ENERGY CONSERVATION IN *Methylophilus methylotrophus*

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Abbreviations

ADP	Adenosine-5′-diphosphate
AMP	Adenosine-5′-monophosphate
ATP	Adenosine-5′-triphosphate
CTP	Cytidine-5′-triphosphate
DEAE-cellulose	Diethylaminoethyl cellulose
DCPIP	2,6-dichlorophenol indophenol
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FPLC	Fast protein liquid chromatography
GTP	Guanosine-5′-triphosphate
HQNO	2-heptyl 4-hydroxyquinoline-N-oxide
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
PMS	Phenazine methosulphate
P_i	Inorganic orthophosphate
PP_i	Inorganic pyrophosphate
TMPD	N,N,N′,N′ tetramethyl-p-phenylene diamine
UTP	Uridine-5′-triphosphate

CHAPTER ONE

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CHAPTER ONE

INTRODUCTION

1.1 Microbiology of the methylotrophic bacteria

Methylotrophs are microorganisms which are able to grow at the expense of reduced carbon compounds which contain one or more carbon atoms but no carbon-carbon bonds (Anthony, 1982). The classification of methylotrophs has been reviewed by Anthony (1982) and is discussed only briefly here. The methylotrophic bacteria may be divided into three groups:

1) Methanotrophs
2) Methylotrophs unable to use methane
3) Facultative methylotrophs

This division reflects the range of substrates used by an organism rather than a taxonomic division; the taxonomy of the methylotrophs remains a difficult area. Separation of the facultative methylotrophs from the obligate methylotrophs can be substantiated on the basis of their different lipid contents and DNA compositions (Byrom, 1981).

1.1.1 Methanotrophs

The methanotrophs are mainly Gram-negative, strictly aerobic organisms which are able to grow on methane, methanol and other C_1 compounds. They exhibit a variety of morphological shapes and all possess a complex arrangement of internal membranes. They have been divided into two groups largely on the basis of their internal membrane structure and their carbon assimilation pathways (see Colby et al., 1975). The type I methanotrophs assimilate carbon via the ribulose monophosphate pathway and have their internal membranes arranged as bundles of vesicular discs. Type II methanotrophs utilise the serine

pathway of carbon assimilation and have paired membranes around the cell periphery.

1.1.2 Methylotrophs unable to use methane

The obligate methylotrophs which are unable to grow on methane are Gram-negative, rod-shaped organisms which assimilate carbon via the ribulose monophosphate pathway and contain predominantly C_{16} fatty acids. These organisms thus possess some features in common with the type I methanotrophs, but differ in that they do not grow on methane, possess internal membranes ,form spores or fix atmospheric nitrogen, and all grow well on methanol. The genera to which obligate methylotrophs unable to use methane have been assigned include Pseudomonas (eg. Pseudomonas C), Methanomonas, Methylomonas, Methylobacillus, Methylophilus and Achromobacter. Despite this diversity of names, many of the properties of these organisms appear similar and it has been suggested that they could be included in a single genus (Urakami and Konogata, 1979). However, more recent work indicates that these organisms are more properly divisible into two separate genera, Methylobacillus and Methylophilus (Byrom, 1981; Jenkins et al., 1984).

1.1.3 Facultative methylotrophs

The facultative methylotrophs are able to utilise multicarbon compounds in addition to methanol and other C_1 compounds. The majority of facultative methylotrophs which have been isolated by growth on methanol are also able to grow on methylamine, but the opposite does not appear to be true (Levering et al., 1981). There is a great deal of diversity amongst this group and they are represented by many genera including Pseudomonas, (eg. Pseudomonas AM1), Arthrobacter, Paracoccus, Rhodopseudomonas and Hyphomicrobium. The facultative methylotrophs may assimilate carbon via the serine pathway, ribulose

monophosphate pathway or ribulose bisphosphate pathway.

1.2 Carbon assimilation pathways of the methylotrophs

The methylotrophic bacteria utilise one (or possibly more; see Colby et al., 1979; Whittenbury, 1981) of three basic pathways for the conversion of C_1 compounds to C_3 compounds, thereafter their metabolism appears to be similar to that of heterotrophic bacteria. The 'true' methylotrophs assimilate carbon as formaldehyde via the ribulose monophosphate pathway or the serine pathway, whereas the 'pseudo-methylotrophs' initially oxidise the carbon source to carbon dioxide and assimilate this via the ribulose bisphosphate pathway (Zatman, 1981). These pathways of carbon assimilation have been extensively reviewed by Anthony (1982).

1.2.1 The ribulose monophosphate pathway

This pathway of carbon assimilation occurs in Type I methanotrophs and obligate methylotrophs, including M.methylotrophus, as well as in a number of facultative methylotrophs. It has been suggested that the ribulose monophosphate pathway may have been an evolutionary precursor of the ribulose bisphosphate pathway of carbon dioxide fixation (Quayle and Ferenci, 1978). The ribulose monophosphate cycle fixes carbon at the level of formaldehyde by condensing the latter with ribulose 5-phosphate to give hexulose 6-phosphate, which is then isomerised to fructose 6-phosphate. The two key enzymes involved in this fixation step, hexulose phosphate synthase and hexulose phosphate isomerase, are characteristic of the ribulose monophosphate pathway of carbon assimilation. These enzymes catalyse reactions whose equilibria lie far over towards hexose phosphte synthesis. The enzymes themselves possess very high affinities for their substrates and very high specific activities in crude cell extracts, and may thus form a highly efficient system for trapping free formaldehyde, a potentially toxic product of

methylotrophic growth (see Attwood and Quayle, 1984).

The subsequent reactions of the ribulose monophosphate pathway may be divided into two stages, cleavage and rearrangement, each of which has two possible variations. There are, therefore, four potential variants of this pathway. The cleavage and rearrangement parts of the pathway regenerate ribulose 5-phosphate, and overall one molecule of triose phosphate or pyruvate is formed from three molecules of formaldehyde (Fig. 1.1 and 1.2)

Fructose 6-phosphate may be cleaved either via the Entner-Doudoroff pathway or via the glycolytic pathway. The enzymes of the Entner-Doudoroff pathway, glucose 6-phosphate isomerase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrase sequentially convert fructose 6-phosphate to glucose 6-phosphate, 6-phosphogluconate and finally to 2-keto 3-deoxy 6-phosphogluconate. This is cleaved by an aldolase to form glyceraldehyde 3-phosphate and pyruvate (KDPG variant, Fig. 1.1). The alternative variant uses the enzymes phosphofructokinase and aldolase to phosphorylate fructose 6-phosphate to fructose 1,6-bisphosphate and cleave the latter to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (FBP variant; Fig. 1.2).

In the rearrangement part of the cycle two molecules of fructose 6-phosphate and glyceraldehyde 3-phosphate undergo a series of reactions leading to regeneration of 3 molecules of ribulose 5-phosphate. There are two variants of this pathway, one utilising sedoheptulose bisphosphatase (SBP variant; Fig. 1.2) but not transaldolase, and one involving transaldolase (TA variant; Fig. 1.1) but not sedoheptulose bisphosphatase.

Of the four possible variants, only two appear to be widespread. The KDPG/TA variant (Fig. 1.1) occurs mainly in obligate methylotrophs, including M.methylotrophus (Beardsmore and Quayle, 1978), whilst the

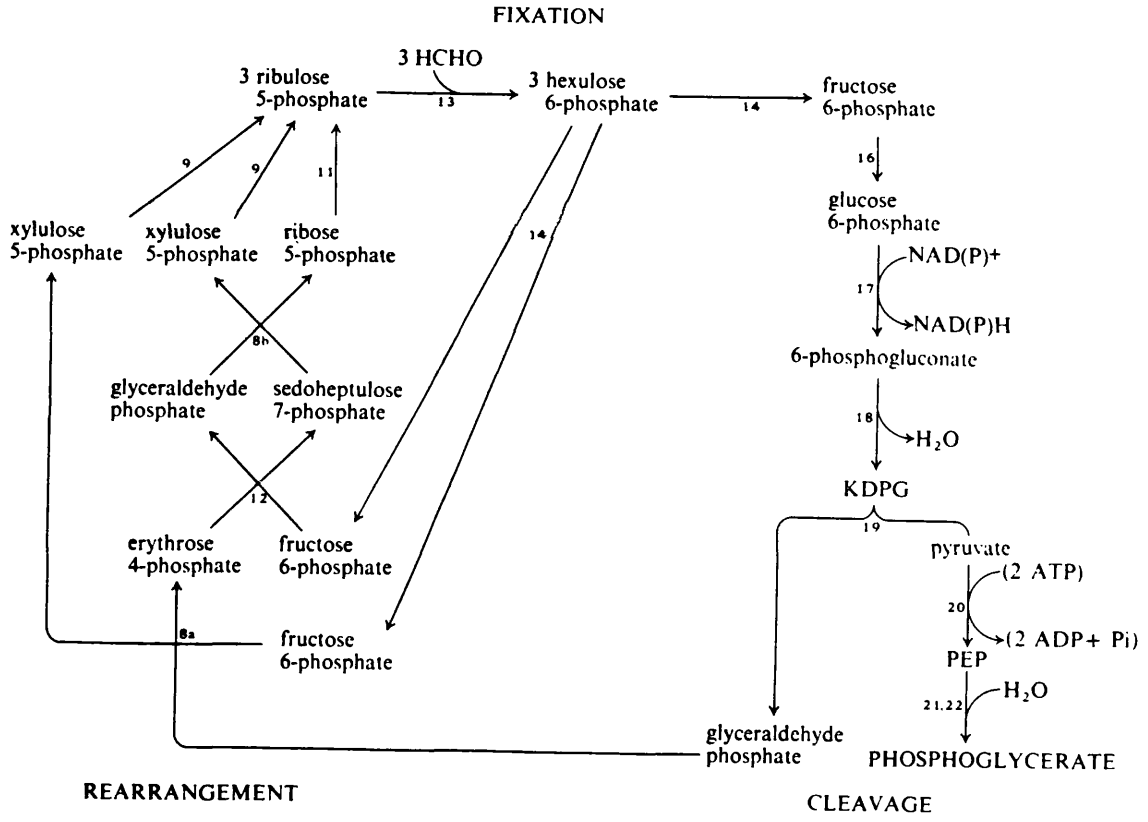


Fig. 1.1 The ribulose monophosphate pathway (KDPG aldolase/ transaldolase variant)

From Anthony (1982). The enzymes are (8) transketolase; (9) pentose phosphate epimerase; (11) pentose phosphate isomerase; (12) transaldolase; (13) hexulose phosphate synthase; (14) hexulose phosphate isomerase; (16) glucose phosphate isomerase; (17) glucose 6-phosphate dehydrogenase; (18) 6-phosphogluconate dehydrase; (19) 2-keto, 3-deoxy,6-phosphogluconate (KDPG) aldolase; (20) phospho-enolpyruvate synthetase; (21) enolase; (22) phosphoglyceromutase.

The KDPGA/TA variant pathway may be summarised thus:

$$3HCHO + NAD^+ + 2ATP \longrightarrow \text{phosphoglycerate} + NADH + H^+ + 2ADP + P_i$$

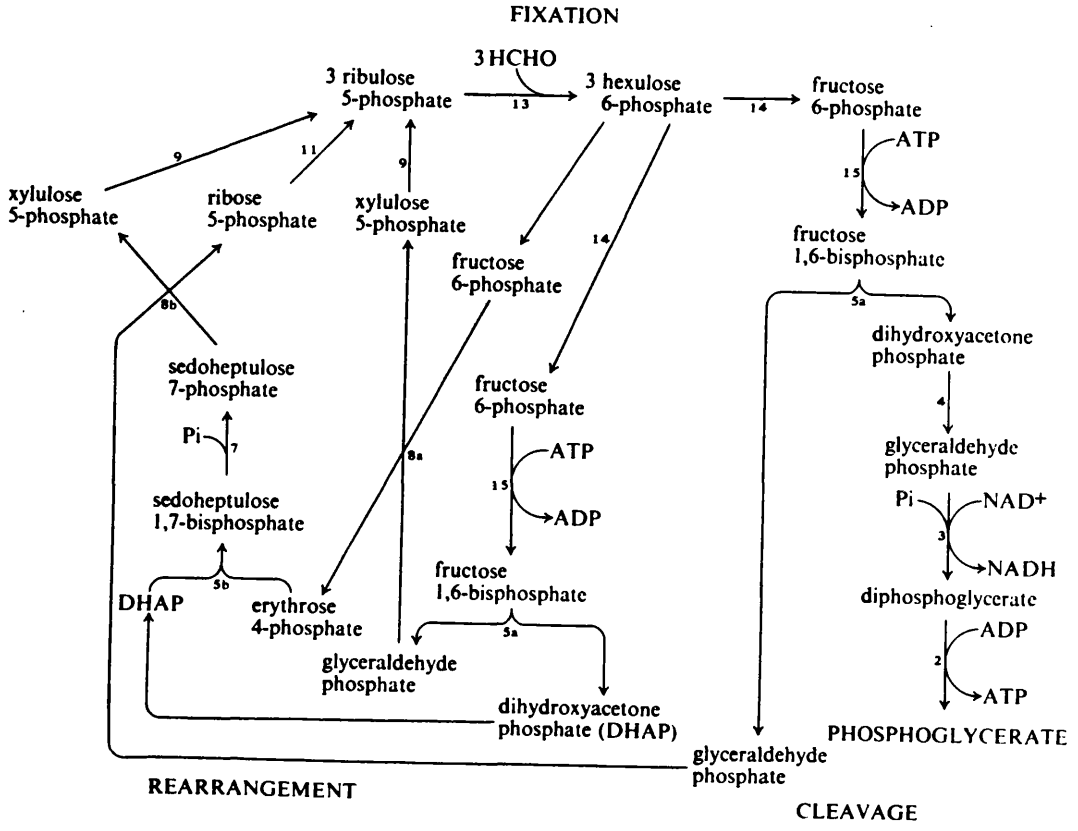


Fig. 1.2 The ribulose monophosphate pathway (fructose bisphosphate/ sedoheptulose bisphosphate variant)

From Anthony (1982). The enzymes are (2) phosphoglycerate kinase; (3) glyceraldehyde phosphate dehydrogenase; (4) triose phosphate isomerase; (5) aldolase; (7) sedoheptulose bisphosphatase; (8) transketolase; (9) pentose phosphate epimerase; (11) pentose phosphate isomerase; (13) hexulose phosphate synthase; (14) hexulose phosphate isomerase; (15) phospho-fructokinase.

The FBP/SBP variant may be summarised thus:

$$3HCHO + NAD^+ + ATP \longrightarrow \text{phosphoglycerate} + NADH + H^+ + ADP$$

FBP/SBP variant (Fig. 1.2) occurs mainly in facultative methylotrophs (Zatman, 1981).

1.2.2 The serine pathway

The first step in the serine pathway is the condensation of formaldehyde with glycine to give serine; this reaction is catalysed by the enzyme serine transhydroxymethylase which uses tetrahydrofolate as a carrier of formaldehyde. Serine is subsequently converted via reduction and phosphorylation reactions to 2-phosphoglycerate, half of which is assimilated into cell material via 3-phosphoglycerate and the remainder is converted to phosphoenolpyruvate which is carboxylated at the expense of carbon dioxide to yield oxaloacetate (Fig. 1.3). Overall, for every two atoms of carbon fixed from formaldehyde, a third atom is fixed from carbon dioxide. Malyl-CoA, derived from oxaloacetate, is cleaved to yield glyoxylate and acetyl-CoA. The acetyl-CoA is converted to glyoxylate, there being two variants in this conversion. In the icl^+ variant acetyl-CoA is oxidised via the glyoxylate cycle and involves the enzyme isocitrate lyase. In the icl^- variant this enzyme is missing and it has been proposed that the oxidation of acetyl-CoA may proceed via homo-isocitrate lyase (Kortsee, 1980; 1981; but see Bellion *et al.*, 1981).

1.2.3 The ribulose bisphosphate pathway

In the ribulose bisphosphate pathway, cell carbon is assimilated at the level of carbon dioxide. Carboxylation of ribulose 1,5-bisphosphate yields two molecules of 3-phosphoglycerate which are subsequently phosphorylated to 1,3-bisphosphoglycerate and then reduced to glyceraldehyde 3-phosphate. One molecule of the latter constitutes the final product of the cycle, whilst a further five molecules undergo rearrangement to regenerate three molecules of ribulose 1,5-bisphosphate. At least two variants of the rearrangement pathway

are known; the TA variant utilises transaldolase (Fig. 1.4), whilst the SBP variant utilises sedoheptulose bisphosphatase.

The balance of energy and reductant for the assimilation of carbon by the various pathways are summarised in Table 1.1. The pathways are normalised for formaldehyde as the substrate and 3-phosphoglycerate as the product; it is assumed that the oxidation of formaldehyde to carbon dioxide prior to assimilation of the latter via the ribulose bisphosphate pathway yields two molecules of NAD(P)H, and that NADH and NADPH are energetically equivalent. It can be seen that the relative energetic efficiencies of the pathways are in the order RMP > serine > RBP, and these theoretical predictions are supported by growth yield studies (Goldberg et al., 1976; Goldberg, 1977). Theoretical studies of predicted growth yields of methylotrophs have been made (van Dijken and Harder, 1975; Anthony, 1978).

1.3 Carbon dissimilation pathways of the methylotrophs

1.3.1 Methane oxidation

The oxidation of methane by methane mono-oxygenase has been extensively reviewed (Dalton, 1981; Higgins et al., 1981; Dalton et al., 1984) and is discussed only briefly here. Methane mono-oxygenase has a broad substrate specificity and appears to be of two types, particulate and soluble. It appears that the soluble form of the enzyme, for which NAD(P)H is the reductant, is produced in response to low concentrations of copper in the growth medium, thus suggesting that a copper protein may be necessary for optimal functioning of the methane mono-oxygenase system (see Dalton et al., 1984). Interestingly, ethanol appears to act as an electron donor for the particulate methane mono-oxygenase in Methylomonas methanica, even though no NAD^+-linked alcohol dehydrogenase could be detected in cell extracts (Leak and Dalton, 1983), and for whole cell activity in Methylomonas capsulata Bath grown in high copper

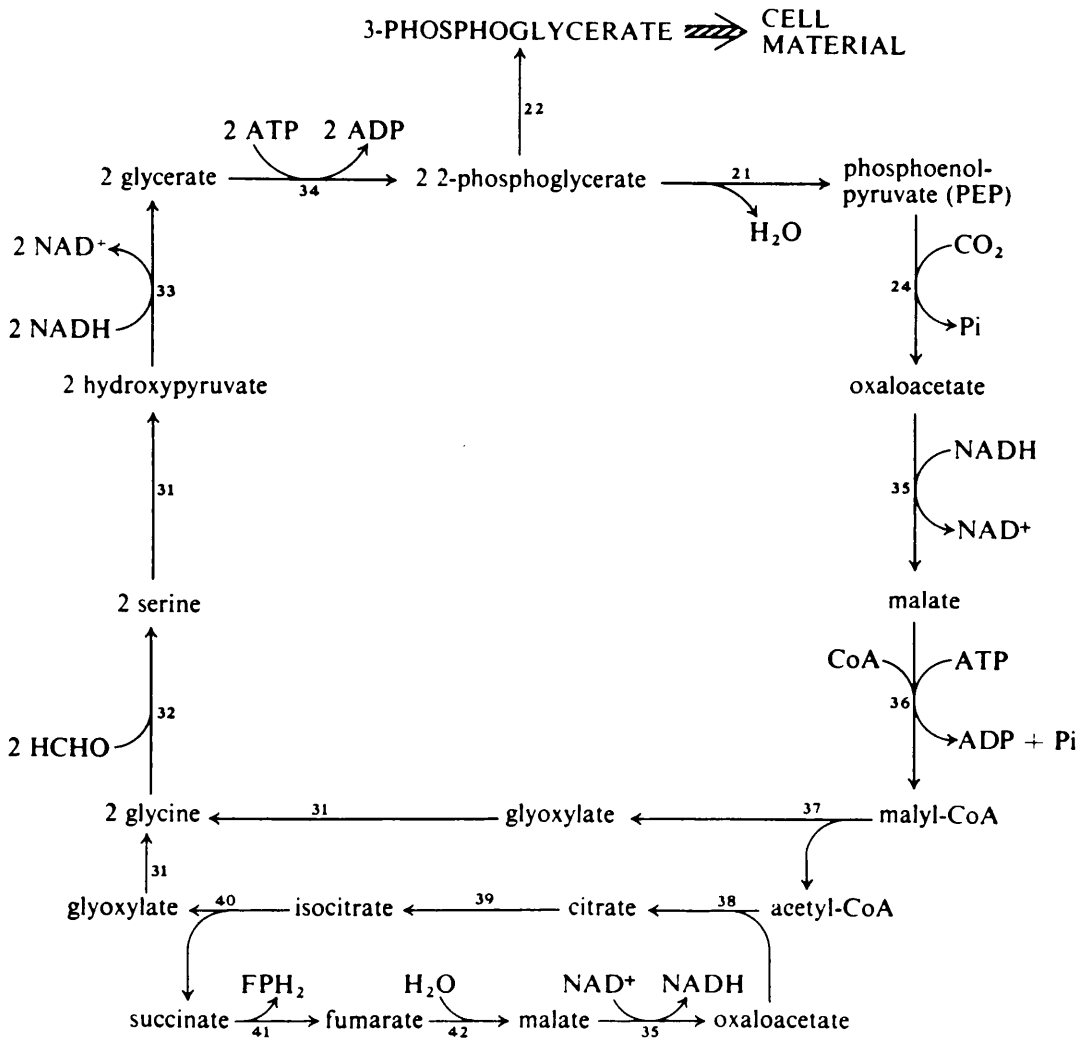


Fig. 1.3 The serine pathway

From Anthony (1982). The enzymes numbered are: (21) enolase; (22) phosphoglycerate mutase; (24) phosphoenol pyruvate carboxylase; (31) serine-glyoxylate amino transferase; (32) serine transhydroxymethylase; (33) hydroxy pyruvate reductase; (34) glycerate kinase; (35) malate dehydrogenase; (36) malate thiokinase; (37) malyl-CoA lyase; (38) citrate synthase; (39) aconitase; (40) isocitrate lyase; (41) succinate dehydrogenase; (42) fumarase.

The serine pathway may be summarised thus:

$$CO_2 + 2HCHO + 2NADH + 2H^+ + 3ATP \rightarrow 3\text{-phosphoglycerate} + 2NAD^+ + 3ADP + 2P_i + FPH_2$$

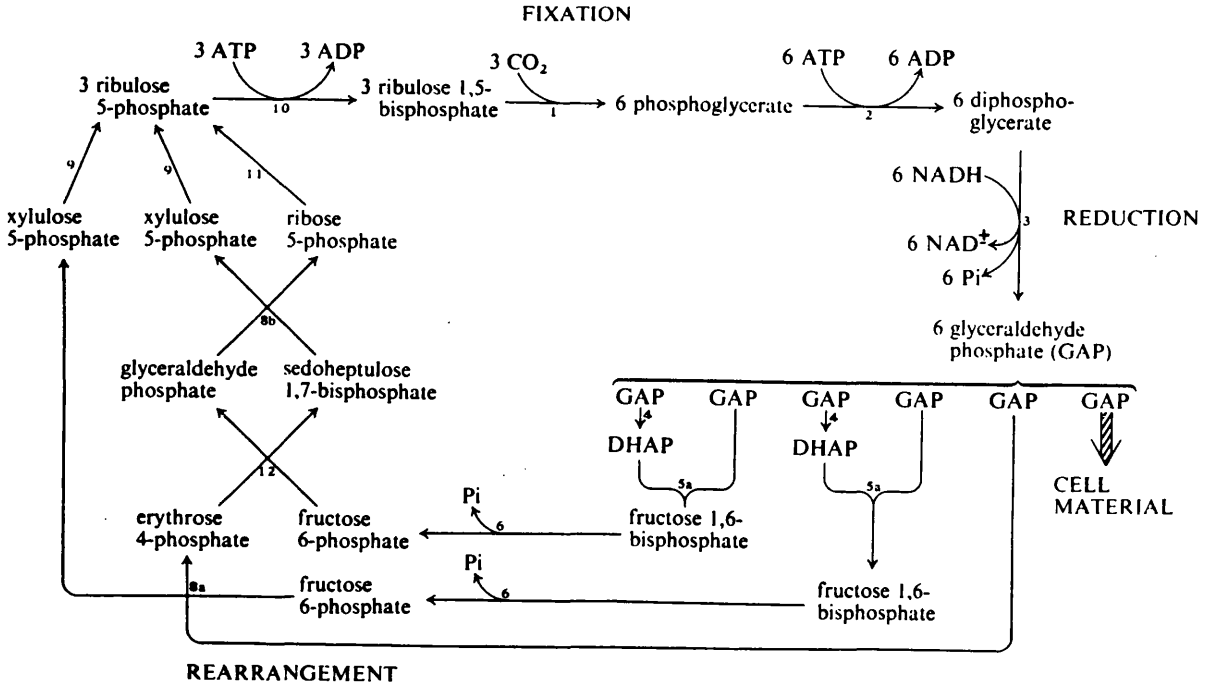


Fig. 1.4 The ribulose bisphosphate pathway (transaldolase variant)

From Anthony (1982). (1) Ribulose bisphosphate carboxylase; (2) phosphoglycerate kinase; (3) glyceraldehyde phosphate dehydrogenase; (4) triose phosphate isomerase; (5a,b) aldolase; (6) fructose bisphosphatase; (8) transketolase; (9) pentose phosphate epimerase; (10) phosphoribulokinase; (11) pentose phosphate isomerase; (12) transaldolase. The ribulose bisphosphate pathway may be summarised thus:

$$3CO_2 + 6NAD(P)H + 6H^+ + 9ATP \longrightarrow \text{glyceraldehyde 3-phosphate} + 6\ NAD(P)^+ + 9ADP + 8P_i$$

Table 1.1 Energy and reductant balances of the carbon assimilation pathways used by methylotrophs

Pathway	ATP	NAD(P)H	FH_2
Ribulose monophosphate			
KDPG/TA variant	-2	+1	0
FBP/SBP variant	-1	+1	0
FBP/TA variant	0	+1	0
KDPG/SBP variant	-3	+1	0
Ribulose bisphosphate	-8	+1	0
Serine	-3	0	+1

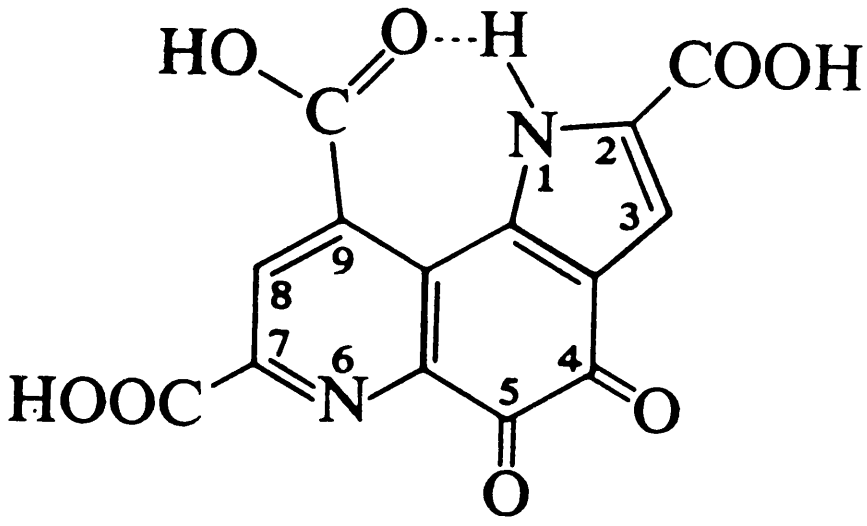
The energy and reductant balances are normalised for formaldehyde as the substrate and 3-phosphoglycerate as the product. The oxidation of formaldehyde to carbon dioxide prior to assimilation of the latter via the ribulose biphosphate pathway or serine pathway yields two molecules of NAD(P)H.

medium (when all the enzyme was in the particulate form), but not when grown in low copper medium such that the enzyme was in the soluble form. Thus the physiological reductant for the particulate methane mono-oxygenase may be generated by electron transfer from ethanol without the involvement of NADH (see Dalton et al., 1984).

