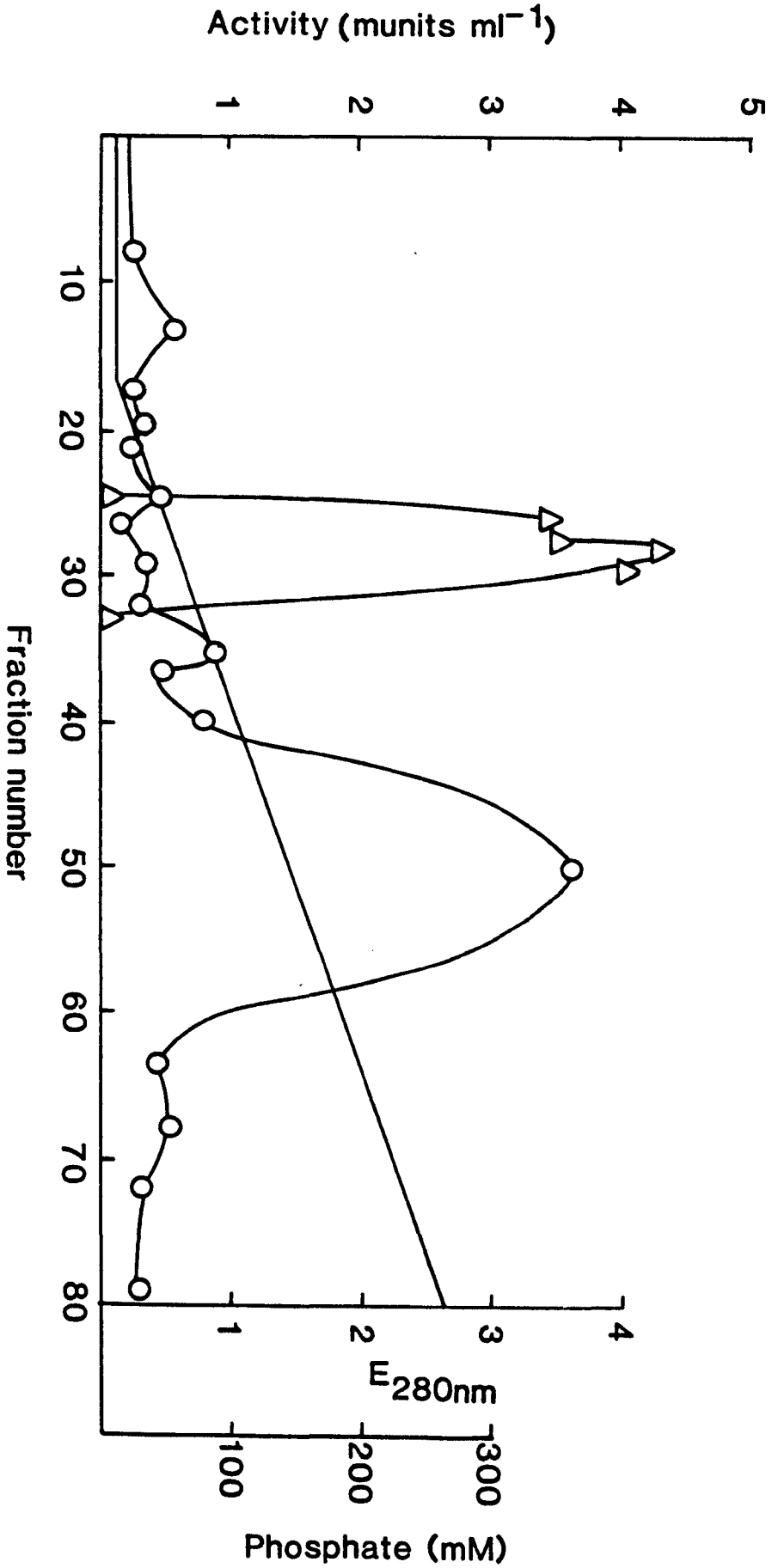
Key:

○ = Extinction at 280 nm

△ = Formaldehyde dehydrogenase activity

—— = Phosphate gradient

Fraction volume 5 ml.


Activity (munits ml^{-1})
1
2
3
4
5
10
20
30
40
50
60
70
80
Fraction number
1
2
3
4
E_{280nm}
100
200
300
Phosphate (mM)

of 3%. The purified enzyme was free from any NADH oxidase activity.

Properties of formaldehyde dehydrogenase

The properties of the formaldehyde dehydrogenase were established using samples of the partially purified enzyme.

Stability

The enzyme was very stable to raised temperatures, this property being used to advantage during the purification of the enzyme when a heat step was employed, the extract being subjected to a temperature of 50°C for ten minutes and suffering no loss of activity. The enzyme was reasonably stable when stored at 0°C, taking a week to lose all activity. When stored frozen at -15°C the enzyme maintained activity for well over a month. However, repeated freezing and thawing reduced the activity considerably.

pH optimum

The buffer used for this determination was tris-HCl, over a range of 7.5 to 8.7. The continuous NAD linked spectrophotometric assay described in the Materials and Methods section was used throughout the investigation. The pH of the buffer was checked before each assay and the pH of the complete assay mixture was tested afterwards to verify that the pH had remained the same throughout. The results obtained are shown in Figure 50. The pH optimum was situated on a broad peak at 8.0.

Metal ions

The continuous assay described in the Materials and

FIGURE 50 pH optimum of formaldehyde dehydrogenase from Methylophilus methylotrophus

The assay used was the continuous NAD-linked assay described in the Materials and Methods section.