1.3.2 Methanol oxidation

Methanol oxidation occurs via an $NAD(P)^+$ independent methanol dehydrogenase, the properties of which have been extensively reviewed (Duine and Frank, 1981b; Anthony, 1982; Duine et al., 1984b; Beardmore-Gray and Anthony, 1984). The purified enzyme has a subunit molecular weight of 60000 to 70000 and exists either as a monomer or a dimer (Bamforth and Quayle, 1978; Colby et al., 1979). The purified enzyme exhibits maximum activity when linked to the electron acceptor phenazine methosulphate at pH 9 in the presence of either ammonium ions or a primary amine as an activator. In whole cells, or methanol dehydrogenase purified from Hyphomicrobium under anaerobic conditions, there is no such requirement for an activator (Duine et al., 1979). The enzyme has a broad substrate specificity and can oxidise a wide variety of primary alcohols in addition to methanol. It can also oxidise its physiological product formaldehyde to formate, probably because the hydrated form of formaldehyde resembles methanol (Sperl et al., 1974). The K_m for methanol is typically very low at around 10 to 20 μM, although the methanol dehydrogenase of Rhodopseudomonas acidophila exhibits a K_m of 145 mM (Bamforth and Quayle, 1978).

The prosthetic group of methanol dehydrogenase has been purified and shown to be a nitrogen containing orthoquinone (Fig. 1.5) which has been given the semi-systematic name of pyrollo-quinoline quinone (PQQ; Duine et al., 1980). It has been suggested from electron spin resonance studies that methanol dehydrogenase contains the semi-quinone form of



PQQ
(quinone)

Fig. 1.5 The structure of Pyrollo-quinoline quinone

From Anthony (1982)

pyrollo-quinoline quinone (PQQH) and this is supported by the observation that methanol dehydrogenase can be converted to the fully reduced form ($PQQH_2$) by a one electron donor (deBeer et al., 1983).

The redox potential of the $PQQ/PQQH_2$ couple is +120 mV (Duine and Frank, 1981b), which is compatible with the coupling of methanol dehydrogenase to the respiratory chain at the level of cytochrome c. This coupling is easily destroyed, although a functional coupling of methanol dehydrogenase to cytochrome c has been demonstrated using a preparation from Hyphomicrobium made under anaerobic conditions, and a methanol-dependent reduction of pure cytochrome c_L by methanol dehydrogenase purified from M.methylotrophus has also been demonstrated (Beardmore-Gray et al., 1983). A mechanism for the reduction of cytochrome c by methanol dehydrogenase which involves an intramolecular auto-reduction has been proposed (O'Keeffe and Anthony, 1980; Beardmore-Gray et al., 1982).

Interestingly, an NAD^+-dependent, PQQ-containing methanol dehydrogenase has recently been reported (Duine et al., 1984a), but confirmation of this finding has not yet been published from other laboratories.

1.3.3 Formaldehyde oxidation

Formaldehyde produced by the oxidation of methanol or methylamine is either assimilated into cell material as described above or oxidised completely to carbon dioxide. This oxidation may be accomplished by several routes.

1) The dissimilatory ribulose monophosphate cycle

The presence of the enzyme 6-phosphogluconate dehydrogenase in organisms possessing the KDPG/TA variant of the ribulose monophosphate pathway allows these organisms to generate ribulose 5-phosphate from 6-phosphogluconate with the production of NAD(P)H and carbon dioxide.

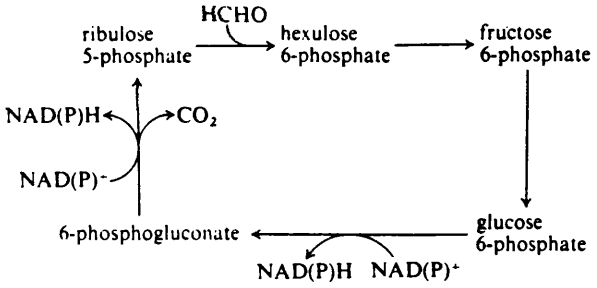


Fig. 1.6 The dissimilatory ribulose monophosphate pathway

From Anthony (1982)

This effects the complete oxidation of formaldehyde to carbon dioxide (Fig. 1.6).

The enzyme 6-phosphogluconate dehydrogenase is situated at an important point of carbon metabolism since it determines whether carbon is assimilated into cell material or dissimilated to carbon dioxide. The activity of this enzyme is controlled by the intracellular $NAD(P)H/NAD(P)^+$ and ATP/ADP/AMP ratios (Beardsmore et al., 1982).

The enzyme 6-phosphogluconate dehydrogenase has been found in a number of organisms using the KDPG/TA variant of the ribulose monophosphate pathway, including M.methylotrophus (Beardsmore et al., 1982) which uses the latter as its main route of carbon dissimilation.

2) Linear oxidation via formate

A number of enzymes which are capable of oxidising formaldehyde to formate have been identified in methylotrophic bacteria.

(a) Methanol dehydrogenase

Although methanol dehydrogenase will oxidise formaldehyde to formate in vitro, there is no evidence that this occurs to any great extent in vivo (see Hepinstall and Quayle, 1970; Dunstan et al., 1972), presumably because the affinity of the enzyme for methanol is very high and formaldehyde can be readily removed by alternative formaldehdye-utilising enzymes. Interestingly, a modifier protein (M-protein) has recently been reported from methylotrophic bacteria (Ford et al., 1985; Page and Anthony, 1986). M-protein from Pseudomonas AM1 and M.methylotrophus significantly alters the K_m and/or V_{max} of methanol dehydrogenase for various substrates, and consequently stimulates the oxidation of methanol and inhibits the oxidation of formaldehyde. Such a mechanism may play an important role in determining the fate of carbon and preventing the wasteful oxidation of formaldehyde by methanol dehydrogenase.

(b) Dye-linked aldehyde dehydrogenases

These dehydrogenases, like methanol dehydrogenase, are assayed using artifical electron acceptors such as phenazine methosulphate. The measured activities are usually low, and they are not generally induced to higher levels by growth of facultative methylotrophs on C_1 compounds (Marison and Attwood, 1980). They are therefore probably only of very limited importance in formaldehyde dissimilation.

(c) NAD^+-linked aldehyde dehydrogenases

Several types of $NAD(P)^+$-linked aldehyde dehydrogenase have been distinguished on the basis of their cofactor requirements and whether or not they are specific for formaldehyde. Some require glutathione or tetrahydrofolate as activators, whilst others have no such requirement (see Anthony, 1982). M.methylotrophus possesses a specific $NAD(P)^+$-linked formaldehyde dehydrogenase which requires glutathione for activity (Large and Haywood, 1981) but which is present in low activity.

3) Oxidation of formate

Formate is oxidised by an NAD^+-linked formate dehydrogenase in the majority of methylotrophic bacteria, including M.methylotrophus (see Johnson and Quayle, 1964).

4) Linear oxidation via tetrahydrofolate derivatives

The presence of enzymes capable of catalysing the linear oxidation of formaldehyde to carbon dioxide via tetrahydrofolate derivatives has been demonstrated in Pseudomonas AM1, and it has been suggested that this pathway might occur in all methylotrophs which utilise the serine pathway (Large and Quayle, 1963; Johnson and Quayle, 1964).

5) Oxidation via the serine pathway

It has been suggested that the oxidation of formaldehyde to

carbon dioxide may be achieved by a route which involves the enzymes of the serine pathway generating phosphoenol pyruvate which may be converted to pyruvate by pyruvate kinase. Pyruvate may be converted to acetyl CoA by pyruvate dehydrogenase and the acetyl CoA dissimilated via the tricarboxylic acid cycle (Newaz and Hersh, 1975).

1.4 Redox centres of the respiratory chain

The respiratory chain transfers electrons through a redox potential span of 1.1 V under standard conditions from the NAD^+/NADH couple ($E'\theta$ = -320 mV) to the 1/2 O_2/H_2O couple ($E'\theta$ = +820 mV). NADH acts as a mobile shuttle of reducing equivalents between the soluble NAD^+-linked dehydrogenases (eg. formate dehydrogenase, 6-phosphogluconate dehydrogenase) and the respiratory chain. It should be noted, however, that many dehydrogenases interact directly with the respiratory chain, eg. methanol dehydrogenase and methylamine dehydrogenase (both quinoproteins) interact with the respiratory chain at the level of cytochrome c, whilst trimethylamine dehydrogenase (an iron-sulphur flavoprotein) interacts with the respiratory chain at the level of the quinone pool. Thus reducing equivalents may enter at all levels of the respiratory chain. The components of the respiratory chain (dehydrogenases, quinones, cytochromes and cytochrome oxidases) act over a wide range of redox potentials and serve sequentially to transfer reducing equivalents to a terminal electron acceptor, this being oxygen in an aerobic respiratory system. These various respiratory chain components contain redox centres which are either metallic (eg. iron-sulphur, haem, copper) or organic (flavin, quinone). The structure, redox properties and spatial organisation of these redox centres have been extensively reviewed (See Ragan, 1976; Hatefi, 1985; Hauska et al., 1983; Poole, 1983; Jones and Poole, 1985, Wikström et al., 1981).

1.5 The mechanism of respiration-linked energy conservation

For many years it was believed that the the free energy released as a result of substrate oxidation by the respiratory chain was coupled to the formation of ATP via a covalent 'high energy intermediate' analogous to those involved in substrate level phosphorylation during glycolysis (Slater, 1953). Acceptance of this so-called chemical coupling hypothesis was impeded by failure to detect any such high energy intermediates or to explain the apparent need for an intact membrane.

The conformational change theory of Boyer (1965) suggested that the free energy released by substrate oxidation was stored as conformational changes induced in membrane proteins by redox activity, these conformational changes being somehow coupled to the formation of ATP.

Modern theories on the coupling between respiration and ATP synthesis postulate the importance of the proton as an intermediary between these two processes. The role of the proton in respiration-linked energy conservation has been stressed in the chemiosmotic hypothesis (Mitchell, 1961) and in the localised proton hypothesis (Williams, 1961). Modern ideas have tended to reconcile aspects of these hypotheses to form a more unified model of respiration-linked energy conservation (eg. Westerhoff *et al.*, 1984).

The chemiosmotic hypothesis was based on four fundamental postulates corresponding to four structural and functional systems (Mitchell, 1961; 1966).

1) The ATP synthase is a membrane-located, reversible proton-translocating ATPase which has a characteristic stoichiometry of proton-translocation for the synthesis of ATP.

2) The respiratory (or photoredox) chain is a membrane-located,

proton-translocating system which has a characteristic stoichiometry of proton translocation during electron transport and exhibits the same polarity of proton translocation across the membrane during normal (forward) redox activity as the ATPase does during ATP hydrolysis.

3) Proton-linked (or hydroxyl-linked) solute porter systems are present in the membrane to effect osmotic stabilisation and metabolite transport.

4) Systems 1 to 3 are plugged through a topologically-closed, insulating membrane that has a non-aqueous osmotic-barrier phase which offers a low permeability to solutes in general, and to protons and hydroxyl ions in particular.

The localised proton hypothesis (Williams, 1961; 1962; 1978a,b) has some features in common with chemiosmosis. However, the localised proton model considers the protons which couple the processes of respiration and ATP synthesis, active transport etc. to be localised within or on the membrane, rather than in the bulk phase aqueous media as envisaged by chemiosmosis. It is suggested that a kinetic barrier prevents equilibration of protons between the membrane and the bulk phases, and that protons may be transported along a network of acid/base centres (Williams, 1978b). Kell (1979) suggested that structured water at the membrane interface presented a medium of lower free energy for proton conduction than does bulk phase water. The localised proton hypothesis allows for better control of the energy-transducing systems with the possibility that energy is partitioned between ATP synthesis and other energy-requiring processes. A more recent refinement of these models, which reconciles some aspects of the localised proton and chemiosmotic theories, is the mosaic protonic coupling hypothesis (Westerhoff et al., 1984). This hypothesis retains the four postulates of the chemiosmotic theory, but in addition states that although single

primary proton-pumps do not share with other primary proton-pumps the domain into which they pump protons, they do share these domains with other individual secondary ATP-driven proton-pumps (or with a few of them). This implies that a barrier exists to the free diffusion of protons between the domains of the individual coupling units and the bulk phase of the organelle. The individual coupling units thus act independently. This model bears similarities to the localised proton model of Williams; however, the latter considers that the electrical potential is not the predominant component of the proton free energy, whilst the mosaic protonic coupling hypothesis retains the electrical potential as providing a basic contribution to the proton free energy and accepts the possibility that some energy coupling can occur via the proton electrochemical potential in the bulk phase.

The four postulates of the chemiosmotic theory have received extensive experimental attention and as a result it has been shown that (i) the translocation of protons across the membrane does accompany respiration (eg. see Harold, 1972; Haddock and Jones, 1977), (ii) electrochemical gradients of protons are formed across energy-transducing membranes (Rottenberg, 1975, 1979), (iii) an artificially-induced electrochemical gradient is able to drive ATP synthesis (Thayer and Hinkle, 1975) and (iv) the effective proton conductance of the mitochondrial membrane has been shown to be very low (Mitchell and Moyle, 1967b). However, these observations are not precluded by the localised proton hypothesis, and there are a number of observations which are more readily explained by the local proton hypothesis than by chemiosmosis (see Kell, 1979; Ferguson, 1985).

First, a transmembrane proton gradient requires a closed vesicular system if it is to exist across the bulk phases as described in the chemiosmotic hypothesis. Whilst the majority of reports indicate

the requirement for a closed membrane system for energy conservation, there have been some reports of energy conservation in non-vesicular systems (eg. Cole and Aleem, 1973; Knobloch, 1978; Storey et al., 1980).

Second, according to the chemiosmotic hypothesis, the respiration-linked translocation of protons forms a trans-membrane electrochemical potential difference of protons ($\Delta\bar{\mu}_{H^+}$; mV) which is composed of an electrical component ($\Delta\psi$) and a chemical component (ΔpH) according to the relationship

$$\Delta\bar{\mu}_{H^+} = \Delta\psi - 2.303 \frac{RT}{F} . \Delta pH$$

where R is the gas constant (8.31 $J.K.^{-1}mol^{-1}$) T is the absolute temperature and F is the Faraday constant (96487 $C.mol^{-1}$). Under equilibrium conditions the relationship between the electrochemical potential difference and the phosphorylation potential (ΔGp) is given by

$$\Delta\bar{\mu}_{H^+} = \frac{\Delta Gp}{\rightarrow H^+/ATP . F}$$

where $\rightarrow H^+/ATP$ is the stoichiometry of proton-translocation for ATP synthesis. Thus asuming a constant value of $\rightarrow H^+/ATP$, ΔGp should vary linearly with $\Delta\bar{\mu}_{H^+}$. Over a limited range of $\Delta\mu$ this behaviour was observed in brown fat mitochondria (Nicholls and Benson, 1977) and in chromatophores from Rhodopseudomonas capsulata (Baccarini-Melandri et al., 1977). However, a number of discrepancies between $\Delta\bar{\mu}_{H^+}$ and ΔGp have been observed (see Kell, 1979; Ferguson, 1985). In bovine heart submitochondrial particles the value of $\Delta\bar{\mu}_{H^+}$ varied with the incubation medium and was not accompanied by a corresponding change in ΔGp (Sorgato et al., 1978). Also, addition of an uncoupler to liver mitochondria reduced $\Delta\bar{\mu}_{H^+}$ considerably more than ΔGp (Holian and Wilson, 1980; Azzone et al., 1978). Independence of $\Delta\bar{\mu}_{H^+}$ and ΔGp has also been observed in

vesicles of Paracoccus denitrificans (Kell et al., 1978). Similarly, work with Halobacter halobium has shown that in the presence of an uncoupler there is poor correspondence between changes in $\Delta\bar{\mu}_{H^+}$ and ATP synthesis; indeed, ATP synthesis was observed under illuminated conditions in which the measured $\Delta\bar{\mu}_{H^+}$ was zero (Michels and Oesterhelt, 1980). The localised proton hypothesis attributes these observations to the measured bulk phase $\Delta\bar{\mu}_{H^+}$ being a variable under-estimate of the true driving force of ATP synthesis. Alternatively the $\rightarrow H^+$/ATP quotient may be variable; whilst this may appear an unattractive alternative, there is some evidence that the $\rightarrow H^+$/ATP quotient exhibited by the ATP-driven proton pump in the plasma membrane of Neurospora crassa may range from one to two depending on metabolic conditions (Warncke and Slayman, 1980).

Third, extreme alkalophiles grow in an environment which imposes a ΔpH across the membrane (inside acid) which is the reverse of that predicted by the chemiosmotic hypothesis, even though they are able to maintain an internal pH as high as 9.5 (see Ferguson, 1982). In order to offset this reversed ΔpH and establish a conventional protonmotive force across the membrane, the $\Delta\psi$ term would have to be extremely large. However, in spite of maintaining a relatively high internal pH, the overall measured $\Delta\bar{\mu}_{H^+}$ is still low; eg. in Bacillus alkalophilus growing at pH 11.0 the internal pH was 9.0 and the $\Delta\psi$ was 135 mV, giving a $\Delta\bar{\mu}_{H^+}$ of only 15 mV (Guffanti et al., 1978). Measurement of ATP synthesis showed that the measured $\Delta\bar{\mu}_{H^+}$ was not thermodynamically competent to account for the observed ΔGp unless the value of the $\rightarrow H^+$/ATP quotient was at least 6. However, these problems are avoided by the localised proton hypothesis (Garland, 1977; Guffanti et al., 1978).

Finally, in the absence of $\Delta\psi$ collapsing agents, a $\Delta\psi$ can be established and ATP synthesis can occur without measurable proton

translocation (Archbold et al., 1979; Conover and Azzone, 1980).

Thus although some systems probably are chemiosmotic, a localised proton gradient may be more successful in accounting for some observations. The chemiosmotic theory is, however, more amenable to experimental investigation and has therefore been widely adopted as a framework for the interpretation of experimental data. It should be noted, however, that the mosaic protonic coupling hypothesis (Westerhoff et al., 1984) goes some way towards reconciling some of the differences between these two models.

The stoichiometry of respiration-linked proton translocation ($\rightarrow H^+/O$; g-ion H^+/g-atom O), the stoichiometry of proton-translocation associated with ATP synthesis ($\rightarrow H^+$/ATP; g-ion H^+/mol ATP) and the stoichiometry of respiration-linked ATP synthesis (ATP/O; mol ATP/g-atom O) are important parameters of the chemiosmotic hypothesis which provide a measure of the efficiency of respiration-linked energy conservation. The measurement of these parameters are discussed below.

1.6 Determination of the ATP/O quotient

The efficiency with which the respiratory chain conserves energy in the form of ATP may be expressed as the number of molecules of ATP synthesised from ADP and inorganic phosphate for each atom of oxygen consumed (the ATP/O quotient). The value of the ATP/O quotient reflects the efficiency with which the respiratory chain translocates protons during substrate oxidation (the $\rightarrow H^+/O$ quotient) to generate a transmembrane electrochemical potential difference of protons, and the number of protons required to drive the synthesis of each molecule of ATP from ADP and inorganic phosphate (the $\rightarrow H^+$/ATP quotient). Three methods have been used to determine the ATP/O quotient, viz. (i) by comparing the $\rightarrow H^+/O$ and $\rightarrow H^+$/ATP quotients, (ii) by determining growth yields, and (iii) by direct measurement of ATP synthesis and oxygen

consumption. These methods are discussed below.

1.6.1 Indirect determination of ATP/O quotients by comparing $\rightarrow H^+/O$ and $\rightarrow H^+/ATP$ quotients

As the ATP/O quotient is related to the $\rightarrow H^+/O$ and $\rightarrow H^+/ATP$ quotients by the equation

$$ATP/O = \frac{\rightarrow H^+/O \quad \text{(g-equiv/g-atom)}}{\rightarrow H^+/ATP \quad \text{(g-equiv/mol)}}$$

it can be determined indirectly by measuring the $\rightarrow H^+/O$ and $\rightarrow H^+/ATP$ quotients.

(a) Determination of the $\rightarrow H^+/O$ quotient

The number of protons translocated across the membrane during electron transport has been measured in mitochondria and bacteria by three methods, viz. the oxygen pulse technique, the initial rate method and the steady-state method.

The oxygen pulse technique measures the acidification of a lightly-buffered, anaerobic suspension of respiratory organelles upon the addition of a small pulse of oxygen (Mitchell and Moyle, 1965; 1967a). The reaction is carried out in the presence of a permeant anion (eg. SCN^-) or cation (eg. K^+/valinomycin) in order to collapse the $\Delta\psi$ component of the protonmotive force and thus maximise ΔpH. The oxygen pulse technique has been criticised on several grounds, and other techniques have yielded higher values of the $\rightarrow H^+/O$ quotient. Brand et al. (1976a) suggested that the $\rightarrow H^+/2e^-$ quotient has been systematically underestimated due to the dissipation of the ΔpH by the uptake of phosphate on the $H_2PO_4^-:OH^-$ antiporter. Indeed, an average $\rightarrow H^+$/site quotient of 3 was obtained for the respiration of succinate in the presence of N-ethylmaleimide to inhibit phosphate uptake, compared with only 2 in the absence of the inhibitor. However, Mitchell (1979) has argued that the effect of N-ethylmaleimide is to increase the $\rightarrow H^+/O$

quotient towards a maximum value of 8 for the oxidation of NADH by preventing the access of reducing equivalents to the respiratory chain at the level of ubiquinone or NAD^+, thus enhancing the participation of the transhydrogenase in respiration (Mitchell, 1979; Mitchell and Moyle, 1979). It has been suggested (Heinz et al., 1981) that extrapolation of the decay of the ΔpH to the point of half-maximal pH underestimates the rate of back-leakage and thus causes an underestimate of the $\rightarrow H^+/O$ quotient.

The initial-rate method relies on the determination of the initial rate of H^+ ejection and oxygen consumption following the addition of substrate to a lightly-buffered, aerobic suspension of respiratory organelles (Reynefarje et al., 1976). Measurement of counterion movement (K^+/valinomycin) with ion-specific electrodes allow the detemination of the → charge/O quotient (Reynefarje and Lehninger, 1978; Vercesi et al., 1978). This method has yielded higher values of the $\rightarrow H^+/O$ quotient than the oxygen pulse technique, with an average $\rightarrow H^+$/site quotient of approximately 3 in mitochondria which increases to 4 in the presence of N-ethylmaleimide (see Lehninger et al., 1979). Similar results have been obtained upon initiation of respiration and proton-translocation by collapsing Δψ using K^+/valinomycin or Ca^{2+} (Azzone et al., 1979; Pozzan et al., 1979). It has, however, been suggested that with this method the high Δψ will drive super-stoichiometric uptake of K^+ or Ca^{2+} upon the addition of the Δψ collapsing agent (Mitchell, 1972).

Measurement of the $\rightarrow$charge/$2e^-$ quotient during steady-state respiration has been achieved by varying the respiration rate and measuring Δψ in the presence of sub-optimal concentrations of uncoupler. In the presence of nigericin, K^+ and acetate, ΔpH is minimised and Δψ is thus approximately equal to $\Delta\bar{\mu}_{H^+}$. Under these conditions the magnitude

of $\Delta\psi$ should be proportional to the rate of charge translocation and thus the respiration rate. Substrates coupled to the respiratory chain at different sites give different slopes in a plot of $\Delta\psi$ against respiration rate. As the slope is proportional to the →charge/O quotient, relative →charge/O quotients may be determined (Brand et al., 1978).

The $\rightarrow H^+/O$ quotient has been widely employed as an index of the efficiency of respiration-linked energy conservation in whole bacteria, and $\rightarrow H^+/O$ quotients have been measured for many species. By starving the cells and then supplying defined substrates which are capable of donating reducing equivalents to different respiratory carriers, the location of different proton-translocating sites within the respiratory chain has been determined (see Jones, 1977; also Table 1.2). These experiments indicate that the bacterial respiratory chain is organised into two, three or four proton-translocating segments. The presence of these proton-translocating segments is associated with various components of the respiratory chain. Sites I and II are present in the majority of bacteria, whilst sites O and III are dependent upon the presence of a membrane-bound nicotinamide nucleotide transhydrogenase and a high-potential cytochrome c respectively (Jones et al., 1975). Bacterial respiratory chains show a wide diversity of composition and this is reflected in the diversity of their energy conserving properties (see section 1.10). The majority of measurements have used the oxygen pulse technique and have yielded results consistent with energy conservation at site O ($\rightarrow H^+/O = 2$, →charge/O = -2), site I ($\rightarrow H^+/O = 2$, →charge/O = -2), site II, $\rightarrow H^+/O = 4$, →charge/O = -2) and site III ($\rightarrow H^+/O = 0$, →charge/O = -2 or $\rightarrow H^+/O = 2$, →charge/O =-4). Where measurements have been performed using the initial rate method, results have been consistent with oxygen pulse data (Dawson and Jones, 1981a,b; 1982).

Table 1.2 $\rightarrow H^+/O$ or $\rightarrow K^+/O$ quotients (g-ion/min/mg cells) of whole bacteria during substrate oxidation

State of cells	Substrate	Electron donor	$\rightarrow H^+/O$ and/or $\rightarrow$charge/O quotients				
			E.coli	Acinetobacter lwoffi	Pseudomonas ovalis (ng-ion/ng-atom)	Alcaligenes eutrophus	M.methylotrophus
Unstarved	Endogenous	-	-	5.69	6.59	7.82	-
Starved	Endogenous	-	3.36	4.40	5.90	6.45	-
	+ isocitrate	NADPH	-	-	-	7.62	-
	+ glutamate	(NADPH+NADH	-	5.44	-	-	-
		(NADH	-	-	5.82	-	
	+ malate	NADH	3.86	4.49	4.49±0.26(4)	-	-
	+ pyruvate	NADH	-	-	-	6.06	-
	+ formate	NADH	-	-	-	-	6.27
	+ lactate	F_pH_2	2.26	-	-	-	-
	+ glycerol	F_pH_2	-	-	3.9	-	-
	+ succinate	F_pH_2	-	-	-	4.49	-
	+ ascorbate-TMPD	cytochrome c	-	-	2.55	2.56	1.78
Energy conserving sites			I,II	0,I,II	0,I,II,III	0,I,II,III	I,II,III

From Jones (1977); Dawson and Jones (1981a,b)

However, a $\rightarrow H^+/2e^-$ up to 4 for site I and an $\rightarrow H^+/2e^-$ of 2 for site III (a H^+ pumping cytochrome $\underline{aa}_3$) have been claimed (Van Verseveld et al., 1981).

(b) Determination of the $\rightarrow H^+$/ATP quotient

A value for the stoichiometry of proton translocation during ATP synthesis or hydrolysis may be obtained either by direct kinetic measurement or indirectly by comparing the thermodynamic parameters of chmemiosmosis ($\Delta\bar{\mu}_{H^+}$ and ΔGp).

Measurement of H^+ uptake during ATP synthesis in whole bacteria gives $\rightarrow H^+$/ATP quotients of greater than 5 g-ion H^+/mol ATP (Maloney, 1977; 1978). These values are, however, almost certainly overestimated due to consumption of ATP by cellular reactions and by uptake of H^+ via other transport systems.

Measurement of H^+ uptake by inverted membrane vesicles should overcome many of these problems. However, experiments using E.coli vesicles yielded a value of only 0.6 g-ion H^+/mol ATP, presumably because of poor coupling (West and Mitchell, 1974). A value of 2 g-ion H^+/mol ATP has, however, been obtained using the ATP phosphohydrolase of the thermophile PS3 incorporated into liposomes at a very high phospholipid:protein ratio which ensures a low passive permeability to protons (Sone et al., 1976). Direct measurement of ATP-driven proton uptake into submitochondrial particles suggested an $\rightarrow H^+$/ATP quotient of 2 g-ion H^+/mol ATP (Thayer and Hinkle, 1973), whilst in whole mitochondria a value of 3 g-ion H^+/mol ATP was obtained (Alexandre et al., 1978) which increased to 4 when calculated by comparing $\rightarrow H^+$/O and ATP/O quotients (Brand et al., 1976a,b). Part of this proton translocation is due to the combined action of the adenine nucleotide antiporter, which exchanges ATP^{4-} for ADP^{3-} across the mitochondrial membrane, and the uptake of $H_2PO_4^-$.

Values of the $\rightarrow H^+/ATP$ quotient have been obtained indirectly by comparing the measured electrochemical potential difference of protons ($\Delta\bar{\mu}_{H^+}$) and the phosphorylation potential ΔGp. In a perfectly coupled system at equilibrium,

$$\frac{-2\ \Delta E_h}{\rightarrow H^+/2e^-} = \Delta\bar{\mu}_{H^+} = \frac{\Delta Gp}{F.\ \rightarrow H^+/ATP}$$

where ΔE_h is the difference in redox potential between the oxidising and reducing couples, $\rightarrow H^+/2e^-$ is the stoichiometry of respiration-linked proton translocation, and F is the Faraday constant. ΔGp is the phosphorylation potential as defined by the equation

$$\Delta Gp = \Delta G^{\theta\prime} - 2.303\ \frac{RT}{F}\ \log_{10}\ \frac{[ATP]}{[ADP].[P_i]}$$

where $\Delta G^{\theta\prime}$ is the standard free energy change for ATP hydrolysis. Comparison of the values of $\Delta\bar{\mu}_{H^+}$ and ΔGp have been made for both mitochondria and bacteria.

The electrochemical potential difference of protons (the protonmotive force; $\Delta\bar{\mu}_{H^+}$) is composed of an electrical component ($\Delta\psi$) and a chemical component (ΔpH):

$$\Delta\bar{\mu}_{H^+} = \Delta\psi - 2.303\ \frac{RT}{F}\ \Delta pH$$

Measurement of $\Delta\psi$ has been achieved by four principle methods; viz. using micro-electrodes, measuring the distribution of permeant ions in response to $\Delta\psi$, measuring the changes in ion distribution on de-energisation, and using fluorescent probes. The principle techniques available for the determination of ΔpH are the measurement of the distribution of permeant weak acids or bases in response to ΔpH, the use of indicator dyes and ^{31}P-nuclear magnetic resonance spectrometry.

These techniques have been extensively reviewed (Rottenberg, 1979; Ferguson and Sorgato, 1982; Ferguson, 1985). Measurements of the protonmotive force have been made in several species of mesophilic bacteria (Table 1.3).

The values of $\Delta\psi$, ΔpH and thus $\Delta\bar{\mu}_{H^+}$ obtained can depend upon the technique used; many of these techniques have possible disadvantages (see Ferguson and Sorgato, 1982).

The use of micro-electrodes appears to be the most direct method for measuring $\Delta\psi$; however, this method causes disruption of the cell membrane during the insertion of the electrode, and the small size of bacteria generally precludes this technique. Despite these shortcomings a value of $\Delta\psi$ of 150 mV has been obtained in giant cells of E.coli by the use of microelectrodes (Felle et al., 1980), a value similar to that obtained by ion distribution methods. The latter method has been criticised on the grounds that permeant ions used to measure $\Delta\psi$ and ΔpH may bind to cell components (Holian and Wilson, 1980). Measurement of $\Delta\psi$ by ion distribution methods or by changes in pH and pK ($-\log[K^+]$) have given values of approximately 150 to 230 mV in whole bacteria and membrane vesicles. In contrast, measurement of the membrane potential by means of the shift in the absorption spectrum of the carotenoids in photosynthetic bacteria in response to the membrane potential (the electrochromic shift) tends to give higher values of $\Delta\psi$. A comparison of the values of $\Delta\bar{\mu}_{H^+}$ obtained by different methods in chromatophores of Rhodopseudomonas capsulata under conditions where ΔpH was negligible, has yielded a $\Delta\psi$ of 160 mV when measured by the ion distribution method and 290 mV from the electrochromic shift (Clarke and Jackson, 1981). The latter method has been criticised on the grounds that calibration of the electrochromic shift by the imposition of a potassium diffusion potential may only be carried out in the range -150 to +150 mV, thus

Table 1.3 The Protonmotive Force in Bacteria

Organism	Preparation	$\Delta\psi$ (mV)	$-z\Delta pH$ (mV)	$\Delta\mu_{H^+}$ (mV)	$\Delta\psi$ Methods	ΔGp (kJ/mol)	References
E.coli	whole cell	150			electrode		Felle *et al.*, 1980
Streptococcus aureus	whole cells	134	67	211	(pH and pK change		Collins and Hamilton, 1976
E.coli	sphaeroplasts	132	98	230	(on de-energisation		
M.methylotrophus	whole cells	144	0	144	ion distribution	45.8	Dawson and Jones, 1982
Paracoccus denitrificans	vesicles	145	<30	145	ion distribution	53.5	Kell *et al.*,1978
Rhodopseudomonas capsulata	chromatophores	160			ion distribution		
"	"	290			electrochromic shift		Clarke and Jackson, 1981
"	"	206	198	419	electrochromic shift	65.4	Casadio *et al.*, 1974

measurement of higher potentials requires extrapolation of the calibration curve with the assumption of linearity (Baccarini-Melandri et al., 1982). It has also been suggested that when membrane potentials are generated by light, the perturbation of the local electric field is possibly quite different from the perturbations which occur following the imposition of a diffusion potential (Rottenberg, 1979).

Comparison of $\Delta\bar{\mu}_{H^+}$ measured in membrane vesicles with ΔGp indicates a minimum $\rightarrow H^+/ATP$ quotient of 3 g-ion H^+/mol ATP (eg. Kell et al., 1978). In contrast, the higher values of $\Delta\bar{\mu}_{H^+}$ obtained when $\Delta\psi$ is measured by the electrochromic shift are consistent with an $\rightarrow H^+/ATP$ quotient of 2 g-ion H^+/mol ATP (Casadio et al., 1974; Baccarini-Melandri et al., 1977).

Measurements of the stoichiometry of proton and charge translocation in bacteria indicate an average $\rightarrow H^+$/site quotient of 2. The value of the $\rightarrow H^+/ATP$ quotient is less clear, with the majority of measurements suggesting a value of 2 or 3, but possibly as high as 4 g-ion H^+/mol ATP. From these values an average ATP/site quotient of 1, 0.66 or 0.5 may be calculated. Technical difficulties in the measurement of $\rightarrow H^+/ATP$ quotients precludes greater precision than this.

1.6.2 Prediction of the ATP/O quotient from growth yields

If both the ATP yield and the mass of cells produced during growth are known, then the cell yield with respect to ATP can be calculated (Y_{ATP} g cells/mol ATP; Bauchop and Elsden, 1960). Conversely, if Y_{ATP} were known it would be possible to predict the ATP yield. Early work in this area was carried out with batch cultures, and thus no account was taken of the effects of growth rate and maintenance energy on cell yields. The later use of continuous culture has largely enabled these faults to be rectified (see Stouthamer and Bettenhausen, 1973).

The growth yield of an anaerobic, glucose-limited culture is described by the equation

$$\frac{1}{Y_{glu}} = \frac{1}{Y_{glu}^{max}} + \frac{M_{glu}}{\mu}$$

where Y_{glu}^{max} is the maximum molar growth yield for glucose (g cells/mol glucose), M_{glu} is the maintenance requirement (mol glucose/g cells/h) and μ is the specific growth rate (h^{-1}; equivalent to the dilution rate, D). Thus Y_{glu}^{max} may be obtained from the intercept of the plot of $1/Y_{glu}$ against $1/\mu$. As the ATP yield during the fermentation of glucose by any given pathway is well known (nmol ATP/mol glu), the value of Y_{glu}^{max} may be used to calculate a value for the maximum molar growth yield for ATP (Y_{ATP}^{max} g cells/mol ATP) since $Y_{glu}^{max} = Y_{ATP}^{max} . n.$ Y_{ATP}^{max} values have been measured for a large number of organisms (see Stouthamer, 1979), and although it has been shown that various environmental parameters and the nature of the carbon source both affect Y_{ATP}^{max}, the values reported for most glucose-limited cultures are between 10 and 12 g cells/mol ATP, eg. a value of Y_{ATP}^{max} of 12.4 g cells/mol ATP has been determined for glucose-limited, anaerobic cultures of E.coli (Stouthamer and Bettenhausen, 1975).

In aerobic organisms growing under energy-limited conditions, Y_{ATP}^{max} and the overall ATP/O quotient (equivalent to the respiratory chain ATP/O quotient if the relatively small yield of ATP from substrate-level phosphorylation is ignored) are related by the equation

$$Y_{O_2}^{max} = Y_{ATP}^{max} . 2(ATP/O)$$

where $Y_{O_2}^{max}$ is the maximum molar growth yield for oxygen corrected for maintenance (g cells/mol O_2). By obtaining the Y_{ATP}^{max} value for growth under anaerobic conditions and applying this value to a measured value of $Y_{O_2}^{max}$, a value for the ATP/O quotient has been obtained. This method

has yielded ATP/O quotients of 1.23-1.40 and 2.4 respectively for glucose-limited cultures of the facultative anaerobes Aerobacter aerogenes (Stouthamer and Bettenhausen, 1975) and E.coli (Hempfling and Mainzer, 1975). By comparing these values with whole cell $\rightarrow H^+/O$ quotients, it has been suggested that the membrane-bound ATP synthase exhibits an $\rightarrow H^+/ATP$ quotient of approximately 2 (Jones, 1977). This approach requires the assumption that Y_{ATP}^{max} for a facultative anaerobe will be the same under anaerobic and aerobic conditions. However, it has recently been shown that the efflux of fermentation products down their concentration gradients may be coupled to energy conservation (Michels et al., 1979; Otto et al., 1980). Also for an obligate aerobe it is necessary to use an average value of Y_{ATP}^{max} obtained from anaerobic organisms. Furthermore, analysis of growth yield in terms of ATP is based on the assumption that growth is energy-limited; whilst this may be true for anaerobes in complex media, under aerobic conditions where the carbon substrate serves as both a carbon and energy source growth may be either carbon-limited or energy-limited (Tempest and Neijssel, 1984; Anthony, 1982). Thus growth on methanol (CH_4O) is possibly carbon-limited, whilst growth on succinate [$(CH_{1.5}O)_4$] is probably energy-limited, and the latter type of substrate may be the best to study growth yields with respect to energy conservation efficiencies.

Theoretical values of Y_{ATP}^{max} have been calculated from the macromolecular composition of the cells and the amount of ATP required to make cell material of this composition. A theoretical value for Y_{ATP}^{max} of 24.6 g cells/mol ATP has been calculated for Klebsiella aerogenes growing at high dilution rate, compared with an experimental value of 11.7 g cells/mol ATP (Tempest and Neijssel, 1984). This discrepancy between experimentally-determined and theoretical values of Y_{ATP}^{max} is widespread (see Stouthamer, 1979). In order to account for this

difference, the use of a 'coupling factor (K)' has been proposed:

$$Y_{ATP}^{max} = K.\ \text{theoretical}\ Y_{ATP}^{max}$$

This equation indicates that not all the energy generated during substrate oxidation is used for biosynthesis and growth-rate independent maintenance, but that part of the energy is lost. It has been suggested that the latter is due to a certain amount of uncoupling between energy generation and growth (Harder and van Dijken, 1976), or that the chemiosmotic reactions involved in ATP synthesis are not fully coupled (Harder et al., 1981; Tempest and Neijssel, 1984). Calculations of ATP/O quotients from growth yield studies are unable to differentiate between these two possibilities.

1.6.3 Direct measurement of the ATP/O quotient

Direct measurement of ATP synthesis concomitant with oxygen consumption provides a means of measuring the ATP/O quotient which avoids many pitfalls in the techniques previously described. Such measurements have been attempted using both membrane vesicles and whole bacteria and the results are discussed more fully in chapter 4.

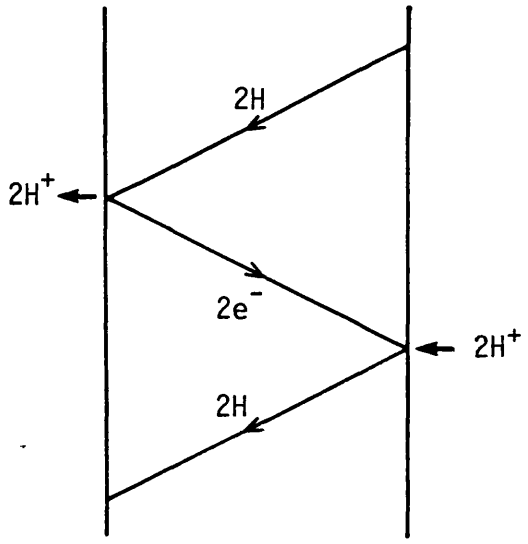
1.7 The mechanism of respiration-linked proton translocation

The stoichiometry of respiration-linked proton-translocation is an important consideration in determining the mechanism of this process. The original chemiosmotic hypothesis envisaged proton translocation as occurring by a redox loop mechanism, each loop consisting of the outward transfer of two hydrogen atoms followed by the inward transfer of two electrons (Fig. 1.7a; Mitchell, 1966; 1968). This mechanism was later modified to include a protonmotive quinone cycle at site II (Fig. 1.7b; Mitchell, 1975a,b). Site III formed a redox arm which allowed electron transfer inwards to be coupled to proton consumption (Fig. 1.7c).

These mechanisms predict fixed stoichiometries of proton translocation (ie. loop $\rightarrow H^+/2e^-$ = 2; quinone cycle $\rightarrow H^+/2e^-$ = 4; redox

arm $\rightarrow H^+/2e^- = 0$). In contrast, a proton pump makes no predictions on the stoichiometry of respiration-linked proton translocation. The proton pump mechanism proposes a pumping of protons across the membrane rather than the effective proton translocation resulting from opposite movements of H^+ and e^- (Fig. 1.7d). A Bohr-like mechanism for proton pumping has been proposed (Papa, 1976) by analogy with haemoglobin; reduction of a metallic redox centre causes an increase in the pK_a of an acidic group on the protein at the inner surface of the membrane resulting in protonation of this group. Reoxidation of the redox centre causes a decrease in the pK_a of an acidic group at the outer surface of the membrane and thus proton release to the external medium. Translocation of a proton between the two acidic groups, or movement of one acidic group between the two positions, is brought about by the opening of an assymetric channel via a conformational change. The stoichiometry of proton translocation by a Bohr-like proton pump will be pH-dependent unless the acidic group is exposed to the inner phase only in the deprotonated form and to the outer phase only in the protonated form.

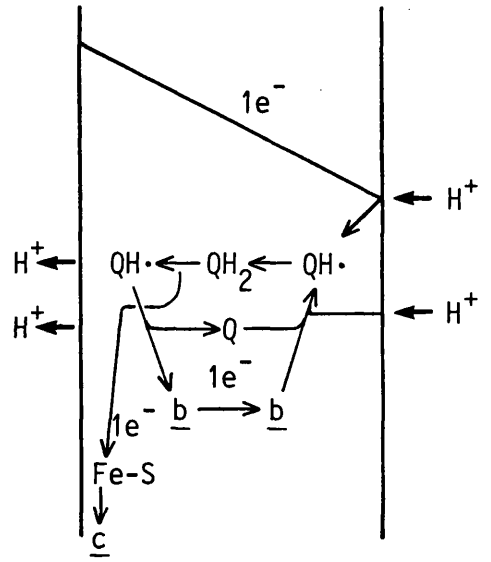
A refinement of the proton pumping mechanism has recently been proposed (see Wikström et al., 1981). In the reciprocating-site mechanism proposed for the proton pumping activity of mitochondrial cytochrome aa_3, the dimeric nature of the cytochrome $(aa_3)_2$ is considered an integral part of the mechanism. One half of the dimer attains a state where cytochrome a is in redox contact with cytochrome c on the c-side of the membrane and the cytochrome a haem or a group in its immediate vicinity may become protonated on the M-side of the membrane. The other half of the dimer is in a state where cytochrome a is in redox contact with the cytochrome a_3/Cu_B centre and the cytochrome a haem or a group in its immediate vicinity may become de-protonated at



(a)

→$H^+/2e^-$ = 2

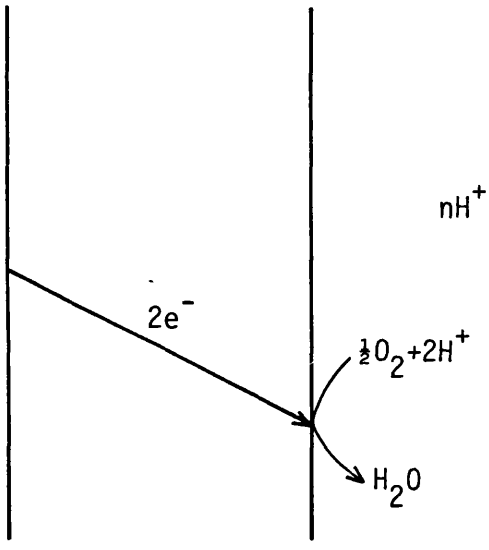
→charge/$2e^-$ = -2



(b)

→$H^+/2e^-$ = 4

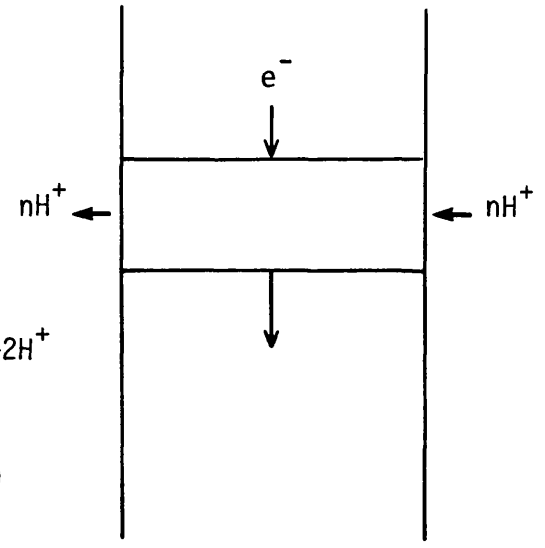
→charge/$2e^-$ = -2



(c)

→$H^+/0$ = 0

→charge/0 = -2



→$H^+/2e^-$ = n

Fig. 1.7 Mechanisms of proton and charge translocation

(a) Protonmotive redox loop

(b) Protonmotive quinone cycle

(c) Redox arm

(d) Proton pump

the M-side. Completion of the redox reactions in both halves of the cytochrome $(aa_3)_2$ dimer results in transition to the alternative state by both cytochrome $\underline{aa_3}$ complexes.

1.8 Utilisation of the protonmotive force

The protonmotive force generated by respiration may be used to drive a number of energy-dependent processes such as ATP synthesis, solute transport, pyrophosphate synthesis, motility and reversed electron transfer.

1.8.1 ATP synthesis

The protonmotive force drives ATP synthesis via the membrane-bound ATP synthase. The structure of both the mitochondrial and bacterial enzymes has been extensively reviewed (Penefsky, 1979 Jones, 1979; Downie et al., 1979; Munoz, 1982; Cox et al., 1984). The ATP synthase may be separated into two components, a hydrophilic F_1 component and a hydrophobic F_o component. The soluble F_1 component catalyses the rapid hydrolysis of ATP and may be dissociated into five subunits. These subunits are believed to be in the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$. The F_1 complex is believed to be the catalytic subunit of the ATP synthase, the ATP hydrolysis reaction being reversed by the protonmotive force. The F_o complex consists of hydrophobic proteins embedded in the membrane to form a proton conductor across the membrane to the catalytic site of F_1. The mechanism by which the protonmotive force drives ATP synthesis remains to be resolved. Modern ideas emphasise the importance of substrate binding in lowering the free energy of hydrolysis of the phosphate anhydride bond in the enzyme-ATP complex, and a possible role of protons in causing a conformational change in the enzyme, resulting in release of ATP, this being the major energy-requiring step in the synthesis of ATP (Cross, 1981; Harris, 1982; Cox et al., 1984). The number of protons required to drive ATP

synthesis, the $\rightarrow H^+$/ATP quotient, remains an area of contention (see section 1.6).

1.8.2 Solute transport

Some small uncharged molecules (eg. CO_2, O_2) may enter the cell in an energy-independent manner by simple diffusion. No carrier molecule is required in this process so saturation of uptake does not occur at high concentrations of solute. In some cases (eg. glycerol) diffusion may be facilitated by a carrier molecule, and in this case saturation kinetics are observed. Neither simple nor facilitated diffusion allows substrates to be accumulated within the cell. However, many substrates can be accumulated within the cell by energy-dependent, active transport processes (see Harold, 1972; Hamilton, 1977).

(i) Group translocation. In this process the solute undergoes chemical modification during transport. For example, the uptake of glucose into obligate and facultative anaerobes by the membrane bound phosphotransferse system results overall in phosphorylation of the glucose to glucose-6-phosphate at the expense of phosphoenol pyruvate.

(ii) $\Delta\bar{\mu}_{H^+}$-dependent solute transport. The accumulation of various substrates may be driven by ΔpH and/or $\Delta\psi$.

a) $S:H^+$ symport. The accumulation of various uncharged molecules is driven by both the $\Delta\psi$ and ΔpH components of the protonmotive force during co-transport with a proton (or anti-transport with an hydroxyl ion, the thermodynamic equivalent) eg. lactose permease of E.coli.

b) $S^-:H^+$ symport. The accumulation of a negatively charged solute by co-transport with a proton is an electroneutral event and is thus driven by ΔpH alone eg. lactate flux in Streptococcus faecalis, phosphate uptake in Paracoccus denitrificans.

c) S^+ uniport. Accumulation of cationic substrates is an

electrogenic event and is thus driven by $\Delta\psi$ alone in whole cells (inside negative) eg. uptake of proline and glycine in E.coli.

d) $H^+:S^+$ antiport. The ejection of a cation coupled to the uptake of a proton is an electroneutral event and is thus driven by ΔpH alone eg. the proton:sodium antiport may catalyse the extrusion of Na^+ from E.coli.