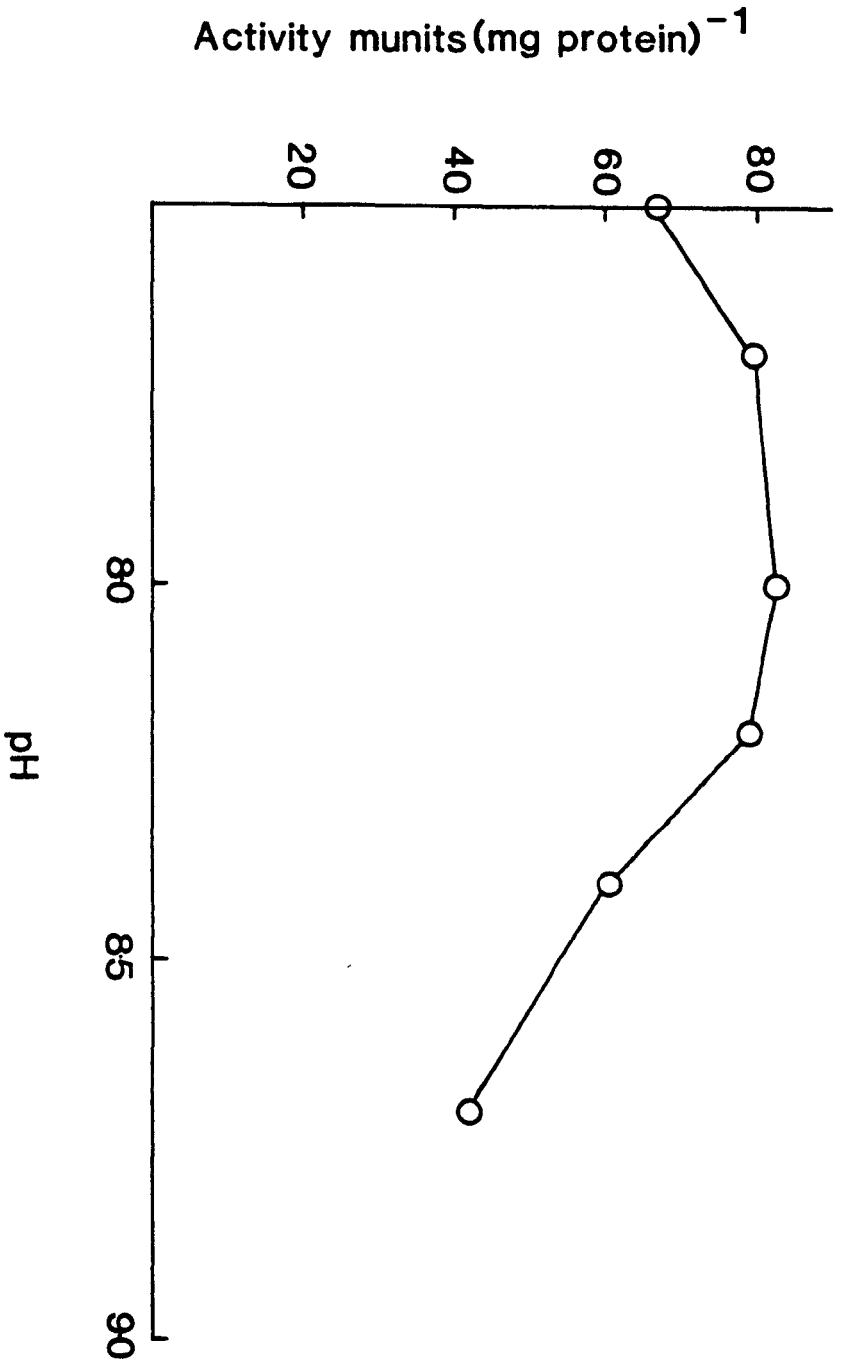

Activity munits(mg protein)$^{-1}$
20
40
60
80
pH
8.0
8.5
9.0

TABLE 26 Activity of formaldehyde dehydrogenase of Methylophilus methylotrophus in the presence of divalent metal ions.

The activity of the enzyme in the presence of the cation is expressed as a percentage of the activity measured in the absence of any metal ion.

Metal ion	Concentration (mM)	Activity (%)
None	-	100
Mg	1	94
Mn	1	88
Cu	1	76
Zn	1	41

TABLE 27 Substrate specificity of formaldehyde dehydrogenase from Methylophilus methylotrophus

The substrate specificity was tested by including the possible substrate at a concentration of 10 mM in the continuous NAD-linked assay described in the Materials and Methods section.

Substrate	Activity %
Formaldehyde	100
Acetaldehyde	69
Propionaldehyde	66
n-Butyraldehyde	33
iso-Butyraldehyde	60
Glycolaldehyde	69
Glyoxal	45
Benzaldehyde	30
Glyoxylate	39
Methanol	0
Formate	0

Methods section was used throughout this investigation. The assay was supplemented by the addition of various metal ions at a concentration of 1 mM. The activities recorded when the metal ions were present were compared to those when ions were absent, and expressed as a percentage of the latter figure. The results are shown in Table 26. Generally all of the metal ions tested inhibited the enzyme; $MgSo_4$ inhibited only slightly, as did $MnCl_2$. However, the activity obtained in the presence of 1 mM $ZnSO_4$ was only 41% of that measured when no ion was present.

Substrate specificity

The substrate specificity was tested by placing a sample of partially purified enzyme into the continuous assay mixture containing the appropriate possible substrate. The results obtained are shown in Table 27. They are expressed as percentages of the activity present in assays containing formaldehyde as a substrate. The results show that although formaldehyde gives the highest activity of any of the substrates tested, the enzyme has a wide range of possible aldehyde substrates.

Effect of -SH groups

Freshly produced cell free extract showed a decrease in activity of between 40% and 50% when GSH was omitted from the assay. As the purification proceeded however, the absence of GSH proved to have a greater effect, the final partially purified enzyme showing activity only when GSH was present in the assay mixture. It seemed likely that the enzyme had a strict requirement for GSH in order to show

activity. The crude cell free extract was active in the absence of externally added GSH, because of the presence of GSH or other compounds containing -SH groups within the extract. As the enzyme was purified the concentration of the compounds containing -SH groups were reduced, thus increasing the stimulatory effect of the externally added GSH. In order to test if this theory was valid, assays of the partially purified enzyme were executed in which varying concentrations of GSH were added. The high concentrations of GSH would mimic conditions present in the crude cell free extract, where contaminating compounds containing -SH groups would be present. Reducing the GSH concentration in the assay would represent various stages in the purification of the enzyme when the contaminating compounds were reduced, and it would therefore be expected that the activity of the enzyme would be concomitantly reduced. The results shown in Figure 51 prove that by increasing the GSH concentration in the formaldehyde dehydrogenase assay, the activity of the enzyme is also increased and that maximum formaldehyde dehydrogenase activity is obtained in the presence of 5.5 mM GSH.

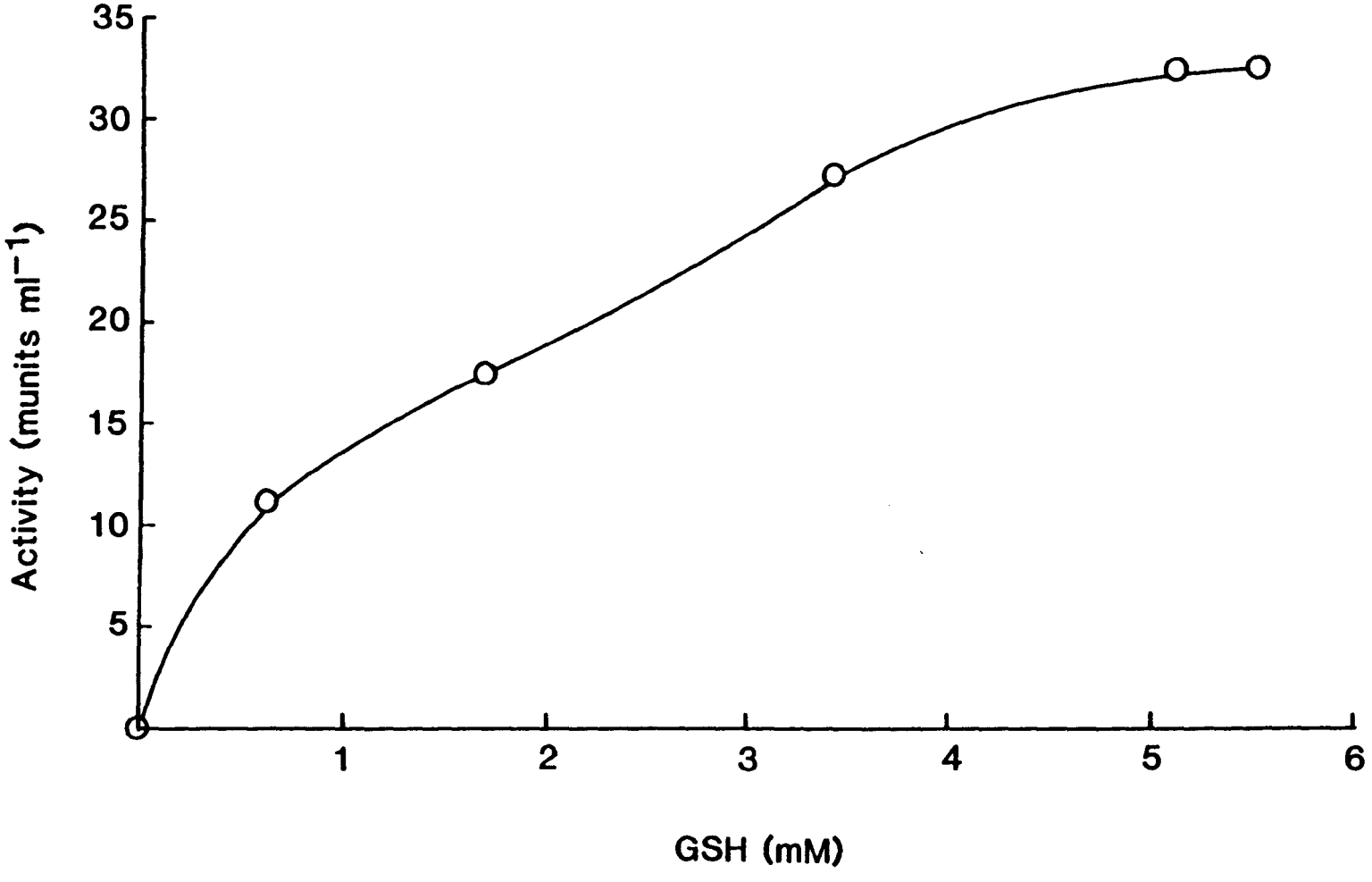
In order to assess whether other compounds containing -SH groups could play a similar role to GSH in activating the enzyme, an assay mixture was produced containing 6.8 mM cysteine in place of GSH. The results from such an assay show that the inclusion of cysteine in the assay gave 68% of the formaldehyde dehydrogenase activity present using a similar concentration of GSH.

Alternative electron acceptors

The partially purified enzyme was assayed for activity

FIGURE 51 Effect of the concentration of GSH on partially purified formaldehyde dehydrogenase from Methylophilus methylotrophus

The enzyme was assayed in the mixture described in the Materials and Methods section, but with differing concentrations of GSH added.


35
30
25
20
15
10
5
Activity (munits ml^{-1})
1
2
3
4
5
6
GSH (mM)

using electron acceptors other than NAD. Those tested were: 1 mM NADP which was added to the standard continuous assay mixture from which NAD had been omitted; 1 mM potassium ferricyanide which was tested in the standard assay mixture, any absorbance decrease at 400 nm being examined; 0.2 mM DCPIP, and 1 mM phenazine ethosulphate plus 0.2 mM DCPIP, which were both assayed in the DCPIP linked formaldehyde dehydrogenase assay described in the Materials and Methods section, the absorbance decrease at 600 nm being followed.

The results showed that the enzyme was only active when NAD was included in the assay.

Determination of the Michaelis constants for formaldehyde dehydrogenase

The Km and Vmax values for formaldehyde and NAD were determined using the method of Eisenthal and Cornish-Bowden (1974). The Km for NAD was calculated by including varying concentrations of NAD in the assay mixture, containing 20 mM formaldehyde and 10 mM GSH. The Km for formaldehyde was determined by the addition of varying quantities of formaldehyde to an assay mixture containing 10 mM NAD and 10 mM GSH.

The results are shown in Figures 52 and 53, the apparent Km and Vmax for NAD being determined as 5.4×10^{-4} M and 49 m units (mg protein)$^{-1}$ respectively, and those for formaldehyde at 1.2×10^{-2} M and 72 m units (mg protein)$^{-1}$ respectively.

FIGURE 52 The determination of the apparent Km of the formaldehyde dehydrogenase of Methylophilus methylotrophus for NAD, using a Cornish-Bowden linear plot.