(iii) $\Delta\bar{\mu}_{Na^+}$-dependent solute transport. The transport of various metabolites may be driven by a gradient of sodium ions across the cell membrane, eg. mellibiose permease in E.coli.

(iv) ATP-dependent solute transport. The accumulation of some substrates requires the presence of periplasmic binding proteins. This process is driven by hydrolysis of ATP derived either from respiration or substrate level phosphorylation, eg. glutamine and arginine transport in E.coli. The $\Delta\bar{\mu}_{H^+}$ across the membrane may be necessary for the interaction between the binding protein-solute complex and a membrane bound protein (see Booth and Hamilton, 1980).

The transport of various solutes and the maintenance of ionic gradients is a major function of the protonmotive force, particularly under conditions of nutrient or ion-limitation where extensive accumulation must occur.

1.8.3 Motility

Many species of bacteria are flagellated and therefore capable of movement. Rotation of bacterial flagella is driven by the protonmotive force (see Macnab and Aizawa, 1984). It has, however, been suggested that the energy used in motility is very small (Drozd, J.W. and Koshland, D.E., personal communication cited in Stouthamer, 1979).

1.8.4 Reversed electron transport

Reduction of NAD^+ by succinate using reversed electron transfer has been demonstrated in many species of bacteria including

P.denitrificans and E.coli. This process is sensitive to uncoupling agents and is a potential means of dissipating the protonmotive force (see Harold, 1972).

1.8.5 Pyrophosphate synthesis

The synthesis of inorganic pyrophosphate may be driven by electron transport in mitochondria (Mansurova et al., 1975) or in chromatophores of Rhodospirillum rubrum (Baltscheffsky and van Stedingk, 1966). In both cases, pyrophosphate synthesis is inhibited by uncoupling agents and increased by inhibition of ATP synthesis (Mansurova et al., 1977; Mansurova et al., 1975, Baltscheffsky and van Stedingk, 1966), indicating that it occurs at the expense of the protonmotive force. This is confirmed by the observation that the hydrolysis of pyrophosphate, like ATP, generates a $\Delta\bar{\mu}_{H^+}$ (Kondrashin et al., 1980). The presence of inorganic pyrophosphate has been reported at a concentration of 0.5 mM in E.coli (Kukko and Heinonen, 1982) although it is not clear whether this arose via direct synthesis from inorganic phosphate or by hydrolysis of phosphorylated compounds such as ATP during amino acid activation.

Synthesis of pyrophosphate may be a widespread property of respiratory organelles, and may thus be a route for dissipation of the protonmotive force. The function of pyrophosphate remains uncleaar, although in some biochemical conversions it may perform a function similar to ATP. It has also been suggested that the PP_i/P_i ratio may control metabolic reactions (Reeves, 1976).

1.9 Organisation of the aerobic respiratory chain

The sequence of electron carriers in the respiratory chain has been established by the use of the oxygen electrode and various spectroscopic techniques in conjunction with specific respiratory chain inhibitors. Measurement of the stoichiometry of respiration-linked

proton translocation has identified four sites of energy conservation, and these sites have been correlated with the presence of discrete respiratory chain complexes. All four sites may occur in bacteria, although many species may lack one or more coupling sites.

1.9.1 Site O, nicotinamide nucleotide transhydrogenase

Transhydrogenases catalyse the transfer of a hydride ion between NADPH and NAD^+. As the redox potentials of the NAD^+/NADH and $NADP^+$/NADPH couples differ by only 4 mV, the equilibrium constant is close to unity. The membrane-bound transhydrogenase is commonly found in a wide range of bacteria (eg. E.coli, Paracoccus denitrificans) and is energy-linked, ie. it utilises the protonmotive force to catalyse reversed electron transfer from NADH to $NADP^+$, and thus links the NAD^+-linked dehydrogenases of catabolism to the supply of NADPH for biosynthesis. This transhydrogenase is specific for the 4A hydrogen atom of NADH and the 4B hydrogen atom of NADPH. The membrane-bound transhydrogenases of bacteria and mitochondria appear to be similar (eg. Asano et al., 1967); both lack obvious redox centres and thus probably act as a Bohr-like proton-pump (section 1.7). The stoichiometry of proton translocation has been claimed to be $\rightarrow 2H^+/2e^-$ in both bacteria and mitochondria (Scholes and Mitchell, 1970; Moyle and Mitchell, 1973).

A soluble, energy-independent transhydrogenase is present in some species of bacteria, eg. Azotobacter vinelandii; this enzyme is a flavoprotein and is specific for the 4B hydrogen atom of both NADH and NADPH.

1.9.2 Site I, NADH : quinone oxidoreductase

The NADH : ubiquinone oxidoreductase complex of mitochondria (complex I) is a large multisubunit protein with at least sixteen polypeptides together with one molecule of FMN and between four and seven iron-sulphur centres. The complex spans the respiratory

membrane, but the oxidation of NADH occurs at the inner surface (see Ragan, 1976; Hatefi, 1985).

NADH dehydrogenases have been purified from several species of bacteria including Acholeplasma laidlawii (Jinks and Matz, 1976), Photobacterium phosphoreum (Imagawa and Nakamura, 1978), Bacillus caldotenax (Kawada et al., 1978) and E.coli (Jaworowski et al., 1981). The bacterial NADH dehydrogenases appear to contain from one to three subunits. The enzymes of the latter three organisms, unlike that of the mitochondrion, contain FAD rather than FMN and are composed of only one subunit. The reconstitution of NADH oxidase activity by the addition of an E.coli NADH : quinone oxidoreductase to membrane vesicles prepared from mutants lacking the enzyme has been demonstrated (Jaworowski et al., 1981). A structural relationship between the NADH dehydrogenases of Paracoccus denitrificans and bovine heart mitochondria has been proposed on the basis of immunological cross-reactivity (George et al., 1986).

1.9.3 Site II, quinol : cytochrome c oxidoreductase

Quinol : cytochrome c reductase of mitochondria (complex III) contains eight subunits including cytochromes b_{562}, b_{566} and c_1 together with at least one high redox potential iron-sulphur protein (the Rieske Fe-S protein; see Trumpower and Katki, 1979; Hauska et al., 1983; Hatefi, 1985). It has been suggested that ubiquinone is bound to specific quinone-binding proteins in the membrane (see Yu and Yu, 1981; Yu et al., 1978). The addition of one such protein to complex III preparation from which it had been removed led to the restoration of quinol : cytochrome c oxidoreductase activity (Wang and King, 1982). It has been suggested from electron paramagnetic resonance studies that binding to proteins may stabilise the semiquinone form of ubiquinone (Yu et al., 1978). Thus it has been proposed that hydrogen flow may proceed

via an ordered series of bound quinones (Salerno et al., 1977). This, together with the observation that ubiquinone reduction and cytochrome c oxidation occur at the inner and outer faces of the membrane respectively, is consistent with the Q-cycle mechanism of proton translocation (see section 1.7). Alternative mechanisms of proton-translocation involving redox-linked proton pumps have, however, been proposed (Papa, 1976; Von Jagow et al., 1978).

Complex III has been isolated from the photosynthetic bacterium Rhodopseudomonas sphaeroides and shown to contain three to five major polypeptides plus one to three small polypeptides. Like the mitochondrial complex, it appears to contain two b-type cytochromes, a high potential c-type cytochrome and a high potential Fe-S protein.

Most bacteria contain quinone and multiple species of cytochrome b (see Jones, 1977). By analogy with mitochondria the quinone may be involved in a protonmotive quinone cycle in organisms possessing a high potential c-type cytochrome (Mitchell, 1975a,b). In bacteria lacking cytochrome c, site II may consist of a simple redox loop (Fig. 1.7).

1.9.4 Site III cytochrome c oxidase

In mitochondria the cytochrome oxidase function is carried out by cytochrome aa_3. In aerobic bacteria the cytochrome oxidases are more diverse with most species possessing branched respiratory chains terminating in different cytochrome oxidases. In bacteria lacking c-type cytochromes the natural electron donor for the cytochrome oxidase will be a b-type cytochrome; thus site III energy conservation will be absent in these organisms. Furthermore, in bacteria which possess c-type cytochromes, in addition to the cytochrome c oxidase there may be alternative oxidases which accept electrons at the level of the b-type cytochromes; thus in these organisms different branches of the respiratory chain may possess different energy conserving properties.

The bacterial cytochrome oxidases have been extensively reviewed (Poole, 1983; Jones and Poole, 1985).

1) Cytochrome aa_3

The mitochondrial cytochrome aa_3 is a multimeric protein comprised of nine or more subunits (see Malmström, 1979; Capaldi et al., 1983; Hatefi, 1985) and four redox centres, viz. two copper atoms and two a-type haems. The two haems function as spectrally-distinct species, a and a_3. By the use of radiolabelled protein functional group reagents it has been shown that cytochrome aa_3 traverses the mitochondrial membrane (Schneider et al., 1972). Cytochrome c, its electron donor, is located on the outer side of the membrane (Schneider et al., 1972) whilst its oxygen reduction site is believed to be on the inner surface of the membrane. Mitochondrial cytochrome c oxidase is believed to be associated with energy conservation via a proton pumping activity (see Wikström and Krab, 1981; Hatefi, 1985).

Bacterial cytochromes aa_3 appears to be considerably simpler than the mitochrondrial enzyme. Cytochrome aa_3 has been purified from several organisms including Paracoccus denitrificans, Thermus thermophilus, Thiobacillus novellus and the thermophilic bacterium PS3, and contained either two or three subunits (see Ludwig, 1980; Poole, 1983). The two major subunits of P.denitrificans appear to be similar to subunits I and II of the mitochondrial enzyme and probably carry the redox centres (Azzi, 1980). In bacteria the electron donor for cytochrome aa_3 may be either a b-type (eg. Bacillus megaterium) or a c-type cytochrome (see Jones, 1977). It has been suggested that cytochrome aa_3 reduces oxygen at the cytoplasmic face of the bacterial membrane, such that the cytochrome c oxidase functions minimally as a redox arm ($\rightarrow H^+/O = 0$, $\rightarrow$charge/O = -2; Hinkle and Mitchell, 1970; see also Jones, 1977). However, there is evidence that cytochrome aa_3 may

function additionally as a proton pump in P.denitrificans (van Verseveld et al., 1981) and Bacillus stearothermophilus (Chicken et al., 1981; $\rightarrow H^+/O = 2$, $\rightarrow charge/O = -4$). Indeed, proton-pumping activity has been observed in purified cytochrome aa_3 of P.denitrificans (Solioz et al., 1982) and PS3 (Sone and Hinkle, 1982) following reconstitution into phospholipid vesicles. In contrast, there is no evidence for proton-pumping via cytochrome aa_3 in whole cells of M.methylotrophus.

2) Cytochromes o and co

Cytochrome oxidases of the o-type have been purified from several bacteria including E.coli (Matsushita et al., 1982; Kita et al., 1984), Pseudomonas aeruginosa (Yang, 1982; Matsushita et al., 1982), Azotobacter vinelandii (Jurtshuk et al., 1981; Yang et al., 1979), Rhodopseudomonas palustris (King and Drews, 1976), Rhodopseudomonas capsulata (Hudig and Drews, 1982a,b) and M.methylotrophus (Carver and Jones, 1983; Froud and Anthony, 1984), and show considerable diversity in terms of their molecular weights, subunit composition and haem contents.

The cytochrome o of E.coli contained two major components, both of which contained b-type haems. Whilst this cytochrome o could oxidise reduced TMPD, it preferred ubiquinol as a substrate (Kita et al., 1984). In A.vinelandii, P.aeruginosa, R.palustris and M.methylotrophus subunit I contains a b-type haem and is accompanied by at least one other subunit which contains haem c, the oxidase thus purifies as a co complex which readily oxidises ascorbate-TMPD. There is no evidence that cytochromes o and co act as proton pumps. In organisms which possess a high potential c-type cytochrome, cytochrome co probably serves as the terminal oxidase and thus allows site III energy conservation ($\rightarrow H^+/O = 0$, $\rightarrow charge/O = -2$). In organisms (or terminal respiratory branches) which lack a high potential c-type cytochrome, cytochrome o accepts

electrons from cytochrome b and there is no energy conservation at site III.

3) Cytochrome d

Cytochrome oxidase d is relatively insensitive to cyanide and is synthesised by several organisms in increased amounts during growth in the presence of cyanide. It has a significantly higher affinity for oxygen than the other cytochrome oxidases. Cytochrome d is the major functional oxidase in Azotobacter sp. where its high activity and its ability to terminate respiration after site II allows its participation in a respiratory protection mechanism for the oxygen-sensitive nitrogenase.

Cytochrome d has been isolated from E.coli, K.aerogenes and Photobacterium phosphoreum and contains two molecules each of haems d and b (see Poole, 1983). A cytochrome oxidase bd complex from E.coli has been reported to be able to catalyse respiration-linked H^+ ejection when reconstituted into proteoliposomes; however this is not supported by whole-cell proton translocation studies and growth yield measurements (see Jones and Poole, 1985).

4) Cytochrome a_1

Relatively little is known about cytochrome oxidase a_1 (see Poole, 1983; Jones and Poole, 1985). Like cytochrome aa_3, it contains two molecules of haem a, but appears to lack copper.

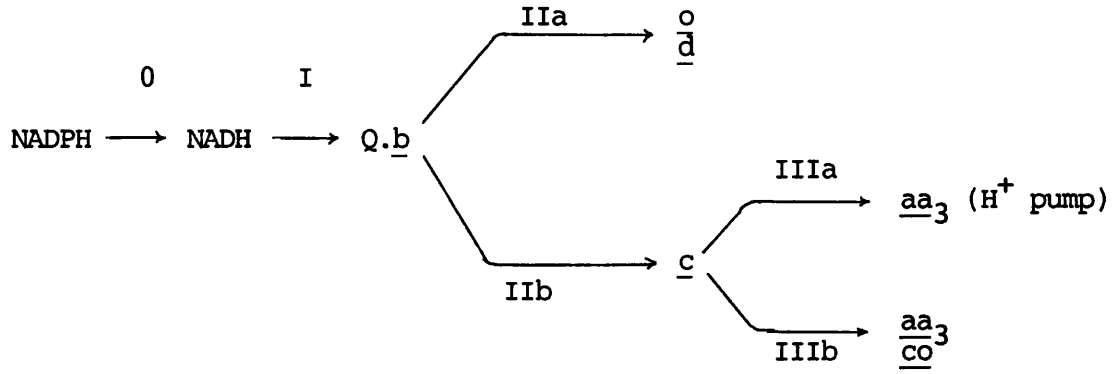
Photodissociation spectra and photochemimcal action spectra have indicated an oxidase role for cytochrome a_1 in Thiobacillus ferro-oxidans and Acetobacter sp.; however, evidence of an oxidase role for cytochrome a_1 in E.coli and Azotobacter vinelandii is conflicting (see Poole, 1983).

1.10 Variation in bacterial respiratory chains

Bacteria live in a wide variety of environments and, unlike

mitochondria, they may be subjected to rapidly changing growth conditions. Thus whilst the general nature of the redox centres in the respiratory chains of mitochondria and bacteria are essentially similar, some species of bacteria (eg. Paracococus denitrificans) have very similar respiratory chains to mitochondria whilst others (eg. E.coli) are very different.

A generalised scheme for bacterial electron transfer pathway from NADPH may be drawn thus:



Inter-species variability of respiratory chains may include the complete absence of one or more sites of energy conservation. Site O, associated with the nicotinamide nucleotide transhydrogenase ($\rightarrow H^+/2e^- = 2$; $\rightarrow charge/2e^- = -2$) is absent from many species of bacteria including M.methylotrophus (Dawson, 1982). In contrast, site I ($\rightarrow H^+/2e^- = 2$; $\rightarrow charge/2e^- = -2$) is generally present, although it may be deleted under certain growth conditions (Haddock and Jones, 1977). In the absence of c-type cytochromes (eg. E.coli) electron transfer from quinones to oxygen via cytochromes o or d results in energy conservation at site IIa ($\rightarrow H^+/2e^- = 2$, $\rightarrow charge/2e^- = -2$). In organisms which possess a high potential c-type cytochrome (eg. Paracoccus denitrificans, M.methylotrophus) energy conservation occurs at site IIb, probably via the action of the Q-cycle ($\rightarrow H^+/2e^- = 4$; $\rightarrow charge/2e^- = -2$). Energy conservation at site III associated with the cytochrome c oxidase may be

via a proton-pumping cytochrome $\underline{aa}_3$ ($\rightarrow H^+/2e^- = 2$; $\rightarrow charge/2e^- = -4$; eg. Paracoccus denitrificans, Bacillus stearothermophilus) or via a non-proton-pumping cytochrome $\underline{aa}_3$ or co ($\rightarrow H^+/2e^- = 0$, $\rightarrow charge/2e^- = -2$; eg. M.methylotrophus). Thus the overall $\rightarrow H^+/O$ and $\rightarrow charge/O$ quotients for the oxidation of endogenous NADPH may vary from 4 in organisms which possess sites I and IIa only, up to possibly as high as 10 in organisms which possess sites 0, I, IIb and IIIa.

In addition to genetic variability, there is considerable phenotypic variability in bacterial respiratory chains. In order to adapt to a changing environment, bacteria are able phenotypically to modify their respiratory chains. The nature and amount of the cytochrome system is particularly responsive to the dissolved oxygen tension; low concentrations of oxygen tend to increase the total cytochrome content (see Jurtshuk et al., 1975) and to bring about a replacement of cytochrome $\underline{aa}_3$ by cytochrome o (eg. P.denitrificans) or co (eg. M.methylotrophus), or of cytochrome o by cytochrome d, eg. E.coli (Sapshead and Wimpenny, 1972; Cross and Anthony, 1980b; see Poole, 1983). In M.methylotrophus cytochrome oxidase co is regulated by the concentration of dissolved oxygen (or an intracellular signal thereof) and is maximally repressed above 2μM oxygen ie. in all methanol-limited cultures and in oxygen-limited cultures at dilution rates close to μ_{max}. In contrast, cytochrome oxidase $\underline{aa}_3$ is regulated by the concentration of methanol (or by its product formaldehyde) and is maximally repressed above approximately 100μM methanol, ie. in oxygen-limited cultures and in methanol-limited cultures at dilution rates approaching μ_{max}. The reason why an o-type oxidase takes over from $\underline{aa}_3$ in oxygen-limited cultures of M.methylotrophus and other organisms is unclear, but could reflect a higher affinity for molecular oxygen (Greenwood and Jones, 1986).

The synthesis of other respiratory chain components may also be affected by the availability of various nutrients. Thus, the growth of cells in iron-limited conditions may result in a decrease in the concentration of cytochromes and iron-sulphur proteins (Rainnie and Bragg, 1973), whilst growth in sulphate-limited conditions may lead to a decrease in the concentrations of iron-sulphur proteins and to the synthesis of alternative cytochromes (Poole and Haddock, 1975).

1.11 The respiratory chain of M.methylotrophus

The respiratory chain of M.methylotrophus contains two b-type cytochromes (E_{m7} = 60 mV and 110 mV), and two soluble c-type cytochromes designated cytochrome c_L (E_{m7} = 310 mV) and cytochrome c_H (E_{m7} = 375 mV) because of their low and high isoelectric points respectively; a third minor c-type cytochrome, cytochrome c_{LM} (E_m = 356 mV) has also been reported, although this may be a degradation product of cytochrome c_L (Cross and Anthony, 1980a,b). The possession of at least one high potential c-type cytochrome appears to be a general property of methanol-utilising bacteria; indeed, mutants of Pseudomonas AM1 (Anthony, 1975; Widdowson and Anthony, 1975) and Paracoccus denitrificans (Willison and John, 1979) which lack cytochrome c no longer either oxidise or grow on methanol or methylamine, thus indicating that cytochrome c is essential for growth on these substrates. An unusual property of the c-type cytochromes of methylotrophs is their ability to bind carbon monoxide; this has led to speculation that the cytochrome c may possess an oxidase function. However, in M.methylotrophus the slow rate of reaction with CO and the high activity of other oxidases makes this unlikely (Cross and Anthony, 1980b).

Cytochrome c_L appears to be the physiological electron acceptor from methanol dehydrogenase, whilst cytochrome c_H is a better reductant

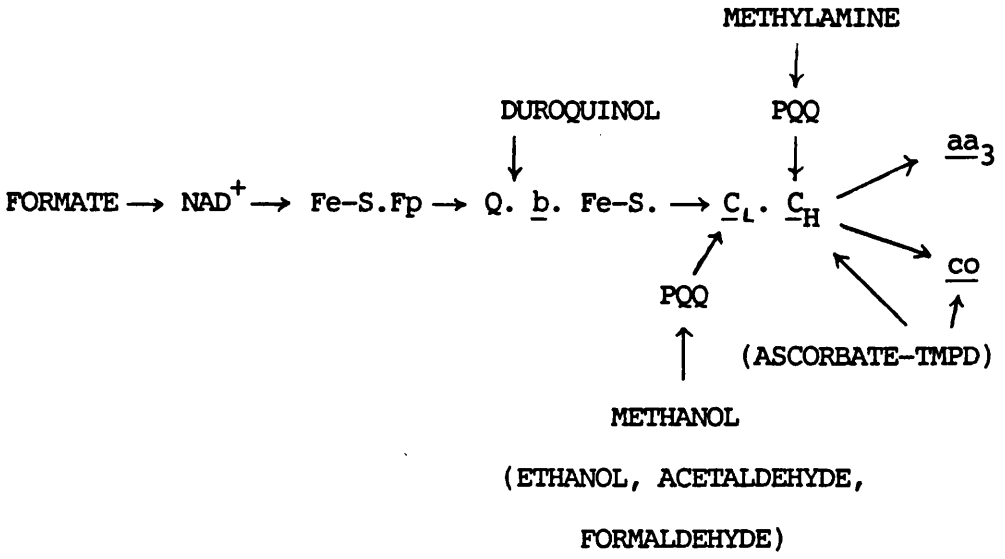
than $\underline{c}_L$ of cytochrome oxidase co; cytochrome $\underline{c}_H$ may thus act as a mediator between methanol dehydrogenase/cytochrome $\underline{c}_L$ and cytochrome oxidases co and $\underline{aa}_3$. Cytochrome $\underline{c}_H$ may also serve to mediate between the cytochrome $\underline{bc}_L$ complex and the ter-minal oxidases during NADH oxidation (Froud and Anthony, 1984a,b; Fig. 1.8).

The respiratory chain of M.methylotrophus contains two cytochrome oxidases, $\underline{aa}_3$ and co, of which only the latter has been purified (Carver and Jones, 1983; Froud and Anthony, 1984). Together with methanol dehydrogenase, cytochromes $\underline{c}_L$ and $\underline{c}_H$ it has been reconstituted into an active methanol oxidase system (Froud and Anthony, 1984). The expression of the cytochrome oxidases is sensitive to the growth conditions. Studies in cells grown at fixed dilution rate indicated that the concentrations of cytochrome oxidases $\underline{aa}_3$ and co vary depending on whether methanol or oxygen is the growth-limiting substrate (see section 1.10).

The first enzyme in the methanol oxidase system, methanol dehydrogenase, is maximally repressed when the standing concentration of methanol exceeds approximately 100μM and thus behaves similarly to cytochrome oxidase $\underline{aa}_3$. Thus the composition and activity of the methanol oxidase system is controlled by the growth environment (Greenwood and Jones, 1986).

Recent reports of a modifier-protein (M-protein) in methylotrophic bacteria (Ford et al., 1985; Page and Anthony, 1986) are of interest to the regulation of methanol dehydrogenase activity. Studies with pure methanol dehydrogenase and M-protein from Pseudomonas AM1 and M.methylotrophus have shown that the M-protein can significantly alter the K_m and/or V_{max} of the dehydrogenase for various substrates, and that it consequently stimulates the oxidation of methanol and inhibits the oxidation of formaldehyde. Such a regulatory mechanism may

Fig. 1.8 The respiratory chain of Methylophilus methylotrophus



be a general means of preventing methanol dehydrogenase from further oxidising to formate the formaldehyde required for carbon assimilation in methylotrophs.

The stoichiometries of proton and charge translocation during the oxidation of methanol, duroquinol and NADH by M.methylotrophus are consistent with three sites of energy conservation, viz. site I (NADH : ubiquinone oxidoreductase; $\rightarrow H^+/2e^- = 2$, $\rightarrow charge/2^- = -2$), site II (Ubiquinol : cytochrome c oxidoreductase; $\rightarrow H^+/2e^- = 4$, $\rightarrow charge/2e^- = -2$) and site III (cytochrome c oxidase; $\rightarrow H^+/O = 0$, $\rightarrow charge/O = -2$; Dawson and Jones, 1981a,b). Only site III is associated with the oxidation of methanol; a range of primary alcohols and aldehydes may also be oxidised via methanol dehydrogenase. Oxidation of methanol yields $\rightarrow H^+/O$ and $\rightarrow charge/O$ quotients of 2 and -2 respectively due to the release of $2H^+$ from the periplasmic side of the membrane during the reduction of cytochrome c_L by reduced methanol dehydrogenase, followed by site III (Fig. 1.9). This redox arm model for the methanol oxidase system is supported by the observation that methanol dehydrogenase is located on the periplasmic side of the respiratory membrane (Jones et al., 1982; Burton et al., 1983; Quilter and Jones, 1984). A similar location has been reported for the methanol dehydrogenase of Paracoccus denitrificans (Alefounder and Ferguson, 1981); however, in this organism oxidation of methanol is associated with the translocation of between three and four protons, which probably reflects the proton-pumping activity of the cytochrome oxidase aa_3 of this organism (van Verseveld and Stouthamer, 1978; van Verseveld et al., 1981).