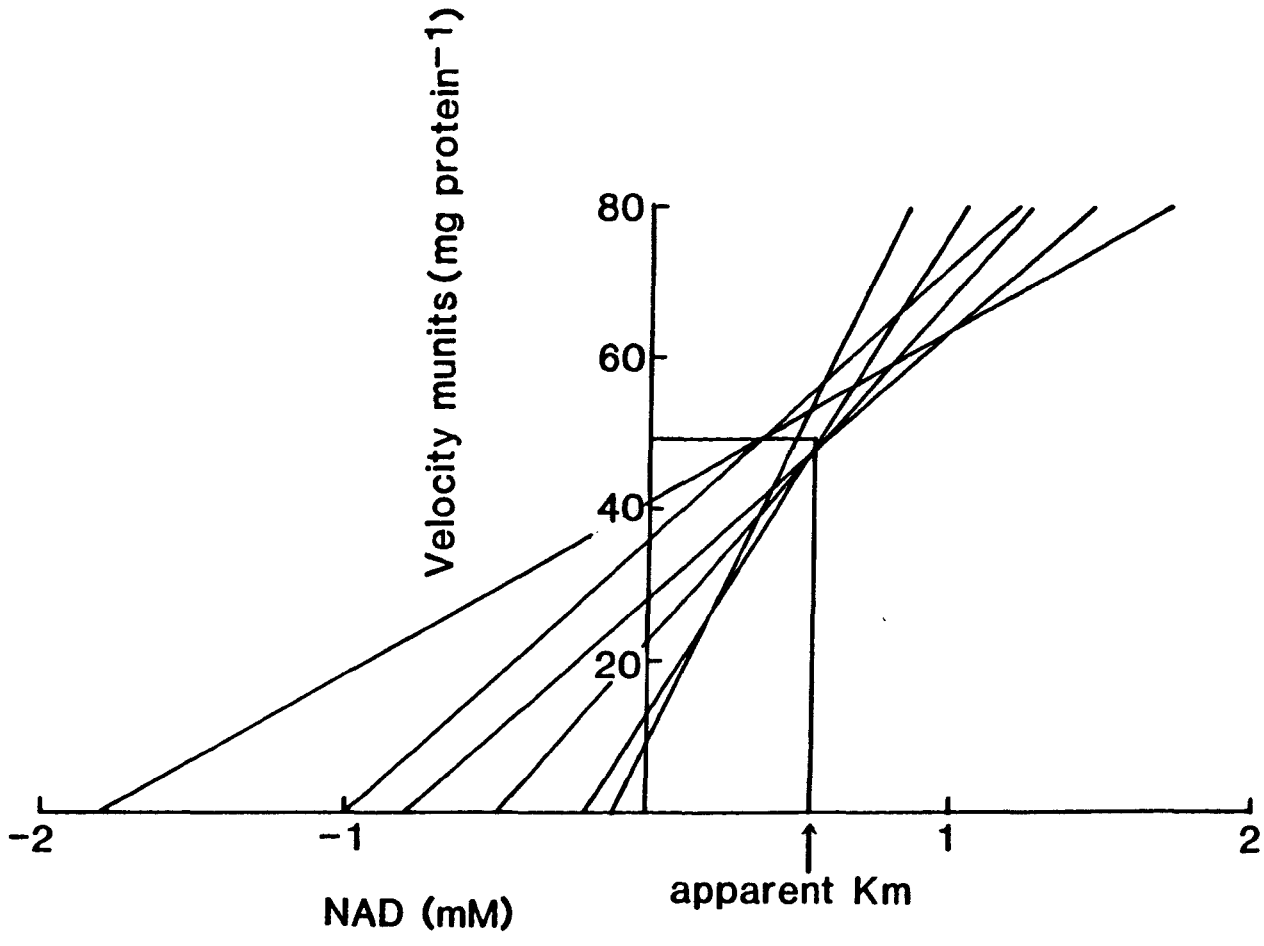
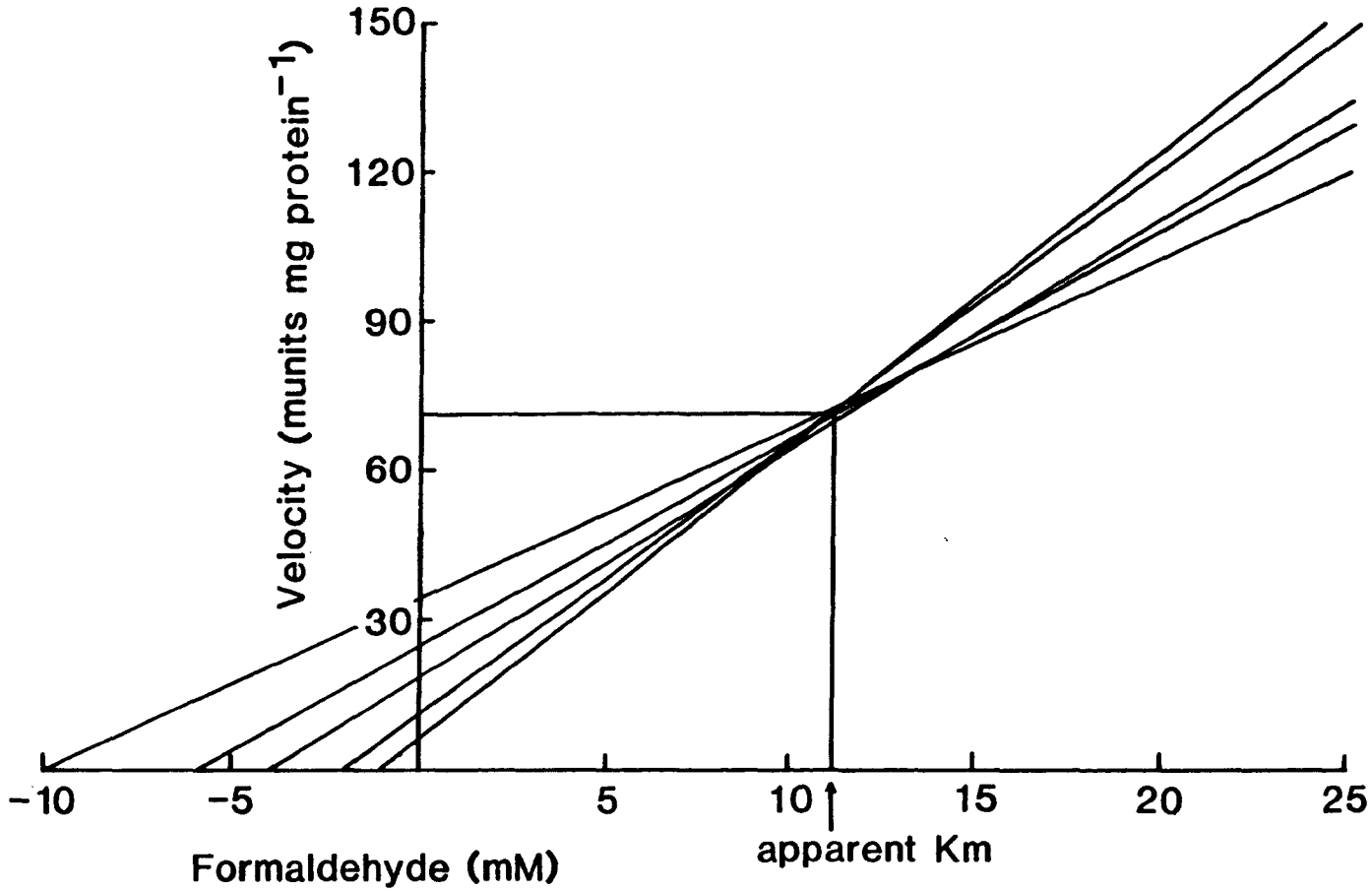

Velocity munits (mg protein^{-1})
80
60
40
20
-2
-1
1
2
NAD (mM)
apparent Km

FIGURE 53 The determination of the apparent Km of formaldehyde dehydrogenase of Methylophilus methylotrophus for formaldehyde using a Cornish-Bowden linear plot.


150
120
90
60
30
Velocity (munits mg protein^{-1})
−10
−5
5
10
15
20
25
Formaldehyde (mM)
apparent Km

Summary of results

The formaldehyde dehydrogenase of *Methylophilus methylotrophus* was purified 17-fold with a yield of 3%. The partially purified enzyme appeared to be an NAD-linked, GSH-dependent, aldehyde dehydrogenase. It could not function as a methanol dehydrogenase or a formate dehydrogenase, and it was not stimulated by any of the metal ions tested.

The enzyme exhibited activity only with NAD and therefore falls into group 1 of the formaldehyde dehydrogenase classification put forward by Stirling and Dalton (1978). The requirement for GSH was not strict, as cysteine could replace it as a source of -SH. However this gave only 68% of the activity, compared to that recorded when GSH was used. A Wide range of aldehydes were utilised by the enzyme; the highest activity however was recorded when using formaldehyde as a substrate, suggesting that this is a major substrate for the enzyme *in vitro*.

The very low specific acitivity of the enzyme present in crude cell free extracts, together with the high apparent Km for formaldehyde, indicate that the enzyme does not carry out a major role in formaldehyde oxidation in *Methylophilus methylotrophus*. The main route of formaldehyde oxidation is probably via the dissimilatory ribulose monophosphate cycle, as high activities of the enzymes required for this pathway to be operable have been found in the organism (Large and Haywood, 1981;Beardsmore *et al.*,1982).

The function of the formaldehyde dehydrogenase in *Methylophilus methylotrophus* is probably one of safety - to oxidise any excess formaldehyde present in the cell which would otherwise damage it. The wide substrate specificity enables it to degrade many other aldehydes which may arise in the cell during growth.

CHAPTER 12

Discussion

Arthrobacter 2B2 displays typical Arthrobacter type rod-coccal pleomorphism when grown in batch culture. During the exponential growth phase the cell morphology is rod shaped, and this changed as the organism entered stationary phase, the cells becoming coccoid. When growing exponentially the rods were visualised under the light microscope as complex 'V' formations, this being characteristic of the organisms contained within the genus Arthrobacter. Several theories have been put forward to explain the change in appearance of the cells: Krulwich and Ensign (1969) concluded that certain chemicals (for example, succinate) were required to induce rod formation and in the absence of such chemicals the cells reverted to cocci. On the other hand, Luscombe and Gray (1971 and 1974), suggested that the morphology of the cell was dependent on growth rate and demonstrated the point by growing organisms at varying growth rates in a chemostat. Perhaps the most widely accepted theory is that the change is due to a decrease in nutrient supply. The organism responds to such a change by increasing the surface to volume ratio of the cell, thus enhancing the uptake capacity (Harder et al., 1984).

During the growth of Arthrobacter 2B2 in the light, a bright yellow pigment is formed which has a characteristic absorption spectrum with peaks at 422 nm, 450 nm and 474 nm. A similar pigment with the same absorption spectrum has been reported in Arthrobacter P1 (Levering et al., 1981), and a suggestion was advanced that the colour was due to a carotenoid like structure. No pigment was produced by cells grown in the dark.

Examination of the enzyme levels of Arthrobacter 2B2

growing in batch culture and using methylamine as a carbon source confirmed that the enzyme responsible for the oxidation of methylamine was methylamine oxidase. Such enzymes are widespread amongst methylotrophic yeasts which can utilise methylamine as a nitrogen source. However, among the bacteria, methylamine oxidase has been found only in members of the genus Arthrobacter.

The pathway adopted by Arthrobacter 2B2 for the assimilation of the C_1 growth substrate is the ribulose monophosphate cycle of formaldehyde fixation. Appreciable activities of the key enzymes of the cycle, hexulose phosphate synthase and phosphohexuloisomerase, were found in crude cell free extracts of the organism whilst growing on methylamine. High activities of the serine pathway enzyme hydroxypyruvate reductase were also discovered in cells grown on methylamine. However, as the activities of other important serine pathway enzymes were negligible, it was concluded that the operation of this pathway during growth on methylamine was unlikely.

The fixation phase of the ribulose monophosphate cycle adopted by Arthrobacter 2B2 involves the use of the Embden-Meyerhof sequence as the C_6 cleavage mechanism. This was indicated by the presence of fructose bisphosphate aldolase and phosphofructokinase in cell free extracts of cells grown on methylamine, together with the absence of the Entner-Doudoroff enzyme 2-keto 3-deoxy 6-phosphogluconate aldolase. The rearrangement phase of the cycle utilises the enzymes transaldolase and transketolase. No sedoheptulose 6-phosphatase activity could be detected. It therefore appears that Arthrobacter 2B2 has adopted the fructose bisphosphate aldolase$^+$ transaldolase$^+$ variant of the ribulose

monophosphate cycle. This variant of the cycle has a net assimilation product from three molecules of formaldehyde of phosphoglycerate and $NADH_2$. This particular variant of the cycle used by Arthrobacter 2B2 is the most energetically favourable of the four possible routes and currently the only other organism reported to use it is Arthrobacter P1 (Levering et al., 1982).

Methylamine-grown Arthrobacter 2B2 contains negligible levels of formaldehyde and formate dehydrogenases, ruling out the operation of a linear pathway as the method of formaldehyde oxidation in this organism. However, cell free extracts of methylamine-grown cells do contain quite high activities of 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase, indicating that the dissimilatory ribulose monophosphate cycle can operate in Arthrobacter 2B2 as the mode of formaldehyde oxidation. In this respect the organism follows the trend described by Zatman (1981), that all non-methane utilising ribulose monophosphate cycle methylotrophs oxidise formaldehyde via an oxidative ribulose monophosphate cycle, whilst methane utilising ribulose monophosphate cycle organisms tend to use a linear oxidation pathway involving formaldehyde and formate dehydrogenases.

An examination of the two key enzymes of the ribulose monophosphate cycle, hexulose phosphate synthase and phospho-hexuloisomerase, was made in order to compare the properties of the enzymes with those isolated previously from other organisms.

The hexulose phosphate synthase purified from Arthrobacter 2B2 is a soluble dimeric protein of molecular weight 155000. It has a requirement for divalent metal ions to maintain

activity and is extremely unstable in the absence of a stabilising mixture containing methanol, ribulose 5-phosphate and ribose 5-phosphate.