During growth on trimethylamine, M.methylotrophus synthesises the enzymes trimethylamine dehydrogenase, dimethylamine mono-oxygenase and methylamine dehydrogenase. Trimethylamine dehydrogenase appears to be associated with the cytoplasmic side of the membrane and donates

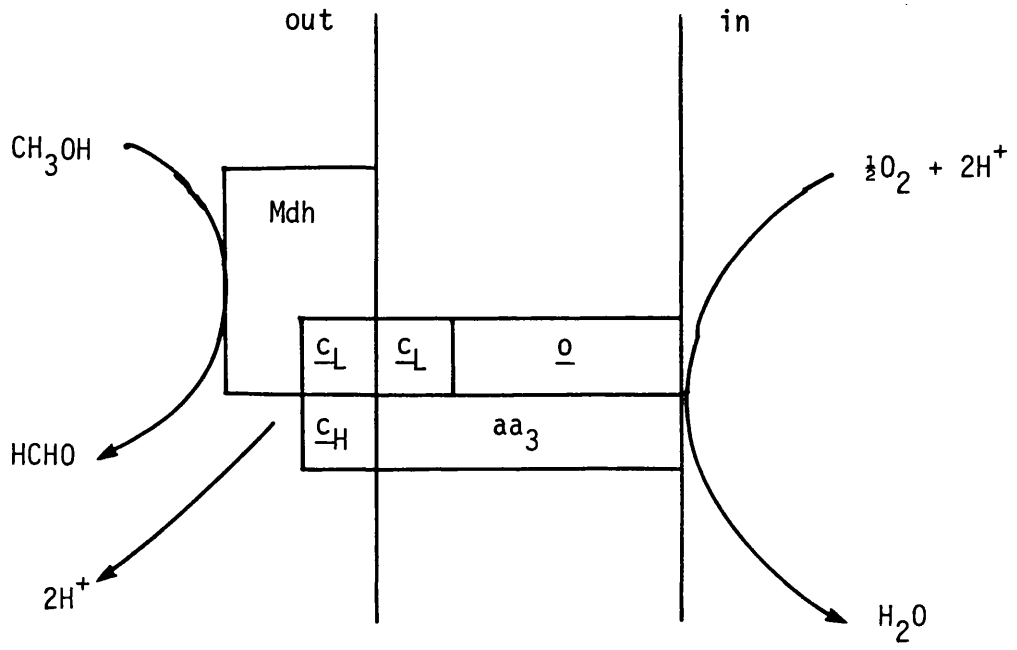


Fig. 1.9. Schematic diagram of the methanol oxidase system in the terminal region of the respiratory chain of M.methylotrophus

From Jones et al. (1984)

reducing equivalents to the respiratory chain at the level of ubiquinone/cytochrome b, whereas methylamine dehydrogenase is attached to the periplasmic side of the membrane and probably donates reducing equivalents to cytochrome c_H (Burton et al., 1983; but see Lawton and Anthony, 1985).

1.12 Single Cell Protein

The growth of microorganisms as a source of single cell protein (SCP) has been intensively studied as a result of projections of an increased world demand for protein. Organisms which have been considered for this purpose include algae, fungi, yeasts and bacteria. Yeasts and bacteria have proved the most popular organisms to use, although a process using the fungus Fusarium graminarium has recently been developed jointly by Rank Hovis McDougall and ICI.

The organism used largely depends on the nature of the chosen carbon source, and it must pass stringent toxicological tests in order to be marketed (see Kharatjan, 1978). Carbon sources may be classified into waste products (eg. whey, molasses), renewable resources (eg. starch, cellulose), or carbon-based energy sources (eg. methane, methanol, ethanol or n-alkanes). The former two classes are dependent upon local agricultural activity, and the large-scale industrial production of SCP has therefore concentrated mainly on the last group, of which methanol has been particularly favoured. Although methanol is more expensive than methane or n-alkanes, it is associated with a lower oxygen demand and a smaller heat output during growth (Goldberg, 1977), both of which are particularly important since energy requirements for supplying compressed air and for cooling are a significant factor in the cost efficiency of any SCP process. Other advantages of methanol include its non-explosive nature, its availability in pure form and its high solubility.

Methylotrophic yeasts offer some advantages over their bacterial counterparts, since their large size allows easier separation from the culture effluent, and they can also grow at low pH and thus reduce the risk of bacterial contamination; indeed, yeasts may be grown non-aseptically at pH 3 to 4 (Litchfield, 1978). In contrast, bacteria possess a higher protein content, and have both a higher growth rate and a higher cell yield. Potential SCP organisms may be grown in pure or mixed culture; in the latter case it has been shown that a heterotroph in a methylotrophic culture may successfully use lysis products from the methylotroph and thus reduce carbon loss (Goldberg, 1977; Harrison, 1978). A mixed culture is also less susceptible to contamination. However, it is more difficult to produce SCP of constant specification from mixed cultures, this being an important requirement for both consumers and regulatory agencies. The nature of the organism may also be determined to some extent by the commercial necessity to produce the organism at a rate of several thousand tonnes per annum. Although the highest production rates may be achieved in carbon-limited continuous culture, the difficulties inherent in mixing large volumes inevitably lead to some heterogeneity of the culture conditions, and hence the organism of choice must be able to tolerate this range of conditionswith little deterioration of yield.

1.12.1 The ICI Single Cell Protein process

The source of SCP in the ICI process (PRUTEEN) is Methylophilus methylotrophus which assimilates carbon at the level of formaldehyde via the Entner-Doudoroff variant of the ribulose monophosphate pathway and is therefore fairly efficient in its utilisation of methanol (see section 1.2). The product has undergone extensive toxicological testing with respect to its acceptability as a food supplement for calves, piglets, hens and turkeys. The development

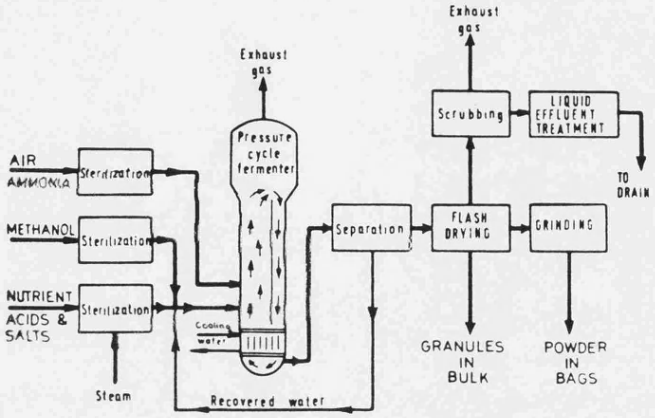
of the ICI single cell protein process has recently been reviewed (Vasey and Powell, 1984).

M,methylotrophus is grown in a large volume pressure-cycle fermenter in methanol-limited continuous culture at 37°C - 40°C at a dilution rate of approximately 0.18 hr^{-1}. The fermenter consists of a concentric riser and downcomer. Compressed air is pumped into the riser, the movement of air maintaining a constant movement of the medium which aids mixing. Nutrient salts, methanol and air are supplied continuously, and ammonia is used both to regulate the pH and to provide the nitrogen source for growth. Cells are recovered from the fermenter effluent by agglomeration nd centrifugation prior to flash drying, and the spent medium is recycled with the addition of fresh nutrients (Fig. 1.10).

In 1981 methanol comprised 59% of the total cost of PRUTEEN production, hence the need for efficient carbon assimilation. Other nutrients comprised 17% of costs, whilst energy accounted for 23% of production costs (Smith, 1981). Energy costs are split between air compression, cooling and product drying. Thus oxygen consumption and heat production are important considerations in the overall economics of PRUTEEN production, and these reflect the efficiency of energy conservation by the organism.

1.13 Objectives

The objective of the work described in this thesis was to study respiration-linked energy conservation in M.methylotrophus. Three facets of this process were studied; viz. (i) the reported ability of M.methylotrophus to oxidise exogenously supplied NADH (Cross and Anthony, 1980), (ii) the stoichiometry of ATP synthesis during substrate oxidation, and (iii) the effect of temperature on respiratory chain energy conservation.



Flow sheet for ICI process.

Fig. 1.10 The I.C.I. single cell protein process.

From Smith (1981)

The results have been reported in several publications (Jones et al., 1984; Carver et al., 1984; Patchett et al., 1985; Patchett and Jones, 1986).

CHAPTER TWO

MATERIALS AND METHODS

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2.1.2 Preparation and assay of stock solutions

2.2 Growth and maintenance of bacterial cultures

2.2.1 Maintenance of stock cultures

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CHAPTER TWO

MATERIALS AND METHODS

2.1 Chemicals

2.1.1 Sources

NADH (grade II), NAD^+ and $NADP^+$ were supplied by Boehringer Corp. (London), Lewes, East Sussex, UK. ATP (grade I), ADP (type II), AMP (grade IV), phosphoenolpyruvate, adenylate kinase (grade III), alcohol dehydrogenase (yeast), GTP (type I), CTP (type IV), UTP (type III), catalase (C-40), pyruvate kinase (type II), carbonic anhydrase, glucose-6-phosphate dehydrogenase (type XV), desiccated firefly lanterns, inorganic pyrophosphatase (yeast), valinomycin, HQNO, hydroxylamine, PMS, DCPIP and TMPD were obtained from Sigma (London) Chemical Co., Poole, Dorset, UK. FCCP was obtained from Fluka Fluorochem Ltd., Peakdale Road, Glossop, Derbyshire, SK13 9XE, UK. Duroquinol was obtained from ICN Pharmaceuticals, Plainview, New York, USA. DEAE cellulose (DE-52) was obtained from Whatman Biochemicals, Springfield Mill, Maidstone, Kent, UK. Oxoid purified agar was obtained from Oxoid Ltd., Basingstoke, Hants, UK. Glycylglycine was obtained from BDH, Poole, Dorset, UK. Whenever possible all chemicals were of ANALAR grade.

2.1.2 Preparation and assay of stock solutions

Stock solutions of FCCP (10 mM), duroquinol (100 mM) and valinomycin (3 mg/ml) were dissolved in NN dimethyl formamide. Solutions of HQNO were prepared by dissolving a few grains of HQNO in dilute potassium hydroxide and then diluting with water. The concentration of HQNO was assayed by absorption at 345 nm (E_{346} 9450; Cornforth and James, 1956). ATP, GTP and UTP for the calibration of

FPLC results were dissolved in 0.01 M potassium phosphate buffer pH 7.0 and assayed by absorption at 260 nm (E_{260} = 15400, 11700 and 9900 mol^{-1} cm^{-1} for ATP, GTP and UTP respectively; see Burton, 1969).

Enzymes were dissolved in the assay buffers described in the relevant assays.

All other reagents were made up in aqueous solution.

2.2 Growth and maintenance of bacterial cultures

2.2.1 Maintenance of stock cultures

M.methylotrophus (formerly Pseudomonas methylotropha; NCIB 10515; Byrom and Ousby, 1975) was obtained from I.C.I. Agricultural Division, Billingham, Cleveland TS23 1LD. Stock cultures were maintained in freeze-dried ampoules and on methanol/salts agar plates containing in 1 litre: K_2HPO_4, 1.9 g; $NaH_2PO_4.2H_2O$, 1.56 g; $(NH_4)_2SO_4$, 1.8 g; $MgSO_4.7H_2O$, 20 mg; $FeCl_3.6H_2O$, 9.7 mg; Fisons Trace Element solution (Cu, 5 ppm; Mn 24-25 ppm; Zn, 22-23 ppm; Ca, 720 ppm), 1 ml; Oxoid purified agar, 15 g; methanol 5 ml; adjusted to pH 7.2 with KOH.

2.2.2 Preparation of inocula

Bacteria used for the inoculation of continuous cultures were grown at 37°C as 150 ml batch cultures in 500 ml flasks which were aerated by shaking on a rotary shaker at approximately 300 rpm. The growth medium used was the methanol/salts solution described above with the methanol concentration increased to 1% (v/v). Bacteria were grown to a final density of approximately 1 g dry weight bacteria/l. and used within 12 hours of the end of the logarithmic growth phase.

2.2.3 Growth of M.methylotrophus in methanol-limited continuous culture

Cultures (approximately 970 ml) were grown in a 1 litre laboratory fermenter 500 Series with magnetic agitation (L.H. Engineering Co. Ltd.). The medium contained in 1 litre: $MgSO_4.7H_2O$,

0.27 g; K_2SO_4, 35 mg; K_2HPO_4, 0.113 g; 1.1 M H_3PO_4, 3.75 ml; Fisons trace elements solution, 6 ml; and methanol, 1.75 ml. Iron was pumped into the fermenter vessel separately as a solution of $FeSO_4$ (0.16 g of $FeSO_4.7H_2O$ plus 2 ml of H_2SO_4 in 1 litre) at a rate of 4 ml/hr. The above medium is acidic (pH ≃ 3.0) and the pH was controlled by automatic addition of 10% (w/v) NH_4OH to maintain a pH of 7.0 ± 0.1. The ammonia also acted as the nitrogen source for growth. All bacteria were grown at a dilution rate of 0.18 h^{-1}. The temperature of growth was 40°C unless otherwise stated. The dissolved oxygen tension was measured by a lead/silver galvanic electrode and maintained above 50% of air saturation. The purity of the culture was checked by plating on to nutrient agar.

2.3 Harvesting and preparation of cell suspensions

Cultures (approx. 200 ml) of M.methylotrophus were taken directly from the chemostat and harvested by centrifugation (12,000 g, 10 min) in an MSE 18 centrifuge (MSE, Sussex Manor Park, Gatwick Park, Crawley, Sussex RH10 QQ). The cells were washed by resuspension in approximately 70 ml of the assay buffer described for each experiment. In experiments measuring the changes in inorganic phosphate concentration this buffer was rendered anoxic by sparging with oxygen-free nitrogen prior to resuspension of the cells. The cell suspension was further sparged prior to centrifugation (12,000g, 10 min) - this procedure assisted in depleting the level of inorganic phosphate present in the cells. The above washing procedure was repeated for a second time and the cells finally resuspended in the assay buffer.

2.4 Determination of maximum specific growth rates

The maximum specific growth rate (μ_{max}) of M.methylotrophus at 26°C was determined by measurement of the optical density at 600 nm with

time of cultures grown in batch culture at 26°C in the methanol/minimal salts medium described in section 2.2.2. The specific growth rate was obtained during the exponential period of growth using the equation

$$\mu_{max} = \frac{0.693}{t_{1/2}}$$

2.5 Determination of dry weights of bacterial suspensions

The dry weight of bacterial suspensions was determined from the optical density at 680 nm measured using a Unicam SP600 spectrophotometer. The optical density at 680 nm is a linear function of the dry weight up to at least an optical density of 1 (≡ 0.63 mg dry weight bacteria/ml) for M.methylotrophus grown at 40°C under methanol-limited continuous culture (Dawson, 1982). In some experiments cell dry weights were determined directly by diluting cell suspensions with water and filtering on to preweighed 0.45μ millipore filters (Millipore, Molsheim, France). The filtered cells were washed with 2 x 5 ml distilled water and the filters dried overnight at approximately 100°C. The filters were then cooled in a desiccator and weighed. Weights were corrected for the weight loss of filters on washing with water. The determination of optical density and cell dry weight for chemostat cultures at 26°C ($D = 0.18\ h^{-1}$) indicated that the relationship between optical density and dry weight was unchanged at the lower growth temperature.

2.6 Preparation of Sphaeroplasts

Washed bacteria were resuspended in 20 mM TRIS-HCl pH 7.5 plus mannitol (0.75 M) to a final density of 5.04 mg dry weight bacteria/ml. 8.5 ml of this bacterial suspension was incubated at 30°C with lysozyme (0.1 ml of 10 mg/ml stock solution) for 12 minutes prior to the addition of ten 0.1 ml aliquots of EDTA (100 mM) at 1 minute intervals. After a further 5 minutes incubation, excess EDTA was removed by the addition of

0.5 ml $MgCl_2$ (1 M). The sphaeroplast preparation was then incubated with 0.1 ml RNAase (10 mg/ml stock solution) and 0.1 ml DNAase (10 mg/ml stock solution) for 2 minutes prior to harvesting by centrifugation (12,000 g, 15 min). After decanting the pink periplasm fraction the sphaeroplasts were gently resuspended in 8.5 ml 20 mM TRIS-HCl buffer pH 7.5 plus mannitol (0.75 M).

2.7 Determination of respiration rates

Respiration rates were measured using a polarographic oxygen electrode (Rank Bros., Bottisham, Cambridge CB5 9DA) fitted with a thin teflon membrane and thermostatted at the described assay temperature. The electrode had a 90% response time to the addition of dithioniate of approximately 2.5 seconds. The output from the oxygen electrode was recorded on a Servoscribe 2S potentiometric recorder (Smith's Industries Ltd., London NW2 7UR). The reaction conditions are described for individual experiments.

The solubility of oxygen at 40°C was taken to be 0.38 μg-atom O/ml (Dawson, 1980a). For experiments performed at other temperatures the solubility of oxygen was determined by the method of Robinson and Cooper (1970). Buffer (3 ml) equilibrated at the required temperature was incubated in a water jacketed Rank oxygen electrode. PMS (10 μl of 2 mg/ml) and catalase (10 μl of 20000 I.U./ml) were added prior to the introduction of μl quantities of NADH solution. NADH is oxidised by PMS:

$$NADH + H^+ + PMS \longrightarrow NAD^+ + PMSH_2$$

The reduced PMS is subsequently oxidised by oxygen:

$$PMSH_2 + O_2 \longrightarrow PMS + H_2O_2$$

Catalase catalyses the decomposition of hydrogen peroxide giving a net reaction of:

$$NADH + H^+ + 1/2\ O_2 \longrightarrow NAD^+ + H_2O$$

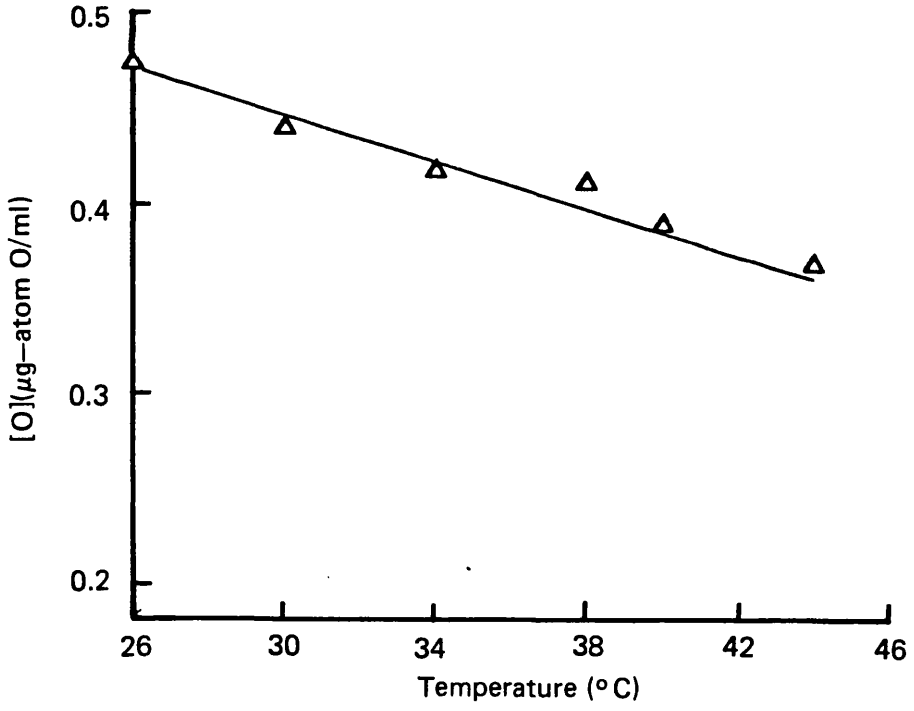


Fig. 2.1 The solubility of oxygen in glycylglycine buffer

Assay of the NADH stock solution by absorption at 340nm and determination of oxygen consumption upon addition of NADH to the reaction mixture allowed calculation of the oxygen content of the buffer (Fig. 2.1).

2.8 Measurement of NADH oxidation rates by ΔA340

The oxidation of NADH was followed by measuring the change in absorbance at 340 nm in a Perkin-Elmer Lambda 5 UV/VIS Spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, Bucks. HP9 1QA). The 1 ml cuvette thermostatted at 40°C containing approximately 0.125 mg dry weight cells in 1 ml 20 mM glycylglycine buffer pH 7.0 was incubated for 2 minutes prior to the addition of NADH (0.2 mM) and the absorbance at 340 nm followed.

2.9 Purification of NADH

A DEAE-cellulose column (bed volume = 1.8 ml) was prewashed with 1.5 mM glycylglycine-KOH buffer pH 7.0. 0.4 ml of 57 mM NADH was applied to the column and eluted successively with 4 ml of 1.5 mM glycylglycine-KOH buffer pH 7.0 and 3 ml 1.5 mM glycylglycine-KOH buffer pH 7.0 plus KCl (0.1 M). Fractions of approximately 0.5 ml were collected and monitored for NADH by absorption at 340 nm.

2.10 Rapid sampling of cells during respiration

Rapid sampling of cell suspensions during the first few seconds of respiration was achieved with a modified version of the apparatus described by Knowles and Smith (1970). The sampling apparatus (Fig. 2.2) consisted of a water jacketed 50 ml glass syringe (Rocket of London, 190 West End Road, London NW6) mounted vertically over the base of a Rank oxygen electrode (Rank Bros. Bottisham, Cambs.) and sealed with epoxy resin. The contents of the reaction chamber were rapidly stirred by a large magnetic flea which rotated on a raised flange to prevent interference with the teflon membrane of the oxygen electrode.

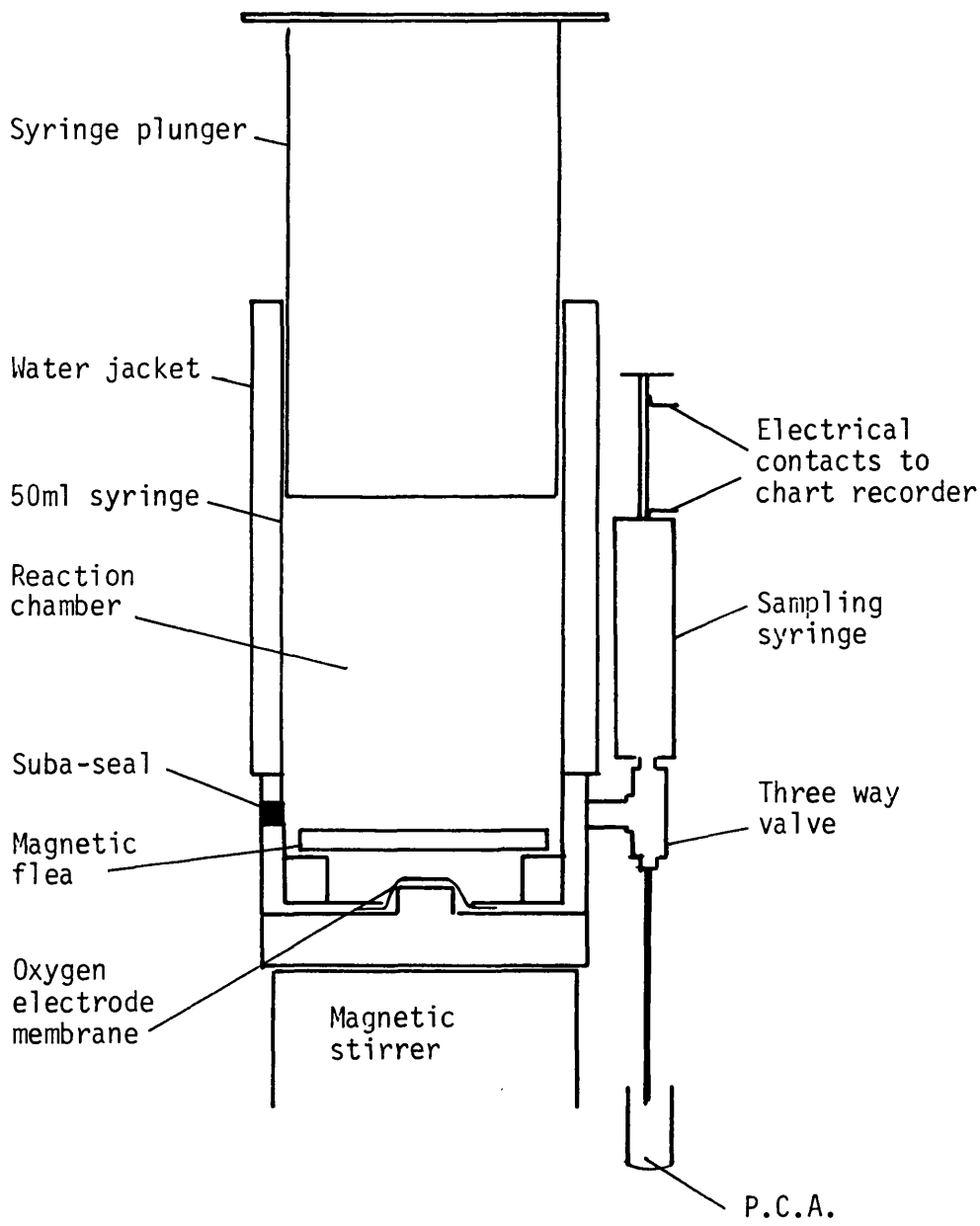


Fig. 2.2 Rapid sampling apparatus

The cell suspension was isolated from the atmosphere by the free-sliding glass barrel of the syringe. A small suba-seal inserted into the side of the syringe enabled the introduction of substrates and inhibitors via micro-syringes. Samples of the reaction mix (1.5 ml) were withdrawn via an exit port using a spring-loaded syringe attached to an automatic 3-way valve (Becton, Dickinson and Co., Rutherford, NJ, USA) and were ejected into 0.48 ml of 25% (v/v) perchloric acid; repeated samples could be taken and quenched every 1.5 to 2.5 seconds. The sampling syringe was fitted with electrical contacts so that the ejection of each sample into the perchloric acid was recorded as a spike on the blue channel of a Servoscribe 2S two-channel potentiometric chart recorder. For initial-rate energisation experiments, approximately 23 ml of 25 mM glycylglycine pH 7.0 plus KCl (140 mM) was equilibrated in the reaction chamber at 40°C for at least 20 minutes and cells (approximately 2 ml) were then added to a final cell density of approximately 3 mg/ml. Substrate (10 mM methanol, ethanol or acetaldehyde, 1 mM duroquinol in DMF or 50 mM formate) was added approximately 2 minutes later and the resultant oxygen uptake was monitored on the red channel of the chart recorder. The solubility of oxygen at 40°C was taken to be 380 ng-atom O/ml (Dawson and Jones, 1981a) and the respiration rate with duroquinol was corrected for auto-oxidation in the absence of cells.

In experiments designed to measure the rate of decay (turnover) of cell energisation, an aerobic whole cell suspension was allowed to attain steady-state energisation by oxidising a given substrate for 15 seconds under the conditions described above. A sample (1.5 ml) was then taken and the uncoupling agent FCCP (10 μM in DMF) was immediately added to prevent further ATP synthesis via respiratory chain phosphorylation. Repeated samples were then taken at suitable intervals until the cells were completely de-energised.

2.11 Assay of intracellular metabolites

2.11.1 Assay of ATP

The perchloric acid extracts were neutralised with a mixture of 6 M KOH and 1.2 M TRIS as described by McKay et al. (1982), and the resultant precipitate of potassium perchlorate was removed by centrifugation (12,000 g for 10 minutes). The ATP concentration in the supernatant was assayed by the luciferin/luciferase method. In the presence of Mg^{2+} and oxygen, the addition of ATP to a mixture of luciferin and the enzyme luciferase results in a flash of light, the intensity of which is proportional to the amount of ATP added (McElroy and Strehler, 1949; Strehler and Trotter, 1952; Kimmich et al., 1975):

$$\text{ATP} + \text{luciferin} \xrightarrow{Mg^{2+},\ \text{luciferase}} \text{adenyl-luciferin} + \text{PP};$$

$$\text{adenyl-luciferin} + O_2 \longrightarrow \text{oxyluciferin} + \text{AMP} + h\nu$$

The flash of light obtained is transient due to complex end-product inhibition (McElroy and DeLuca, 1973; DeLuca, 1976).

The luciferin/luciferase solution was prepared as described by Kimmich et al. (1975). Firefly lanterns (1 g) were ground with 100 ml of ice-cold 20 mM glycylglycine, 20 mM $MgSO_4$, 50 mM sodium arsenate/H_2SO_4 buffer pH 7.4. The residue was removed by centrifugation (12,000 g, 10 min) and the extract was stirred with 1.6 g calcium phosphate for 10 min at room temperature. The calcium phosphate was removed by centrifugation (12,000 g, 2 min) and the supernatant re-treated with calcium phosphate as before. After removing the calcium phosphate by centrifugation (12,000 g, 10 min) the clear, straw-coloured supernatant was frozen in aliquots. The assay mixture consisted of 250 μl of assay buffer (5 mM sodium arsenate, 4 mM $MgSO_4$, 20 mM glycylglycine/NaOH pH 8.0) and a 50 μl sample containing 0-50 pmol ATP within a 6 x 50 mm glass tube. The tube was placed in the reaction

chamber of an Aminco Chem-Glow photometer (American Instruments Co., Maryland 20910, USA) and 100 μl of the luciferin/luciferase solution (equilibrated at room temperature) was injected into the assay mixture with a Hamilton repeating dispenser (Hamilton-Bonaduz, Bonaduz, Switzerland). The peak height of the resultant flash, which has been shown to be proportional to the concentration of ATP (Dawson, 1982), was recorded on a Servoscribe potentiometric recorder.

A number of common anions, including Cl^- and ClO_4^- inhibit the luciferin/luciferase reaction (Strehler and Trotter, 1952; Denburg and McElroy, 1970; Lundin and Thore, 1975), so ATP contents were calibrated using internal standards. Duplicate assays containing 20 pmol of standard ATP were made; the ATP content was calibrated from the difference between the readings for the duplicate assays. A small correction was made for the flash intensity which was obtained following the injection of 100 μl of luciferin/luciferase into ATP-free assay mixture.

2.11.2 Assay of ADP and AMP

ADP and AMP were converted to ATP, then assayed as described above. For the determination of ADP, ADP was converted to ATP with phosphoenol pyruvate and pyruvate, whilst for AMP determination both ADP and AMP were converted to ATP and the AMP concentration obtained by subtraction.

$$ADP + PEP \xrightarrow{\text{Pyruvate kinase}} ATP + \text{pyruvate}$$

$$AMP + ATP \xrightarrow{\text{adenylate kinase}} 2ADP$$

Conversion was achieved in a 500 μl reaction mixture containing 400 μl of sample containing the adenine nucleotides, 1 mM phosphoenol

pyruvate, 50 EU of pyruvate kinase, 50 EU of adenylate kinase (for AMP conversion only), 3 mM $MgSO_4$ and 21 mM Tris/acetate buffer pH 7.3. This mixture was incubated at 30°C for 1 hour and then stored on ice prior to being assayed within a further two hours. It has been demonstrated that this procedure achieves quantitative conversion of AMP and/or ADP into ATP (Dawson, 1982).

2.11.3 Assay of inorganic orthophosphate

Inorganic orthophosphate was determined by the method of Itaya and Ui (1966) in which the phosphate forms a phosphomolybdate complex which is then used to shift the absorption maximum of malachite green. Phosphate was assayed in neutralised cell extracts or in acidic perchloric acid extracts. The colour-developing reagent was made by mixing 1 volume of 4.2% (w/v) ammonium molybdate/5N HCl with 3 volumes of 0.05% (w/v) malachite green. This straw-coloured solution was allowed to stand at room temperature for 30 minutes and then filtered to remove a green precipitate. A sample (0.5 ml) containing 0-50 nmoles of phosphate was added to the reagent (2.5 ml) and the mixture was incubated on ice for 30 minutes prior to addition of 0.1 ml of 1.5% (w/v) Tween 20 and warming to room temperature. After a further 15 minutes the absorbance at 650 nm was measured against a water blank. There is a linear relationship between the absorbance at 650 nm (corrected for the reagent alone) and the phosphate content within the range used. Control experiments have previously confirmed that the conditions under which the cell extract was prepared did not cause the release of inorganic phosphate from AMP, ADP or ATP standards (Dawson, 1982).

2.11.4 Assay of inorganic pyrophosphate

Inorganic pyrophosphate was assayed in neutralised cell extracts according to the method of Gawehn (1974). Pyrophosphate was

digested by incubation for 1 hour at room temperature in a reaction mixture containing 33 mM Tris-HCl pH 7.0, 1.5 mM $MgCl_2$ and 4 units inorganic pyrophosphatase. The hydrolysed samples were assayed for inorganic orthophosphate as described in section 2.9.3 and corrected for the amount of orthophosphate present in the unhydrolysed sample.

2.12 Assay of nucleoside triphosphates by 'Fast Protein Liquid Chromatography'

Neutralised cell extracts were assayed for nucleoside triphosphates by 'fast protein liquid chromatography' (FPLC; Pharmacia, Uppsala, Sweden). Potassium phosphate buffer (1 M) was added to 5 ml samples of cell extracts to attain a final concentration of 0.1 M phosphate and filtered to remove insoluble salts, probably of magnesium, prior to application of the cell extract to a polyanion SI anion-exchange column at a rate of 1 ml/minute. Nucleotides were eluted with a gradient of 0.01 - 1 M potassium phosphate buffer pH 7.0 at a flow rate of 0.4 ml/minute. The eluted material was detected by monitoring the extent of absorbance at 254 nm using a Pharmacia optical monitoring unit, and the results were displayed on a chart recorder. Peaks were identified by comparison with standard mixtures of known nucleotides, and by co-elution with internal standards, and were quantified by comparing the area of each peak with that of a known concentration of a standard.

2.13 Assay of nicotinamide nucleotides

2.13.1 Assay of NAD^+

NAD^+ was assayed using the spectrophotometric method of Klingenberg (1974). The 1 ml reaction mixture contained 0.5 ml of neutralised cell extract and 0.5 ml of a mixture containing 0.10 M tetra-sodium pyrophosphate buffer pH 8.8, 0.5% (w/v) semi-carbazide-HCl and 1.0% (v/v) ethanol. The reaction was started by addition of 5 μl of

alcohol dehydrogenase (1.2 mg/ml), and the change in absorbance at 340 nm measured against a reagent blank using a Perkin-Elmer Lambda 5 spectrophotometer.

2.13.2 Assay of $NADP^+$

$NADP^+$ was assayed spectrophotometrically in a 1.3 ml reaction mixture containing 1 ml of neutralised cell extract and 0.3 ml of a mixture containing 64 mM Tris-HCl pH 7.3, 17 mM $MgCl_2$ and 164 mM glucose-6-phosphate. The reaction was started by the addition of 5 μl of glucose-6-phosphate dehydrogenase (2 mg/ml) and the change in absorbance at 340 nm monitored in a Perkin-Elmer Lambda 5 dual-wavelength spectrophotometer against a reagent blank.

2.14 Assay of methanol dehydrogenase

Methanol dehydrogenase activity was assayed by linking electron transfer to the artificial electron acceptor phenazine methosulphate, and measuring the auto-oxidation of the latter using a Rank oxygen electrode (Carver et al., 1983). The reaction was carried out in a 4 ml reaction volume containing 20 mM glycylglycine buffer pH 7.0, PMS (1 mM) ammonium chloride (15 mM), KCN (1 mM) and approximately 0.5 mg dry weight cells. The reaction was started by the addition of 1 mM methanol. The observed rate of oxygen consumption was corrected for the endogenous rate in the absence of methanol, and since this method leads to the production of hydrogen peroxide and the endogenous catalase activity is totally inhibited under these assay conditions, the observed rates of oxygen consumption were divided by two.

2.15 Determination of the stoichiometry of respiration-linked proton translocation by the oxygen pulse technique

The stoichiometry of respiration-linked proton translocation was assayed by the method of Mitchell and Moyle (1967a). The conditions of measurement of proton translocation favoured the oxidation of endogenous

substrates. The 5 ml reaction mixture contained 1.5 mM glycylglycine pH 5.9, 140 mM KCl, 25–35 mg dry weight of washed cells and 250 μg of carbonic anhydrase. The bacterial suspension was maintained at 40°C in a Rank oxygen electrode chamber and allowed to achieve anaerobiosis by oxidising endogenous substrates. Valinomycin (1 μg/mg cells) was then added and the reagent mix was incubated for a further 30 minutes. The valinomycin and potassium ions were present in order to effect the collapse of the $\Delta\psi$ generated by respiration which would oppose further proton translocation. Carbonic anhydrase was present in order to prevent any pH overshoot resulting from the slow re-equilibration of the CO_2/HCO_3^- couple after a pH change (see Scholes and Mitchell, 1970).

The pH was adjusted to 5.9 with anaerobic KOH or HCl, and oxygen was then introduced in 5–50 μl volumes of air-saturated 140 mM KCl, approximately ten volumes being used for each experiment. The resulting acidifications were measured with a Russell combination micro-electrode (Russell pH Ltd., Auchtermuchty, Fife, Scotland K714 7DP) fitted through the perspex top of the oxygen electrode chamber and attached to a Beckman 4500 pH meter (Beckman Instruments Inc., California 92634, USA). The pH change was recorded on a Servoscribe 2S potentiometric recorder. Acidification was complete within 3–4 seconds of adding oxygen, and ΔpH was estimated by extrapolating back to the time at which half the pH change had occurred (Mitchell and Moyle, 1965). The concentration of oxygen in air-saturated 140 mM KCl at 40°C was taken as 0.380 μg-atom O/ml (Dawson, 1982). The pH change was calibrated by 1–10 μl additions of anaerobic KOH, and the $\rightarrow H^+/O$ quotient was calculated from linear plots of the ΔpH against the amount of oxygen added.

The half-time of decay (t1/2) of the ΔpH resulting from an oxygen pulse was determined from plots of log ΔpH against time.

2.16 Measurement of cytochrome contents

Cytochrome contents of whole cells were measured by recording reduced minus oxidised difference spectra using cell suspensions of approximately 6 mg dry weight/ml. The air-oxidised minus air-oxidised spectrum was first recorded, then the contents of the sample cuvette were reduced with a few grains of dithionite whilst those of the reference cuvette were oxidised with 5 μl 0.1 M potassium ferricyanide, and the dithionite-reduced minus ferricyanide oxidised difference spectrum was recorded. Total c-type cytochromes were estimated from the difference in absorbance between the peak at 552 nm and the trough at 538 nm (ε = 17.3 $mM^{-1}.cm^{-1}$), total b-type cytochromes were estimated from the difference in absorbance between the peak at 558 nm and the trough at 572 nm (ε = 17.5 $mM^{-1}.cm^{-1}$), and cytochromes a + a_3 were estimated from the difference in absorbance between the peak at 630 nm and the trough at 610 nm (ε = 11.7 $mM^{-1}.cm^{-1}$; see Jones and Poole, 1985).

CHAPTER 3

OXIDATION OF EXOGENOUS NADH BY *Methylophilus methylotrophus*

3.1 Introduction

3.2 Results

3.2.1 The stoichiometry of oxygen consumption during the oxidation of exogenous NADH

3.2.2 Methanol and exogenous NADH oxidase activities in *M.methylotrophus* grown on methanol or trimethylamine.

3.2.3 pH profile of methanol oxidase and exogenous NADH oxidase activities

3.2.4 Inhibition of methanol, duroquinol and exogenous NADH oxidase activities by HQNO

3.2.5 Inhibition of methanol and exogenous NADH oxidase activities by hydroxylamine

3.2.6 Inhibition of methanol and exogenous NADH oxidase activities by chelating agents

3.2.7 Further investigation of exogenous NADH oxidation by measuring $\Delta[O_2]$, ΔA_{340} and ΔpH

3.2.8 The oxidation of purified NADH

3.3 Discussion

CHAPTER 3

OXIDATION OF EXOGENOUS NADH BY *Methylophilus methylotrophus*

3.1 Introduction

Oxidation of exogenous NADH by intact mitochondria and bacteria occurs via a respiratory chain-linked NADH dehydrogenase. This implies that in intact mitochondria or bacteria either

(i) the NADH binding site of the dehydrogenase is on the external surface of the respiratory membrane (ie. external oxidation), or

(ii) the NADH is transported across the membrane and subsequently oxidised by a dehydrogenase having its binding site on the internal surface of the respiratory membrane (ie. transport followed by internal oxidation).

Mitochondria possess a double membrane . The outer membrane is relatively permeable to most low molecular weight solutes, whilst the inner membrane, which contains the respiratory chain components, is impermeable to many solutes including NADH (Lehninger, 1975). NADH generated by metabolism within the mitochondrion is oxidised by NADH dehydrogenase, a large multisubunit enzyme which spans the mitochondrial inner membrane and whose binding site for NADH is on the matrix side of the mitochondrial inner membrane (M-side; see section 1.9). However, oxidation of exogenous NADH by rat liver mitochondria in the presence of cytochrome *c* has also been observed (Lehninger, 1951), thus these mitochondria must possess an externally located NADH dehydrogenase or be able to transport reducing equivalents into the mitochondrion. It was subsequently shown that oxidation of exogenous NADH occurred on the mitochondrial outer membrane with the reduction of cytochrome b_5 (Sottocassa *et al.*, 1967). It has been suggested that cytochrome *c* acts

as an electron shuttle between cytochrome b_5 on the mitochondrial outer membrane and cytochrome oxidase in the mitochondrial inner membrane (Bernardi and Azzone, 1981, 1982).

In addition to the conventional respiratory chain NADH dehydrogenase (with its NADH-binding site on the M-side of the membrane) and an NADH dehydrogenase associated with the mitochondrial outer membrane, plant mitochondria have been reported to possess an NADH dehydrogenase associated with the cytoplasmic surface of the mitochondrial inner membrane (C-side; see Palmer and Møller, 1982). However, whereas the outer membrane NADH dehydrogenase of mung bean mitochondria (Phaseolus aureus) is specific for the 4α hydrogen atom of NADH, the NADH dehydrogenase on the C-side of the mitochondrial inner membrane is specific for the 4β hydrogen atom of NADH (Douce et al., 1973).

In Jerusalem artichoke (Helianthus tuberosus) mitochondria, oxidation of NADH at the C-side of the membrane has yielded P/O quotients of 1.8 (ie. similar to those obtained with succinate), compared with a P/O quotient of 3 for the oxidation of internal NADH (Palmer and Passam, 1971). This suggests that coupling of the external NADH dehydrogenase to the respiratory chain is at the level of ubiquinone. The activity of this enzyme is inhibited by chelators such as EDTA and EGTA and may be regulated by micromolar concentrations of Ca^{2+} (Møller et al., 1981; Moore and Akerman, 1982).

The purification of the NADH dehydrogenase from the C-side of the membrane of cuckoo-pint mitochondria (Arum maculatum) has been reported. The purified enzyme contained two major bands of molecular weights 78000 and 65000 plus a minor band of 76000 when examined via SDS-polyacrylamide gel electrophoresis. The enzyme contained non-covalently bound FAD and a small amount of FMN (Cottingham and

Moore, 1984).

The isolation of an NADH dehydrogenase associated with the C-side of the mitochondrial membrane has also been reported for the yeast Candida utilis. The enzyme had a molecular weight of approximately 1.5 x 10^6 and contained two large subunits and eight smaller subunits of molecular weights 270000 and 135000 respectively. Iron and copper were also present but appeared to be contaminants (Mackler et al., 1980).

The physiological significance of the externally-located NADH dehydrogenases remains to be elucidated, although they clearly couple the oxidation of cytosolic NADH to ATP synthesis with a lower efficiency than that exhibited by the conventional respiratory chain enzyme. The significance of this is unknown, but it is possible that the removal of excess reducing power from the cytoplasm may be more important than extra ATP synthesis. Reducing equivalents from cytoplasmic NADH may be indirectly transported into the mitochondria via the malate-aspartate shuttle. Malate, formed by reduction of oxaloacetate by NADH, is taken into the mitochondrion in exchange for α-ketoglutarate. Re-oxidation of malate yields oxaloacetate and NADH within the mitochondrion. Transamination between oxaloacetate and glutamate yields α-ketoglutarate, which may drive further malate uptake, and aspartate which may be transported out of the mitochondria in exchange for glutamate. Cytoplasmic transamination yields oxaloacetate to complete the cycle. It is possible that this transport system may not be particularly fast, such that it is better to oxidise NADH externally with a diminished P/O quotient to yield a higher rate of ATP production.

By contrast, the ability to oxidise exogenous NADH would appear to be superfluous in free-living bacteria. However, this property has been reported for whole cells of Haemophilus parainfluenzae, where it was attributed to a high permeability of the cell envelope to NADH;

unfortunately no attempt was made to confirm this permeability to NADH experimentally (White and Smith, 1964; White and Sinclair, 1971). In addition, right-side-out vesicles of Bacillus subtilis have been reported to oxidise NADH at their outer surface. No uptake of NADH was found and the presence of only one NADH dehydrogenase was demonstrated; this was located on the cytoplasmic side of the membrane (it should be noted that the cytoplasmic side of the bacterial membrane is equivalent to the M-side of the mitochondrial membrane). Solubilisation of 74% of the NADH dehydrogenase with Triton-X-100 decreased the NADH oxidation rate by only 24%. This could not be explained by an increased permeation rate of NADH across the membrane. Membrane vesicles of a menaquinone-deficient mutant of B.subtilis did not oxidise external NADH. A high rate of oxidation was restored when the menaquinone analogue, menadione, was added. It was concluded that NADH donates electrons directly to menaquinone and that an NADH dehydrogenase was not involved (Bergsma et al., 1981).

The ability of whole cells of M.methylotrophus to oxidise exogenous NADH at a rate of 171 ng atom O/min per mg dry weight bacteria has been reported (Cross and Anthony, 1980b). It has further been claimed that the cells are permeable to NADH (Anthony, 1982) and that oxidation of exogenous NADH drives ATP synthesis in an uncoupler-dependent manner but without the mediation of cytochrome b (Dawson, 1982). A novel NADH dehydrogenase having an NADH binding site on the C-side of the membrane would have important implications for energy conservation in M.methylotrophus for such an enzyme might be unable to catalyse H^+ translocation at site 1 depending on the mechanism of this process (eg. protonmotive redox loop cf. pump see section 1.7). Alternatively, an uptake system for NADH would be of unique interest. Further investigations of the apparent ability of M.methylotrophus to

oxidise exogenous NADH were therfore undertaken.

3.2 Results

3.2.1 The stoichiometry of oxygen consumption during the oxidation of exogenous NADH

The stoichiometry of oxygen consumption during the oxidation of exogenous NADH by whole cells of M.methylotrophus was determined using an oxygen electrode (Fig. 3.1). The results indicated a linear relationship over the range tested (0-0.9 μmol NADH) and are commensurate with a stoichiometry of 1 ng-atom O/nmol NADH as predicted by the equation

$$NADH + H^+ + 1/2\ O_2 \longrightarrow NAD^+ + H_2O$$

3.2.2 Methanol and exogenous NADH oxidase activities in M.methylotrophus grown on methanol or trimethylamine

The methanol oxidase and exogenous NADH oxidase activities of whole cells of M.methylotrophus following growth in methanol-limited continuous culture, or in batch culture with trimethylamine carbon source, were measured (Table 3.1). NADH oxidase activities closely paralleled methanol oxidase activities in both methanol-grown and trimethylamine-grown cells. The methanol oxidase activity observed in cells grown in methanol-limited continuous culture was generally rather variable within the range 700 to 1200 ng-atom O/min/mg cells. In these experiments the methanol oxidase activity was at the upper end of this range and was closely matched by the exogenous NADH oxidase activity. Both methanol oxidase and NADH oxidase activities were considerably decreased in cells grown in batch culture with trimethylamine as the carbon source, but again the two activities were very similar to each other. The rate of oxygen uptake in the presence of both methanol and trimethylamine was no faster than with each substrate alone, indicating a common rate-limiting step.

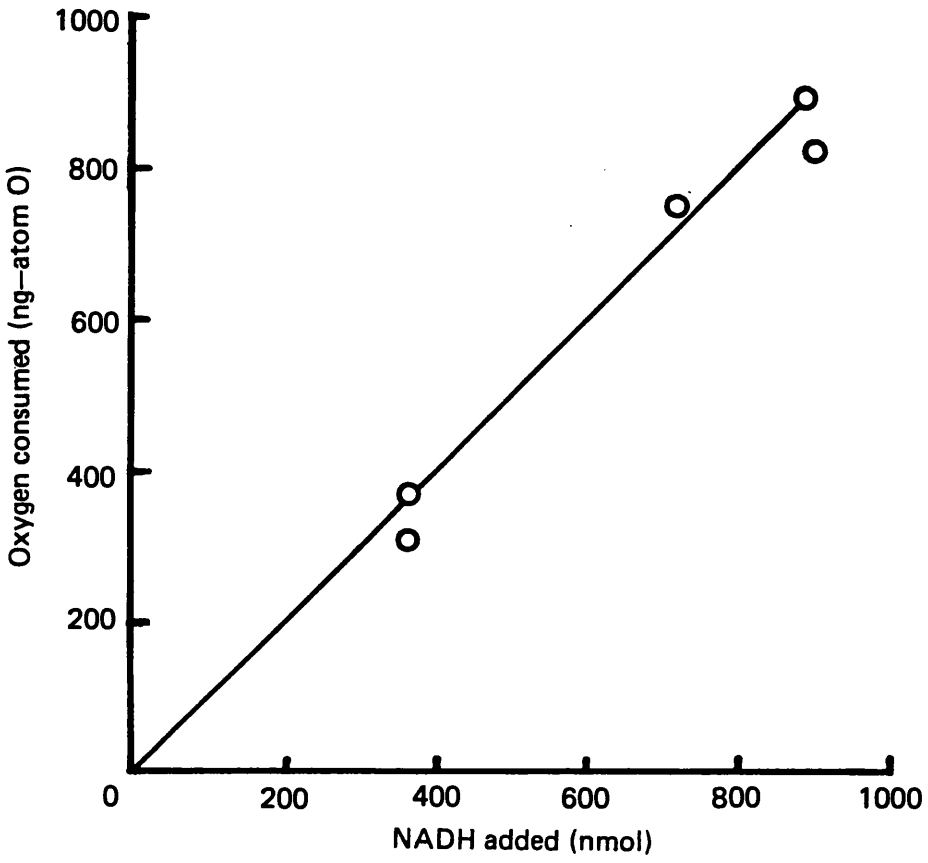


Fig. 3.1 The stoichiometry of oxygen consumption upon the addition of NADH to whole cells of M.methylotrophus

Washed cells of M.methylotrophus (approx. 0.5mg dry weight cells) were incubated at 40°C in 4 mls of 20 mM glycylglycine-KOH buffer pH 7.0. The extent of oxygen consumption upon the addition of aliquots of NADH was measured as described in section 2.7.

Table 3.1 Methanol oxidase and exogenous NADH oxidase activities in *M.methylotrophus* grown on methanol or trimethylamine

Growth conditions	Growth substrate	Respiratory substrate		
		CH_3OH	NADH	CH_3OH + NADH
		(ng-atom O/min/mg)		
Continuous culture	methanol	1189	1127	1157
Batch culture	trimethylamine	580	516	499

M.methylotrophus was grown in continuous culture ($D = 0.18\ h^{-1}$) with methanol as the carbon source, or in batch culture with trimethylamine as the carbon source. Washed cells (approx. 0.5 mg dry weight) were incubated for 2 minutes in 20 mM glycylglycine pH 7.0 at 40°C prior to the introduction of methanol (12.5 mM) and/or NADH (1 mM).

3.2.3 pH profile of methanol oxidase and exogenous NADH oxidase activities

The activities of methanol oxidase and exogenous NADH oxidase in whole cells grown in methanol-limited continuous culture were measured over the pH range 6.5 to 8.0 (Fig. 3.2). The two oxidase activities exhibited a similar pH profile with optimum activity at pH 7.0 to 7.1. In each case respiration at pH 7.0 was more rapid in glycylglycine buffer than in PIPES buffer.