Hexulose phosphate synthases have been previously purified from Methylococcus capsulatus (Bath) (Ferenci et al., 1974), Methylomonas M15 (Sahm et al., 1976b), Methylomonas aminofaciens (Kato et al., 1978), Pseudomonas oleovorans (Sokolov and Trotsenko 1978a, b), Methylophilus methylotrophus (Beardsmore et al., 1982), and Bacterium MB58 (Muller and Babel, 1978). Comparison of the properties of these different enzymes reveals many similarities. As far as has been reported, they are all completely specific with respect to ribulose 5-phosphate, formaldehyde and hexulose 6-phosphate as substrates. The pH optima for the enzymes occur within the range 7 to 8 and they are all absolutely dependent on Mg or Mn ions for activity and stability. The enzymes seem to fall into four groups with respect to their molecular weights. (1) The enzymes from Methylomonas M15, Methylomonas aminofaciens, Methylophilus methylotrophus and Pseudomonas oleovorans have molecular weights of 40000 to 47000. In the case of the first three organisms the enzymes are dimeric, consisting of two identical subunits. The subunit structure of Pseudomonas oleovorans has not been reported. (2) The enzyme from Methylococcus capsulatus (Bath), has a molecular weight of 310000, consisting of six identical subunits which will dissociate in conditions of low pH. (3) The enzyme from Bacterium MB58 has a molecular weight of 80000 and consists of four identical subunits. (4) The enzyme from Arthrobacter 2B2 which has a molecular weight of 155000, consisting of two identical subunits.

Of all the enzymes studied only that from Methylococcus capsulatus appears to be membrane bound. However, it has been suggested that the hexulose phosphate synthase from Methylophilus methylotrophus could have a loose association with the membrane and is dislodged from it by the ultrasonic disruption used to produce the cell free extract (Beardsmore, 1979). The enzyme from Arthrobacter 2B2 shows properties consistent with the theory that it too has a loose association with particulate matter within the cell. It has been proposed (Kato et al., 1978), that the membrane bound enzyme found in Methylococcus capsulatus reflects the complex internal membrane arrangement of this obligate methanotroph. All of the other hexulose phosphate synthase enzymes that have been studied are from non-methane utilising methylotrophs (Pseudomonas oleovorans, Bacterium MB58 and Arthrobacter 2B2 being facultative, the others obligate methylotrophs). These organisms do not possess the complex internal membrane arrangement of methanotrophs and this would be consistent with their hexulose phosphate synthases being soluble rather than membrane bound.

It has been previously reported that the hexulose phosphate synthase enzymes from Pseudomonas oleovorans (Muller and Sokolov, 1979) and Bacterium MB58 (Muller and Babel, 1980), exist as multiple interconvertible forms. It is claimed that the presence of these multiple forms of the enzyme gives rise to deviations in Michaelis-Menten kinetics and could impart properties of regulatory significance on the enzyme. With one exception none of the hexulose phosphate synthases so far examined show any modulation of activity with adenine or pyridine nucleotides. This would make sense in the case of

organisms utilising the dissimilatory ribulose monophosphate cycle as a mode of formaldehyde oxidation as it would seem illogical to regulate an enzyme through which flows the majority of the carbon for both assimilation and dissimilation. A more realistic target in terms of energetics would be the branch point between assimilation and dissimilation. In methanotrophs such as Methylococcus capsulatus which appear to have functional formaldehyde and formate dehydrogenases and use a linear pathway of formaldehyde oxidation, one might expect to find some regulation of hexulose phosphate synthase since this is at the branch point of assimilation and dissimilation of formaldehyde. Such control has not been found in the hexulose phosphate synthase from Methylococcus capsulatus, the enzyme being unaffected by pyridine- or adenine-nucleotides (Ferenci et al., 1974). The one exception which shows an alteration of activity with reduced pyridine nucleotides is the enzyme purified from Pseudomonas oleovorans. It is reported that this enzyme shows inhibition with NADH and NADPH (Sokolov and Trotsenko, 1978a).

It has been recently reported that the synthesis of hexulose phosphate synthase in the facultative methylotroph Arthrobacter P1 is induced by formaldehyde and not the C_1 carbon source methylamine (Levering et al., 1984). This group has found that mutants of Arthrobacter P1 lacking methylamine oxidase do not produce hexulose phosphate synthase when grown on a medium containing methylamine. However, growth of the same mutant on choline, a substrate which yields formaldehyde as it is broken down, resulted in the synthesis of hexulose phosphate synthase.

The hexulose phosphate synthase from Arthrobacter 2B2

showed no appreciable departure from Michaelis-Menten kinetics, which may have been due to multiple interconvertible forms of the enzyme as reported in Bacterium MB58 and Pseudomonas oleovorans. Furthermore, no rate modulation by adenine- or pyridine-nucleotides was observed. Hence the exact method of regulation of the enzyme from Arthrobacter 2B2 remains open.

The phosphohexuloisomerase from Arthrobacter 2B2 was determined to be a soluble trimeric protein with a total molecular weight of 108000. It has a pH optimum of 8.5 and does not require the presence of divalent metal ions to maintain activity. The enzyme was however extremely unstable if placed in solutions of low protein concentration, all activity being lost within a few minutes.

There have been few previous reports of the purification of phosphohexuloisomerase from organisms, probably due to the difficulty in obtaining the substrate for the enzyme D-arabino-3-hexulose 6-phosphate. In the present study stocks of the substrate were produced using the purified hexulose phosphate synthase from Arthrobacter 2B2 as described in the Materials and Methods section.

Phosphohexuloisomerases have previously been purified from Methylococcus capsulatus (Bath) (Ferenci et al., 1974), Methylophilus methylotrophus (Beardsmore et al., 1982), and Methylomonas aminofaciens (Kato et al., 1977b). The enzymes from Methylococcus capsulatus and Methylophilus methylotrophus are very similar in their properties, both being monomeric with molecular weights of 67000 and 70000 respectively, and having respective pH optima of 8.3 and 8.6. No detailed comparison can be made with the enzyme from Methylomonas

aminofaciens as it was only partially purified. However, it does appear similar to the two previously described enzymes with the exception that its pH optimum is lower being 7.5. When the phosphohexuloisomerase from Arthrobacter 2B2 is compared with the three enzymes discussed earlier, it appears quite different. The major distinction is that it is a trimer, whilst the other enzymes are reported to be monomers, although that from Methylophilus methylotrophus has been reported to exist in polymeric forms. The pH optimum resembles those reported for the enzymes from Methylococcus capsulatus and Methylophilus methylotrophus. However, it is a very unstable enzyme, unlike those from the other organisms.