3.2.4 Inhibition of methanol, duroquinol and exogenous NADH oxidase activities by HQNO

2 n-Heptyl-4-hydroxy quinoline-N-oxide (HQNO) inhibited duroquinol oxidation (I_{50} = 1.25 μM) but had no effect on the oxidation of methanol or exogenous NADH (Fig. 3.3). HQNO inhibits respiration at the level of cytochrome b, thus oxidation of duroquinol, which interacts with the respiratory chain at the level of the quinone pool or cytochrome b (Boveris et al., 1971; Kröger and Klingenberg, 1973), was blocked. Methanol dehydrogenase is coupled to the respiratory chain at the level of cytochrome c (Anthony, 1975; Bamforth and Quayle, 1978) and therefore was not inhibited by HQNO. Oxidation of exogenous NADH was not inhibited by HQNO, suggesting coupling of this activity to the respiratory chain after cytochrome b.

3.2.5 Inhibition of methanol and exogenous NADH oxidase activities by hydroxylamine

Hydroxylamine is a structural analogue of methanol (NH_2OH cf CH_3OH) and is a known competitive inhibitor of methanol dehydrogenase (Duine and Frank, 1980). The effect of a competitive inhibitor on an enzyme-catalysed reaction may be described by the equation

$$\frac{1}{v} = \frac{Km}{V_{max} \cdot K_I \cdot [S]} \cdot [I] + \frac{1}{V_{max}} \left(1 + \frac{Km}{[S]} \right)$$

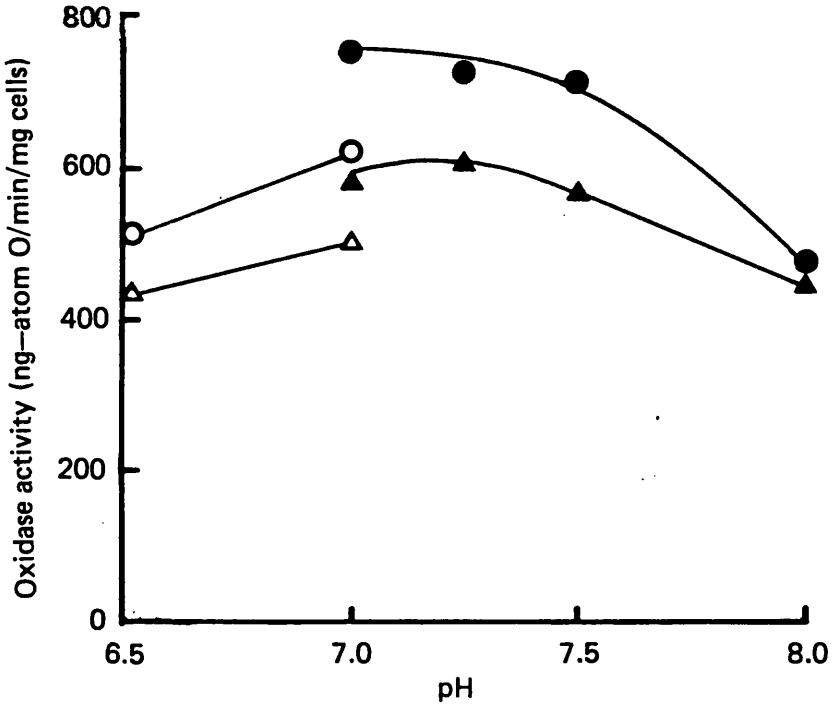


Fig. 3.2 pH Profiles of methanol oxidase and exogenous NADH oxidase activities

Washed cells (approx. 0.5 mg dry weight) were incubated for 2 minutes at 40°C in 4 ml of assay buffer prior to the introduction of methanol (1 mM) or NADH (1 mM). Buffers used were 20 mM PIPES/KOH (pH 6.5 and 7.0) and 20 mM glycylglycine/KOH (pH 7.0 to pH 8.0).

Symbols: Methanol ○ PIPES buffer

● glycylglycine-KOH buffer

NADH △ PIPES buffer

▲ glycylglycine-KOH buffer

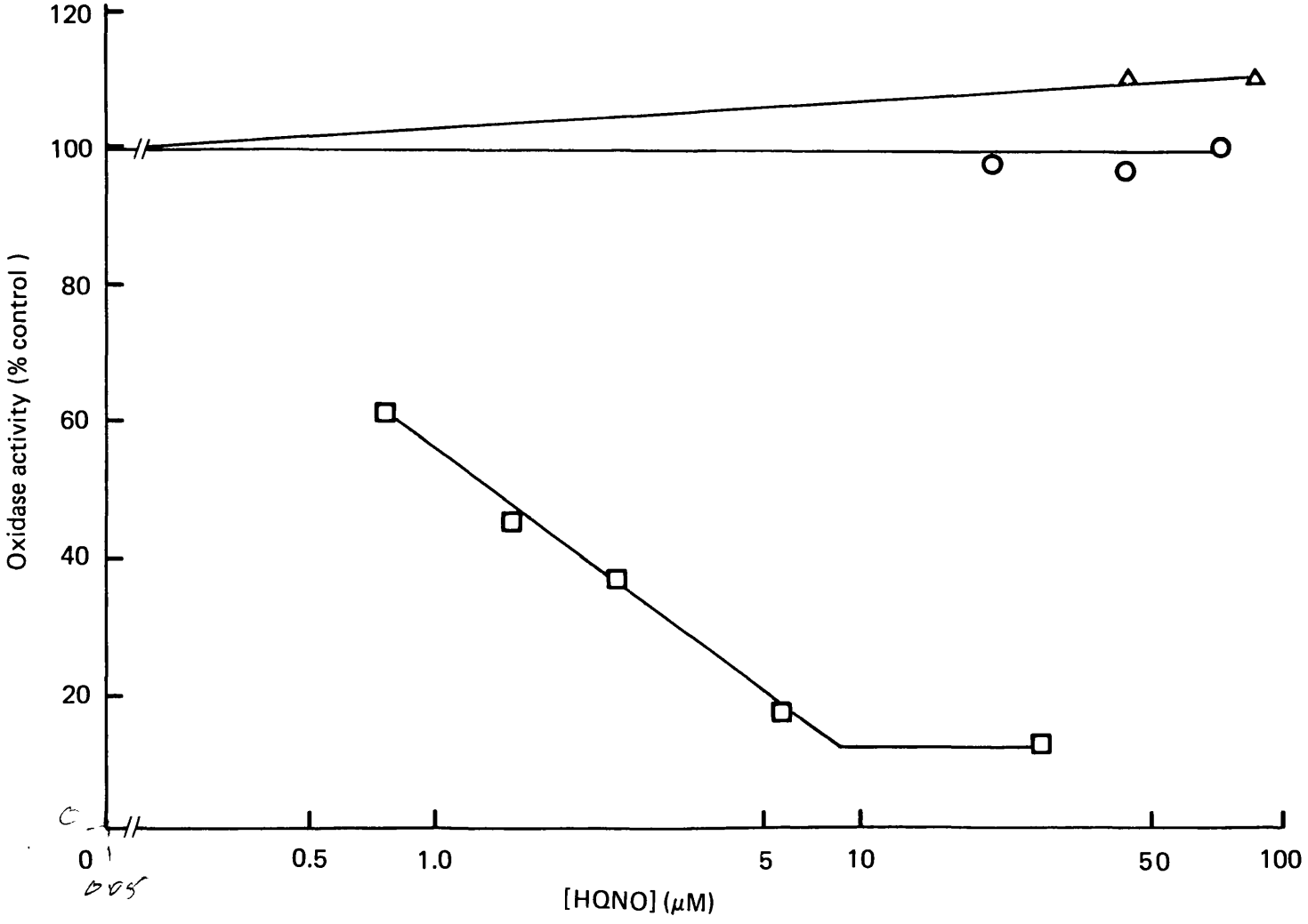

120
100
80
60
40
20
0
0.5
1.0
5
10
50
100
Oxidase activity (% control)
[HQNO] (μM)

Fig. 3.3 Inhibition of methanol oxidase, exogenous NADH oxidase and duroquinol oxidase activities by HQNO

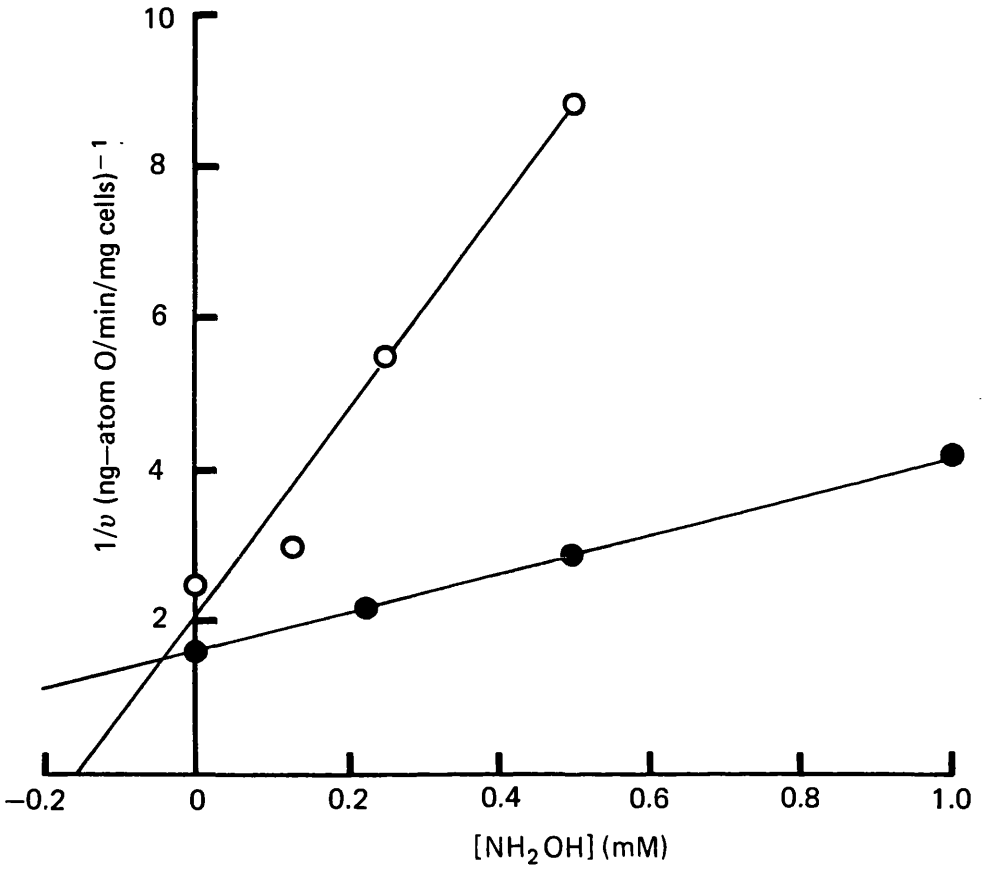
Washed cells (approx. 0.5mg dry weight) were incubated for 2 minutes in 4ml 20mM glycylglycine buffer pH 7.0 with HQNO at 40°C prior to the addition of methanol (1mM; ○), NADH (1mM; △) or duroquinol (1.2mM; □).

This equation predicts that a plot of $\frac{1}{v}$ against inhibitor concentration will yield a straight line with an intercept on the x axis of $-K_I \left(1 + \frac{[S]}{Km}\right)$ and a slope of $\frac{Km}{V_{max} \cdot K_I \cdot [S]}$. Furthermore, if points are plotted with different substrate concentrations, competitive inhibition gives converging lines, the point of convergence giving $-K_I$ on the x axis and $1/V_{max}$ on the y axis (Dixon, 1953). The inhibition of methanol oxidase and exogenous NADH oxidase was investigated using this approach. Dixon plots (1/v versus [I]) indicated competitive inhibition of both methanol oxidase and exogenous NADH oxidase by hydroxylamine with K_I values of 45 μM and 30 μM respectively (Fig. 3.4). A linear mixed-type inhibitor yields the same type of Dixon plot as a pure competitive inhibitor. These forms of inhibition may be distinguished by plotting the slope of the lines from the Dixon plot against 1/[S]. A competitive inhibitor gives a line which intercepts the origin whilst the line from a mixed-type inhibitor does not. Replotting the slopes of the Dixon plots against 1/[S] gave lines with a positive intercept on the y axis for both methanol oxidase and exogenous NADH oxidase activities (not shown). This suggests that a mixed-type of inhibition was occurring; however, this result must be viewed with caution as it was based on a line with only two points. These data do show, however, that the kinetics for the inhibition of methanol dehydrogenase and NADH dehydrogenase by hydroxylamine were remarkably similar.

3.2.6 Inhibition of methanol and exogenous NADH oxidase activities by chelating agents

EDTA is a known inhibitor of methanol oxidation by methylotrophic bacteria (eg. Anthony and Zatman, 1964; Duine et al., 1979). Both methanol oxidase and exogenous NADH oxidase activities were completely inhibited by EDTA and exhibited essentially identical

a)


10
8
6
4
2
1/υ (ng–atom O/min/mg cells)⁻¹
–0.2
0
0.2
0.4
0.6
0.8
1.0
[NH2OH] (mM)

b)

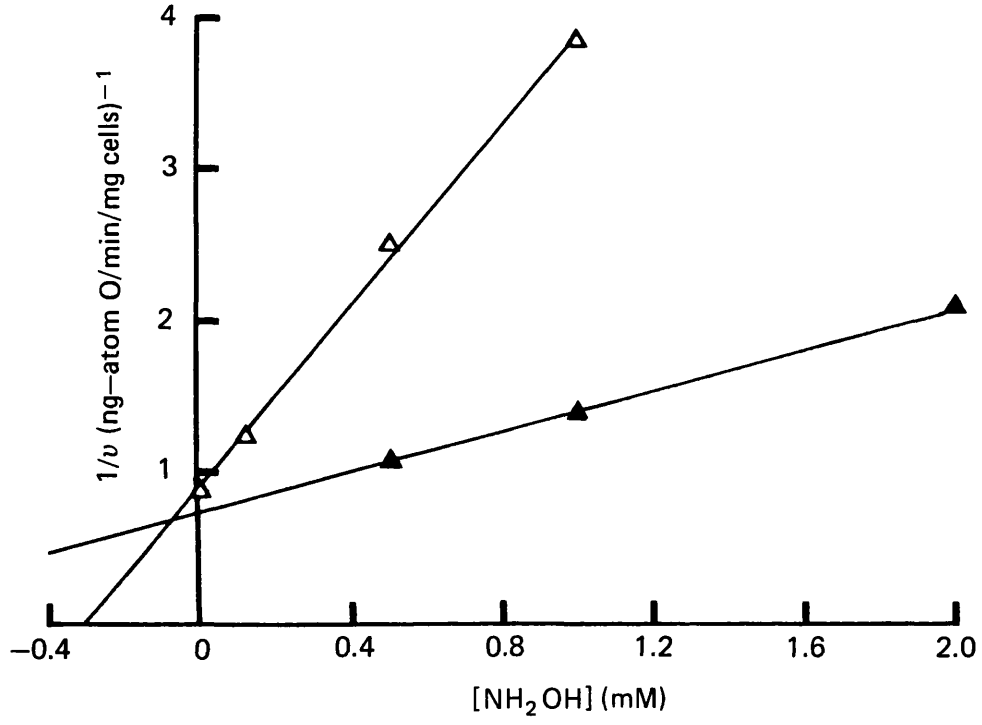

4
3
2
1
1/υ (ng–atom O/min/mg cells)⁻¹
–0.4
0
0.4
0.8
1.2
1.6
2.0
[NH2OH] (mM)

Fig. 3.4 Inhibition of methanol oxidase and exogenous NADH oxidase activities by hydroxylamine

Washed cells (approx. 0.5mg dry weight) were incubated for 1 minute in 4 ml 20mM glycylglycine-KOH buffer pH 7.0 with hydroxylamine at 40°C prior to the introduction of (a) methanol (0.1mM, ○ ; or 0.5mM, ●) or (b) NADH (0.5mM, △ ; or 6mM, ▲).

inhibition curves with 50% inhibition at 15 μM EDTA (Fig. 3.5). In contrast, methanol dehydrogenase activity was not completely inhibited by EDTA and 30% of the original activity remained in the presence of 160 μM EDTA (Fig. 3.6).

The inhibition of methanol oxidase activity by EDTA was found to be partly reversible, ie. the addition of divalent metal ions to EDTA-inhibited cells produced a substantial increase in the oxidation rate (Table 3.2). Thus methanol oxidase activity was inhibited by 79% in the presence of 25 μM EDTA, but this inhibition was decreased to 51% following the addition of Cu^{2+} (25 μM). Further alleviation of inhibition could not be achieved by the addition of a further aliquot of Cu^{2+}. Similar results were obtained with Ca^{2+}, whereas Mg^{2+} was less effective and higher concentrations were required to bring about a similar degree of reactivation. Similar concentrations of sodium chloride and sodium sulphate had no effect on the degree of inhibition by EDTA, indicating that the alleviation of inhibition was not due to activation by the anions SO_4^{2-} and Cl^-.

The ability of Cu^{2+} and Ca^{2+} to bring about maximal reactivation of the EDTA-inhibited methanol oxidase in concentrations equivalent to that of EDTA indicates that these ions form more stable complexes with EDTA than does either Mg^{2+} or the endogenous ion.

The inhibition of methanol oxidase activity by chelating agents was further examined by the use of EGTA. There was a substantial difference between the inhibition curves of methanol oxidase activity produced by EDTA and EGTA (Fig. 3.7). It was concluded that EGTA is a less potent inhibitor of methanol oxidase activity than EDTA (I_{50} 80 μM cf. 15 μM).

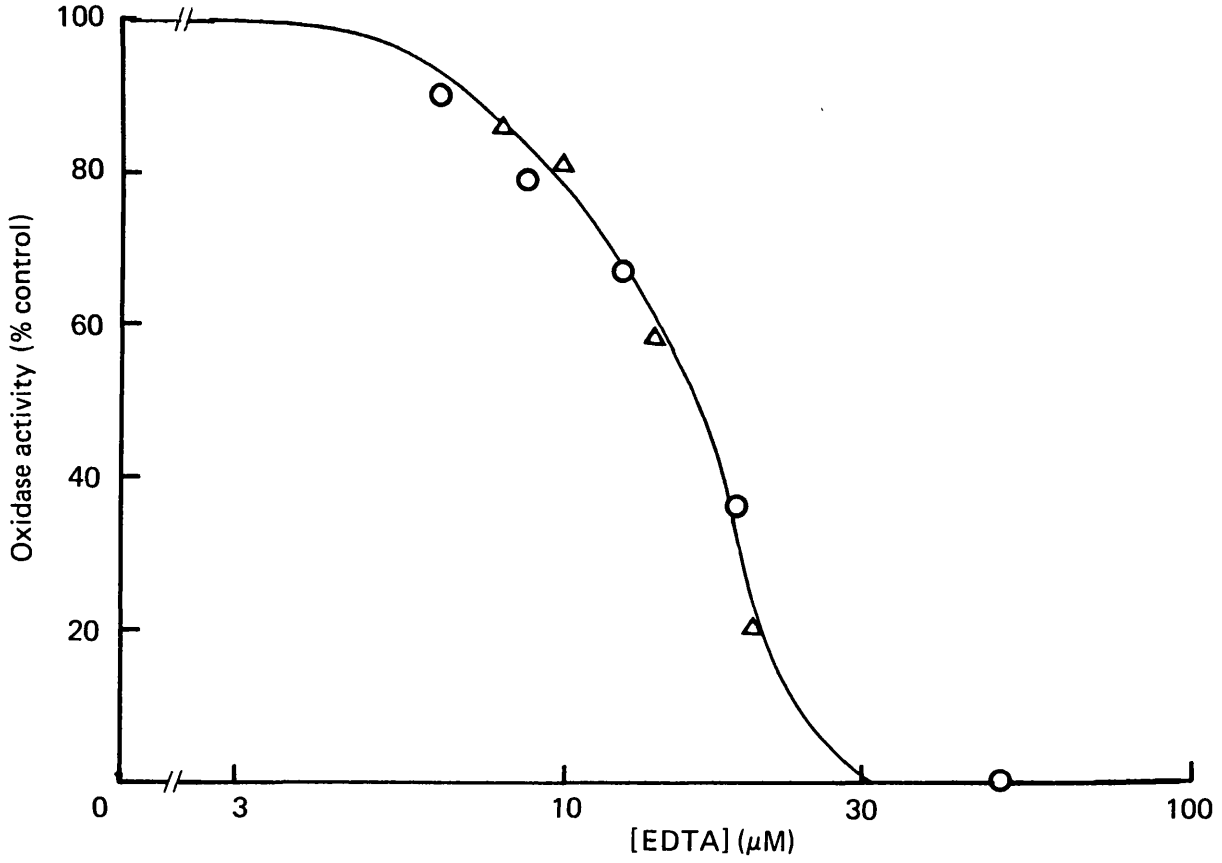

100
80
60
40
20
0
3
10
30
100
Oxidase activity (% control)
[EDTA] (μM)

Fig. 3.5 Inhibition of methanol oxidase and exogenous NADH oxidase activities by EDTA

Washed cells (approx. 0.5mg dry weight) were incubated for 2 minutes in 4ml glycylglycine-KOH buffer pH 7.0 with EDTA at 40°C prior to the introduction of methanol (1mM, ○) or NADH (1mM △).

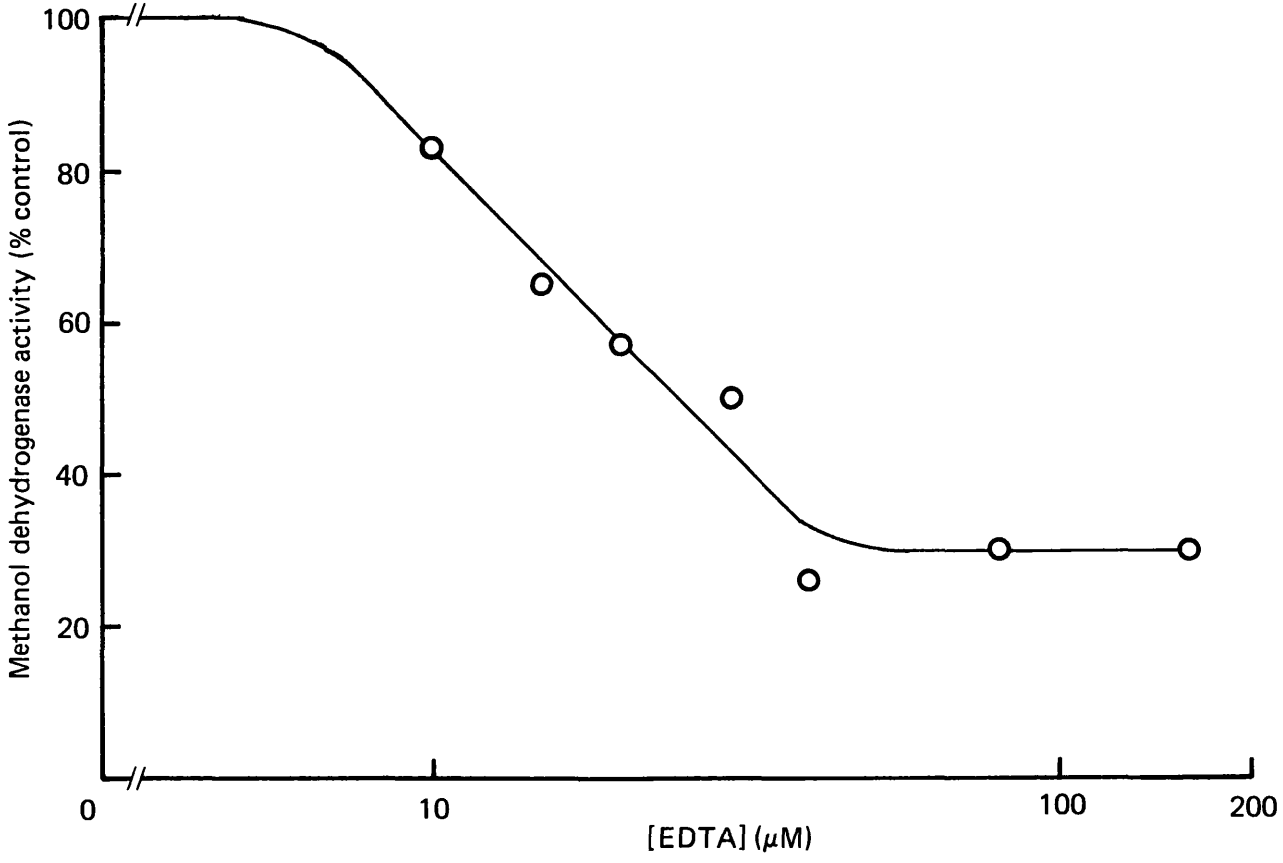

100
80
60
40
20
0
10
100
200
Methanol dehydrogenase activity (% control)
[EDTA] (μM)

Fig. 3.6 Inhibition of methanol dehydrogenase by EDTA

Washed cells (approx. 10μg dry weight) were incubated for 2 minutes in the reaction mixture (0.43mM PMS, 0.2mM DCPIP, 6mM NH_4Cl, 1mM KCN in 4ml 20mM TRIS-HCl buffer pH 8.5 with EDTA prior to the introduction of methanol (1mM).

Table 3.2 Reactivation of EDTA-inhibited methanol oxidase by divalent cations

[EDTA]	[Cation]	Methanol oxidase activity (%)
-	-	100
25 μM	-	21
25 μM	25 μM Cu^{2+}	41
25 μM	50 μM Cu^{2+}	37
25 μM	25 μM Ca^{2+}	53
25 μM	50 μM Ca^{2+}	53
25 μM	25 μM Mg^{2+}	27
25 μM	50 μM Mg^{2+}	39

Washed cells (approx. 0.5 mg dry weight) were incubated in 4 ml 20 mM glycylglycine pH 7.0 at 40°C with or without EDTA for 2 minutes prior to the addition of methanol (1 mM). Respiration rates were recorded for approximately 1.5 minutes prior to the addition of divalent cations ($CuSO_4$, $CaCl_2$ or $MgCl_2$). The above concentrations of these salts had no effect on the respiratory activities in the absence of EDTA.