As was the case with hexulose phosphate synthase, no phosphohexuloisomerase so far isolated has been shown to be affected by pyridine or adenine nucleotides. This would be the expected result as it is the second enxyme in the assimilation route and does not lie at any branch point in the pathway.

One of the reasons for making a detailed study of the phosphohexuloisomerase from Arthrobacter 2B2 was that Arthrobacter P1, a very similar organism to Arthrobacter 2B2, apparently assimilated C_1 compounds via the ribulose monophosphate cycle, and yet no activity of phosphohexuloisomerase could be found. The results obtained from the study of the enzyme from Arthrobacter 2B2 indicate that the reason for the apparent absence of phosphohexuloisomerase from Arthrobacter P1 could be its instability in conditions of low protein concentration. The problem can be alleviated by the inclusion of an inert protein such as bovine serum albumin in the

mixture used to assay the enzyme, and by making the enzyme the final addition used to initiate the assay, thus allowing a linear rate to be established before the effects of inactivation become too great.

It is clear that the mode of carbon assimilation adopted by Arthrobacter 2B2 when growing on methylamine is the ribulose monophosphate cycle of formaldehyde fixation. However, as mentioned earlier, very high activities of an NADPH-linked hydroxypyruvate reductase were also recorded in cells grown on methylamine. Hydroxypyruvate reductase is a key enzyme involved in the serine pathway, and its presence in a ribulose monophosphate cycle organism growing on a C_1 substrate is quite interesting. Initially it was speculated that the organism may be using both the ribulose monophosphate cycle and the serine pathway as assimilatory routes during growth on methylamine. However, the negative results obtained when other key enzymes of the serine pathway were assayed, ruled out this possibility. Consideration of the results obtained by Levering et al., (1981b) using Arthrobacter P1 showed that this organism produced no hydroxypyruvate reductase activity during growth on methylamine. However, when choline was used as sole carbon source, high levels of both NADH and NADPH-linked hydroxypyruvate reductase activity were recorded. Arthrobacter 2B2 utilising choline as a sole source of carbon gave a similar result containing high levels of both NADH and NADPH-linked hydroxypyruvate reductase. The reason for the presence of high levels of hydroxypyruvate reductase in choline grown Arthrobacter P1 has been ascribed to the method of choline metabolism in this organism (Levering et al., 1981b). Choline is oxidised initially by choline oxidase which yields

betaine aldehyde. This is converted to betaine by betaine aldehyde dehydrogenase. Betaine is acted upon by an as yet uncharacterised enzyme yielding dimethylglycine, which is oxidised to sarcosine by dimethylglycine oxidase. Finally glycine is produced by the action of sarcosine oxidase on sarcosine. This pathway of choline metabolism is similar to that known to occur in mammals, fungi and previously studied bacteria. In mammals betaine is demethylated by betaine-homocysteine transmethylase (Greenberg, 1961). However, in previously studied bacteria this enzyme has been ruled out as the mechanism of betaine demethylation (Shieh, 1966). It has been proposed by Levering et al., (1981b), that the glycine resulting from this degradative pathway is condensed with formaldehyde by serine transhydroxymethylase, yielding serine. Serine can then be converted to pyruvate by the action of serine- α ketoglutarate aminotransferase, hydroxypyruvate reductase, glycerate kinase, and finally the glycolytic enzymes phosphoglycerate mutase and phosphoenolpyruvate hydratase. The operation of such a pathway clearly requires the presence of hydroxypyruvate reductase.

Work with Arthrobacter 2B2 has demonstrated that the organism contains all of the enzymes required to oxidise choline to glycine in a similar manner to Arthrobacter P1. Assays for the presence of serine transhydroxymethylase, serine- α ketoglutarate aminotransferase, hydroxypyruvate reductase and glycerate kinase in Arthrobacter 2B2 all proved positive. However, all of the enzymes except hydroxypyruvate reductase were at low levels. An alternative route leading from serine to pyruvate involves the enzyme serine dehydratase and this too has been found in Arthrobacter 2B2. In conclusion

therefore, Arthrobacter 2B2 uses a similar pathway of choline oxidation as that proposed in Arthrobacter P1, the product of the oxidative pathway being glycine. The glycine can be further metabolised to pyruvate by two routes, one using enzymes of the serine pathway, the other using serine dehydratase. Hydroxypyruvate reductase, therefore, has a role in the metabolism of choline as it forms a step in one of the conversion sequences of serine to pyruvate. However, the reason for the relatively high levels of this enzyme present in the organism, compared to the levels of other enzymes in the pathway, is unknown.

During the conversion of choline to glycine, three successive demethylation reactions occur, each producing a molecule of formaldehyde. As formaldehyde is highly toxic it must be removed. Arthrobacter P1 has been reported to lack formaldehyde dehydrogenase; however, during growth on choline hexulose phosphate synthase is produced, indicating that the ribulose monophosphate cycle can operate. This cycle can utilise formaldehyde for assimilation into cell material, or dissimilation to form carbon dioxide. Arthrobacter 2B2 also produces the ribulose monophosphate cycle enzymes hexulose phosphate synthase and phosphohexulo-isomerase during growth on choline. However, they are present at low levels and cannot account for the oxidation of all excess formaldehyde produced by the cell. The organism has been demonstrated to produce high activities of formaldehyde dehydrogenase during growth on choline, and it is anticipated that the latter enzyme accounts for the majority of formaldehyde oxidation. It should be noted that during the growth of Arthrobacter 2B2 on methylamine, formaldehyde dehydrogenase

is not synthesised, indicating that the oxidation of formaldehyde must occur via the dissimilatory ribulose monophosphate cycle.

The first report of hydroxypyruvate reductase in a methylotrophic organism was that of Large and Quayle (1963) in Pseudomonas AM1. The presence of the enzyme has sometimes been used as the sole indicator of the operation of the serine pathway in methylotrophs. However, criticism has been levelled at the practice of assigning organisms as serine pathway operators on the evidence of the presence of this one enzyme (Bamforth and Quayle, 1977). As mentioned previously, Arthrobacter 2B2 when growing on methylamine as a sole source of carbon by the ribulose monophosphate cycle contains a very high specific activity of NADPH-linked hydroxypyruvate reductase. When growing on choline the organism contains NADH/NADPH-linked hydroxypyruvate reductase activity, and NADPH-linked hydroxypyruvate reductase activity, both at high levels. The hydroxypyruvate reductases from methylamine and choline grown Arthrobacter 2B2 were partially purified in order to compare them with each other and with similar enzymes purified from other organisms.

The activity from choline-grown cells was successfully separated by DEAE-cellulose chromatography, clearly demonstrating the presence of two enzymes. One enzyme was strictly linked to NADPH, the other exhibited dual substrate specificity. The ratio of the activity of NADPH to NADH in the dual specificity enzyme was estimated to be 1.06 : 1. When a comparison was made of the characteristics and properties of the strictly NADPH-linked hydroxypyruvate reductases from methylamine and choline-grown cells, they appeared very

similar in all respects. The values of the kinetic constants were alike, as were the molecular weights, and they had a very similar response to anions. The results led to the conclusion that the NADPH-linked hydroxypyruvate reductase activities that were produced during growth on both methylamine and choline were due to the same enzyme. However, a comparison of the properties of this enzyme with those of the NADH/NADPH-linked enzyme showed some major differences.

A comparison of the hydroxypyruvate reductases present in Arthrobacter 2B2 with those previously studied in other organisms show that the NADH/NADPH-linked enzyme produced by choline-grown cells resembles the enzymes studied in Pseudomonas AM1 and Paracoccus denitrificans (Large and Quayle, 1963; Bamforth and Quayle, 1977). All these enzymes have a dual specificity towards pyridine nucleotides and are inhibited by citrate. The strict NADPH-linked hydroxypyruvate reductase on the other hand, differs from those previously described in its strict requirement for NADPH and its reaction to anions. The enzyme from Paracoccus denitrificans is inhibited by citrate, bromide and iodide, whilst the NADPH-linked enzyme shows little inhibition with citrate, but significant inhibition with bromide and iodide.

Consideration should now be made of the role of hydroxypyruvate reductase in cells grown on methylamine as sole source of carbon. As mentioned previously, due to lack of other key enzymes, it cannot be involved in carbon assimilation via the serine pathway. Initially it was considered that the activity was due to a relatively unspecific enzyme which had the ability to reduce hydroxypyruvate to glycerate. However, a consideration of the substrate specificity shows

that other than hydroxypyruvate, only methylglyoxal can be utilised as a substrate by the enzyme, and this is reduced at about one seventh of the rate of hydroxypyruvate. This narrow substrate specificity indicates that hydroxypyruvate is the *in vivo* substrate for the enzyme and therefore that it is a genuine hydroxypyruvate reductase and not an unspecific enzyme.

Various roles have been ascribed to hydroxypyruvate reductases in other organisms which do not use the serine pathway as their major route of C_1 assimilation. It has been suggested by Bamforth and Quayle (1977), that the hydroxypyruvate reductase in *Paracoccus denitrificans* acts as a scavenger enzyme returning debris derived from oxaloglycollate breakdown back into mainstream metabolism. This view, however, is discounted by Colby *et al.*, (1979) and Taylor *et al.*, (1981). These groups have considered the appearance of hydroxypyruvate reductase in methane-grown *Methylococcus capsulatus* (Bath). They have concluded that the oxygenase activity of the low levels of ribulose bisphosphate carboxylase in this organism can convert ribulose bisphosphate to phosphoglycollate, which can be metabolised via glycollate and glyoxylate, yielding glycine. It has been suggested that a C_1 group probably in the form of tetrahydrofolate can combine with glycine to produce serine, which can be incorporated into cell carbon by a method analogous to the serine pathway, and thus involving hydroxypyruvate reductase. Taylor *et al.*, (1981) have assumed the presence of a full serine pathway in *Paracoccus denitrificans* and have assigned the hydroxypyruvate reductase in this organism to the same role as that proposed for it in *Methylococcus capsulates* (Bath).

However, this cannot be the reason for the presence of such an enzyme in methylamine-grown Arthrobacter 2B2 as this organism contains none of the other enzymes of the serine pathway.

The production of two hydroxypyruvate reductases in Arthrobacter 2B2 growing on choline as a source of carbon was initially studied in batch culture. This investigation proved that the two enzymes were synthesised during different phases of the growth cycle. The NADH/NADPH-linked enzyme was produced in early to mid-exponential phase, and its specific activity began to decline in late exponential phase. The strict NADPH-linked enzyme on the other hand, was not produced until late exponential phase, its production continuing into stationary phase. It is proposed that such a change could be due to a build up during the exponential phase of growth of the intermediates of choline metabolism in the growth medium, which could inhibit the production of strict NADPH-linked enzyme. Choline is a complex carbon source which is degraded through a series of intermediates. It is possible that the degradation is not completed in one sequence, but that an intermediate of the catabolic pathway is allowed to build up. This intermediate of choline metabolism could repress the production of the NADPH-linked enzyme. As the choline in the batch culture is used up, the culture becomes 'starved' of carbon and induces enzymes to further degrade the intermediate breakdown product. As the concentration of the intermediate decreases the repression is withdrawn and the NADPH-linked enzyme is produced. This proposal also fits the results obtained in chemostat continuous culture. At low growth rates the organism is under such strict carbon limitation that it is fully degrading choline

without allowing the intermediate to build up. Therefore the NADPH-linked enzyme is not repressed and is present at high activities. At high growth rates, however, the rate-limiting step in metabolism might occur at places other than in the initial oxidation steps of choline resulting in excretion of degradative intermediate(s). The build up of intermediate(s) might then repress formation of the NADPH-linked enzyme.

The growth of the organism in continuous culture using methylamine as a source of carbon indicates that no matter what the growth rate is, only the strict NADPH-linked enzyme is produced. This suggests that during growth on choline the NADH/NADPH-linked enzyme is induced rather than derepressed.

To sum up this section, the role of NADPH-linked hydroxypyruvate reductase is unknown. Its high activity in methylamine grown cells indicates that it must play a role in metabolism during growth on this carbon source. Synthesis of the enzyme appears to be inhibited during the early stages of batch growth on choline, and it is suggested that the inhibition is due to a build up of an intermediate of choline breakdown. The NADH/NADPH-linked enzyme is not produced during growth on methylamine, and is induced during growth on choline. Its levels during growth on choline do change, with more being produced in early to mid-exponential phase than in late exponential to stationary phase. However, its fluctuations are not as pronounced as those of the NADPH-linked enzyme.

Further work is required in order to confirm the hypothesis put forward to explain the role and regulation of the hydroxypyruvate reductase. Initially the inducer of

the NADH/NADPH-linked enzyme could be determined by subjecting cells growing continuously on methylamine to choline and its various breakdown products. Cells could then be harvested and assayed for the enzyme, to determine what compound or compounds would induce the cells to produce the enzyme. Secondly it would be interesting to determine the putative repressor of the NADPH-linked hydroxypyruvate reductase in choline-grown cells. This might be approached by a more detailed analysis of the contents of the growth medium during batch culture in order to detect, possibly at very low concentrations, excretion of intermediates.

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