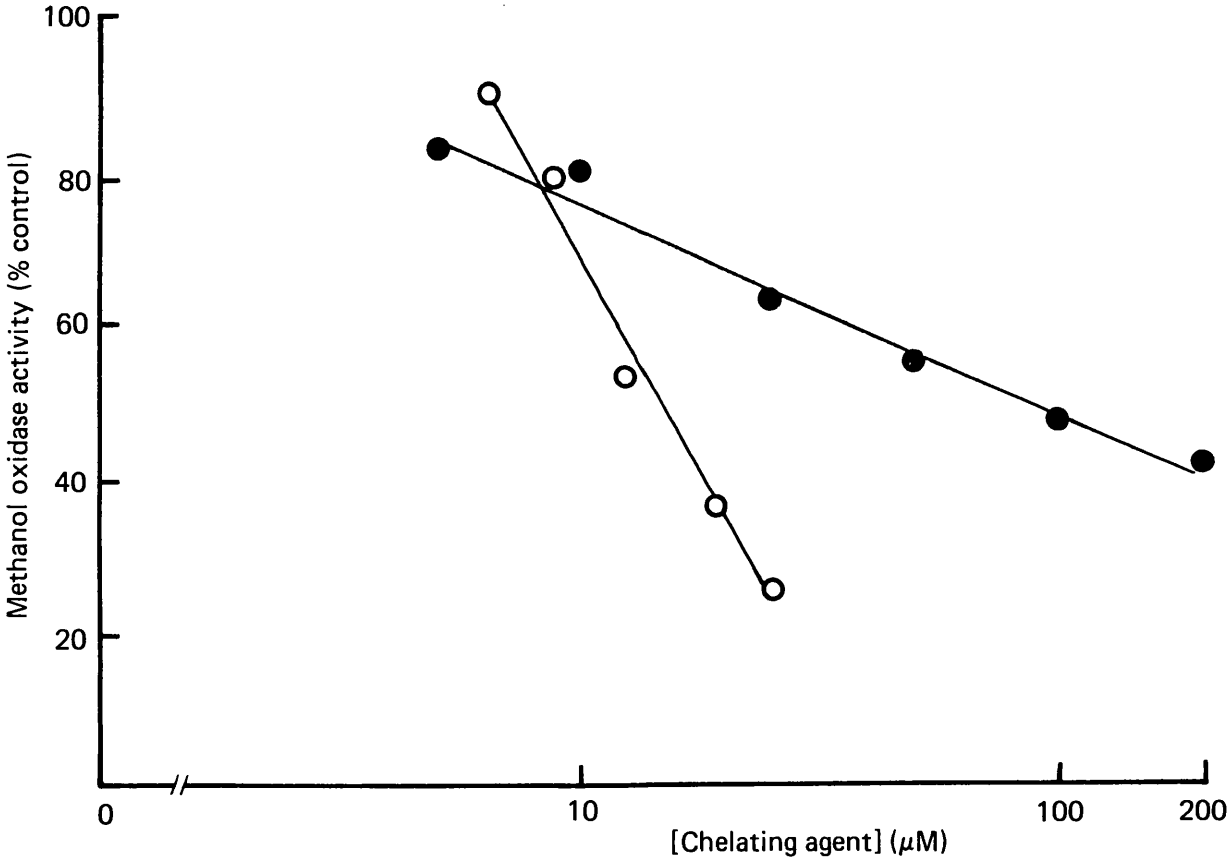

100
80
60
40
20
Methanol oxidase activity (% control)
0
10
100
200
[Chelating agent] (μM)

Fig. 3.7 Inhibition of methanol oxidase by EDTA and EGTA

Washed cells (approx. 0.5mg dry weight) were incubated in 4ml 20mM glycylglycine-KOH buffer pH 7.0 at 40°C with EDTA (○) or EGTA (●) for 2 minutes prior to the introduction of methanol (1mM).

3.2.7 Further investigation of exogenous NADH oxidation by measuring $\Delta[O_2]$, ΔA_{340} and ΔpH

NADH is oxidised according to the equation

$$NADH + H^+ + 1/2\ O_2 \longrightarrow NAD^+ + H_2O$$

This reaction may therefore be examined by measuring the changes in the concentrations of (i) oxygen, (ii) NADH, and (iii) H^+ (ie. pH).

The remarkable similarities in the inhibition patterns of methanol oxidase and exogenous NADH oxidase activities by HQNO, chelating agents and hydroxylamine described above increasingly pointed to the possibility that both activities may conceivably be catalysed by the same initial enzyme, ie. methanol dehydrogenase. If this were so, then methanol and NADH may act as inhibitors of each other's oxidation. With this in mind the oxidation of NADH by whole cells was followed spectrophotometrically by measuring the change in absorbance at 340 nm to see if the NADH oxidation rate was diminished in the presence of methanol. However, upon addition of NADH to whole cells of M.methylotrophus no subsequent decrease in absorbance at 340 nm was observed, despite oxygen consumption being observed in a parallel experiment which was monitored using an oxygen electrode (Table 3.3). In contrast, when NADH was added to sphaeroplasts of M.methylotrophus which had been lysed by resuspension in 20 mM glycylglycine buffer pH 7.0, both oxygen consumption and a decrease in absorbance at 340 nm were observed. These yielded similar specific activities which were more than an order of magnitude lower than the rate of oxygen consumption by whole cells and probably reflected the activity of the conventional NADH oxidase system in the respiratory chain.

The inability of whole cells to catalyse a decrease in absorbance at 340 nm in the presence of NADH indicates that the latter was not being oxidised and, together with the earlier stoichiometry data,

suggests the presence in the NADH of a second oxidisable substrate at a concentration which was similar in molar terms to that of NADH itself.

In a further experiment, a limiting amount of NADH was added to whole cells and oxygen consumption was followed to completion. It was found that the subsequent addition of lysed sphaeroplasts elicited a further burst of oxygen uptake, both periods consuming an amount of oxygen (g-atom) that was approximately equal to the amount of NADH (mole) added (Fig. 3.8).

The equation shown above for the oxidation of NADH predicts a net consumption of both oxygen and protons. The pH change which occurred upon the addition of NADH to whole cells or lysed sphaeroplasts were therefore measured in parallel with oxygen consumption. Upon the addition of a limiting amount of NADH to lysed sphaeroplasts, oxygen consumption was accompanied by alkalinisation of the medium (Fig. 3.9a). This alkalinisation corresponded to a consumption of approximately 1.5 ng-ion H^+/ng-atom O (calculated from the initial rates of oxygen consumption and alkalinisation), compared with a theoretical value of 1 ng-ion H^+/ng-atom O. The measured value is possibly inaccurate due to the low rates of respiration observed in lysed sphaeroplasts. In contrast, following the addition of a limiting amount of NADH to whole cells, oxygen consumption occurred in two phases (Fig. 3.9b). during the initial phase there was a rapid and linear consumption of oxygen with only a very small change in pH. The second phase was linear, but slower, and was accompanied by a substantial acidification which corresponded to approximately 1 g-ion H^+/g-atom O (Table 3.4). These results confirm that whole cells do not oxidise exogenous NADH, but do oxidise a second substrate which is present in the NADH solution and which gives rise to an acidic product.

Table 3.3 Exogenous NADH oxidase activity measured from the rate of change of oxygen concentration and A_{340}

Preparation	Specific activities	
	ΔO_2 (ng-atom O/min/mg cells)	ΔA_{340} (nmol NADH/min/mg cells)
Whole cells	1160	0
Lysed sphaeroplasts	86	97

Washed cells (approx. 0.125 mg dry weight) or lysed sphaeroplasts (equivalent to approx. 0.125 mg dry weight cells) were incubated in 1 ml 20 mM glycylglycine pH 7.0 at 40°C for 2 minutes prior to addition of NADH (0.2 mM). The change in absorbance at 340 nm was measured spectrophotometrically.

To measure oxygen consumption, washed cells (0.5 mg dry weight) or lysed sphaeroplasts (equivalent to 0.5 mg dry weight whole cells) were incubated in 4 ml 20 mM glycylglycine pH 7.0 at 40°C for 2 minutes prior to the addition of NADH (0.2 mM).

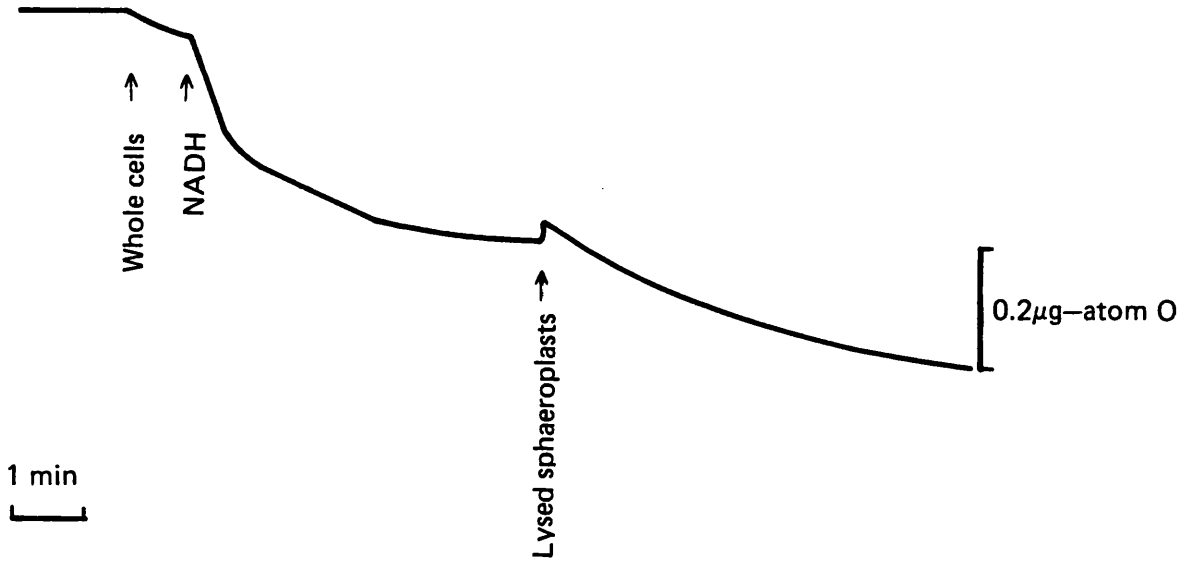


Fig. 3.8 Oxygen consumption by whole cells and lysed sphaeroplasts in the presence of NADH

Whole cells (approx. 0.5mg dry weight) were incubated in 4ml 20mM glycylglycine buffer pH 7.0 at 40°C for 1 minute prior to the addition of 360 nmol NADH. Oxygen consumption was recorded until completion, then lysed sphaeroplasts (equivalent to 0.8mg dry weight whole cells) were added.

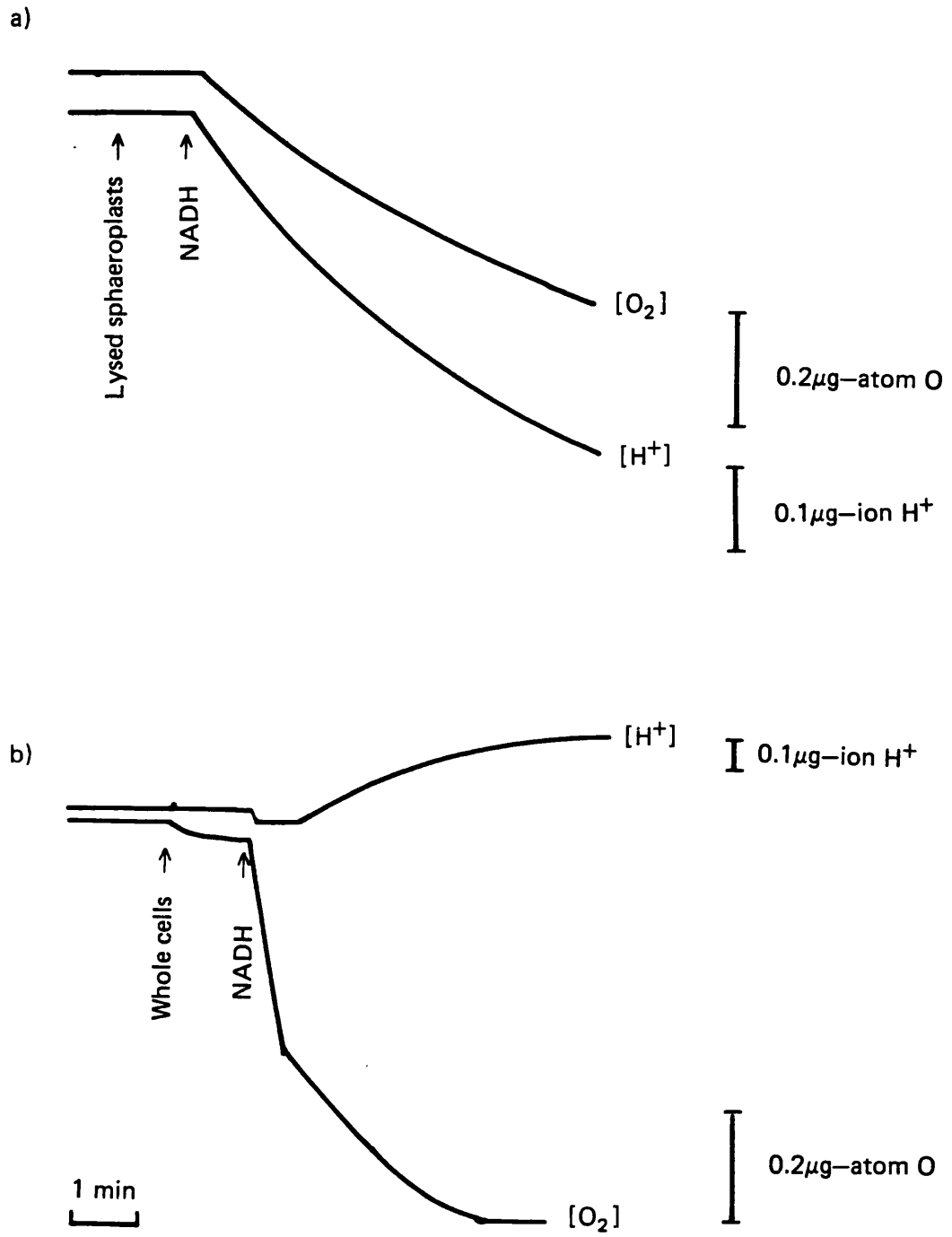


Fig. 3.9 Oxygen consumption and pH change upon the addition of NADH to lysed sphaeroplasts or whole cells

Washed cells (approx. 0.5mg dry weight) or lysed sphaeroplasts (equivalent to approx. 0.5mg dry weight whole cells) were incubated for 2 minutes in 4ml glycylglycine buffer pH 7.0 at 40°C prior to the introduction of NADH (900 nmol)

a) Lysed sphaeroplasts b) Whole cells

Table 3.4 Oxygen consumption and pH change following the addition of NADH to whole cells and lysed sphaeroplasts

Preparation	Phase	Specific activities		H^+/O
		(ng-atom O/min/mg cells)	(ng-ionH^+/min/mg cells)	
Whole cells	1	904 ± 110 (3)	+ 72 ± 38 (3)	0.08
	2	201 ± 5 (3)	191 ± 41 (3)	0.95
Lysed sphaeroplasts		36 ± 4 (4)	-55 ± 11 (4)	-1.5

Washed cells (approx. 0.5 mg dry weight) or sphaeroplasts (equivalent to approx. 0.5 mg dry weight whole cells) were incubated for 2 minutes in 4 ml 1.5 mM glycylglycine buffer pH 7.0 at 40°C prior to the introduction of NADH (900 nmol; see Fig. 3.8).

3.2.8 The oxidation of purified NADH

NADH was purified by ion exchange chromatography on a DEAE-cellulose column (Fig. 3.10). The initial fractions that eluted with 1.5 mM glycylglycine pH 7.0 were bulked and placed in an oxygen electrode reaction chamber. These fractions contained no NADH as measured by absorption at 340 nm, but on addition of whole cells of M.methylotrophus a rapid consumption of oxygen was observed (Table 3.5). In contrast, upon the addition of an aliquot of the later bulked fractions that contained purified NADH to whole cells of M.methylotrophus, no consumption of oxygen was observed, thus confirming that whole cells of M.methylotrophus are unable to oxidise exogenous NADH.

3.3. Discussion

The ability of whole cells of M.methylotrophus to oxidise added NADH (Cross and Anthony, 1980) has been investigated in detail. Although the addition of a solution of NADH (Boehringer) to whole cells indeed resulted in oxygen consumption, when the NADH was purified by passage through a DEAE-cellulose column the purified NADH was unable to elicit respiration. A contaminant was separated from the NADH and was capable of being oxidised. Furthermore, in view of the closely similar properties of methanol oxidase and 'NADH oxidase' (eg. specific activities, pH optima and sensitivities to inhibition by HQNO, hydroxylamine and EDTA) it appears likely that the contaminant is either methanol or a closely related primary alcohol or aldehyde. The known substrate specificities of methanol dehydrogenase suggested that either methanol, ethanol or formaldehyde was the most likely substrate since all three are readily oxidised by methanol dehydrogenase (Anthony and Zatman, 1967) and could yield the acidic products formic acid, or acetic acid. No gas-liquid chromatography was readily available to analyse the

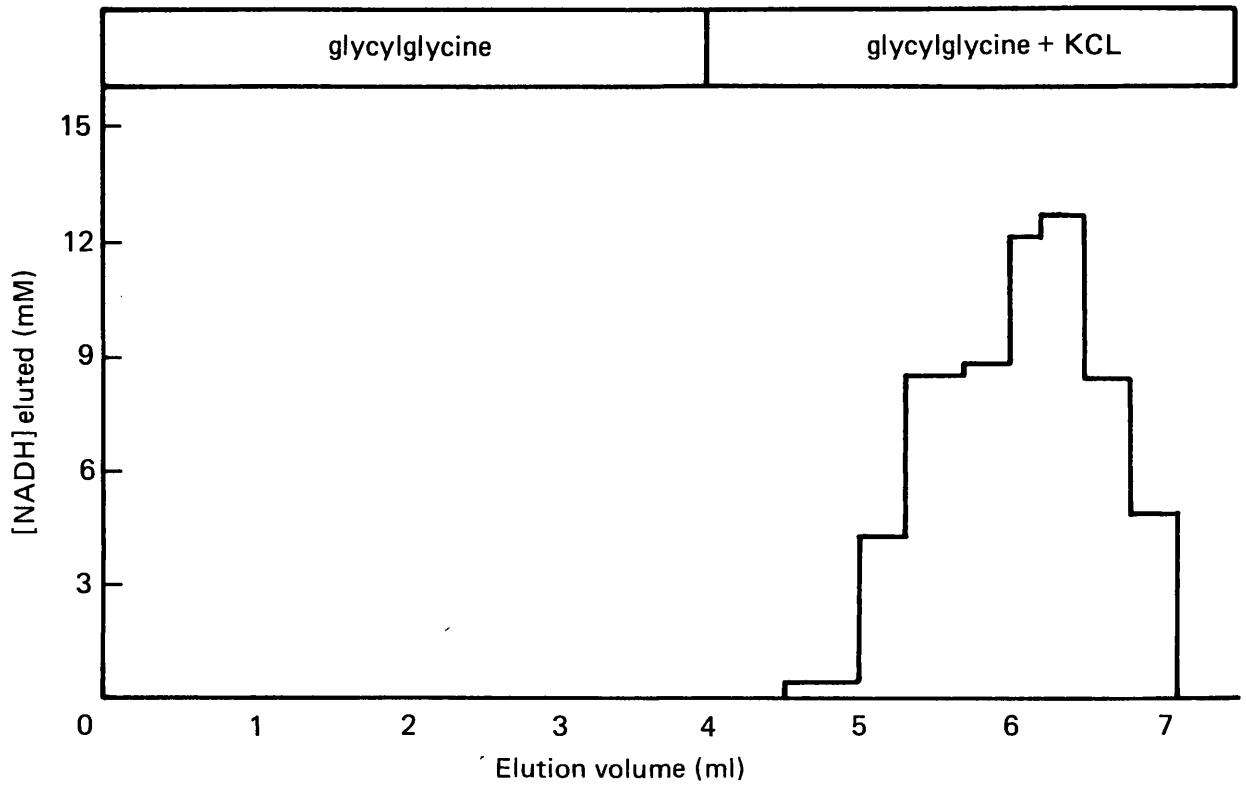
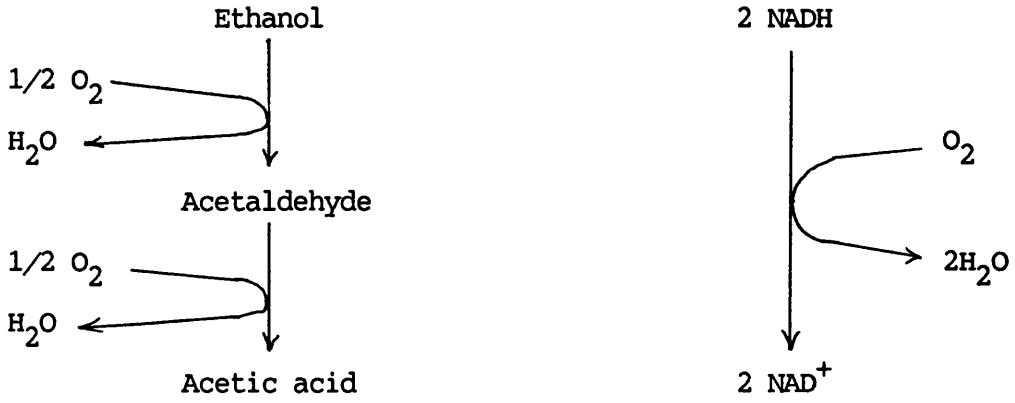

glycylglycine
glycylglycine + KCL
15
12
9
6
3
0
1
2
3
4
5
6
7
[NADH] eluted (mM)
Elution volume (ml)

Fig. 3.10 Purification of NADH

A DEAE cellulose column (bed volume 1.8ml) was prewashed with 1.5mM glycylglycine buffer pH 7.0. 0.4ml of 57mM NADH was applied and eluted successively with 1.5mM glycylglycine buffer pH 7.0 and 1.5mM glycylglycine buffer pH 7.0 plus KCl (0.1M). Fractions were collected and the NADH concentration was determined from the absorbance at 340nm.

contaminant; however representatives from Boehringer eventually admitted that their NADH was contaminated with ethanol to the extent of approximately 3% (w/w). In molar terms this means that commercial NADH contains a 2:1 molar ratio of NADH to ethanol. Thus oxidation of the ethanol in the NADH gave a stoichiometry of oxygen consumption which was consistent with NADH oxidation because the ethanol was oxidised to acetaldehyde and then to acetic acid, both reactions being catalysed by methanol dehydrogenase in association with the terminal cytochrome system



This sequential oxidation of ethanol thus readily explains the observed biphasic rates of oxygen consumption and acidification which were observed in later experiments; the first step occurs rapidly but yields a neutral product whilst the second step occurs more slowly but produces acetic acid. The measurement of pH changes also of course immediately indicated that the expected alkalinisation characteristic of NADH oxidation did not occur.

The inhibition of methanol oxidase by low concentrations of EDTA is of interest as it indicates the involvement of a divalent metal ion in the methanol oxidase system. Comparison of the inhibition curves of methanol oxidase by EDTA and EGTA shows EGTA to be a less effective inhibitor of methanol oxidase activity than EDTA. The stability

constants of EDTA and EGTA with Ca^{2+} are similar ($\log_{10}$ K = 7.3 and 7.1 respectively), whilst Mg^{2+} exhibits significantly different stability constants with EDTA and EGTA $\log_{10}$ K = 5.4 and 2.3 respectively; see Table 3.6) suggesting that the removal of Mg^{2+} may be the mechanism by which chelating agents inhibit methanol oxidase activity. EDTA has been shown to cause a decrease in the aerobic steady state reduction of c-type cytochromes; indicating that EDTA acts between c-type cytochromes and methanol. EDTA has also been shown to inhibit the methanol-dependent reduction of cytochrome c by methanol dehydrogenase in a methanol dehydrogenase/cytochrome c complex isolated anaerobically from Hyphomycrobium (Duine et al., 1979). EDTA also inhibited electron-transfer between methanol dehydrogenase and cytochrome c_L purified from M.methylotrophus (Beardmore-Gray et al., 1983).

Sonic disruption of M.methylotrophus has been shown to cause the methanol dehydrogenase activity to decrease linearly as a function of the decreasing methanol oxidase activity (Carver et al., 1984). When the methanol oxidase activity was completely abolished, about 25% of the original methanol dehydrogenase activity remained. Following high speed centrifugation of the sonicated cell suspension to yield membrane and supernatant fraction, the methanol dehydrogenase activity was found to be entirely in the supernatant fraction and was completely insensitive to EDTA. It was concluded, therefore, that sonication removes methanol dehydrogenase from the periplasmic surface of the respiratory membrane to yield a solubilised, low activity form of the enzyme with no functional coupling to the remainder of the membrane-bound respiratory chain. It was also shown that EDTA caused changes in the relative activities of methanol oxidase and methanol dehydrogenase which were almost identical to those produced by sonication. These results, taken together with the incomplete inhibition of whole cell methanol

Table 3.6 Stability constants of EDTA and EGTA with some divalent cations

Chelating agent	Log_{10} stability constant		
	Cu^{2+}	Ca^{2+}	Mg^{2+}
EDTA	15.5	7.3	5.4
EGTA	12.8	7.1	2.3

Reported stability constants between the chelating agent and divalent metal ions ($K = [\text{chelating agent}.\ M^{2+}]/[\text{chelating agent}].[M^{2+}]$) (Perrin, 1979; O'Sullivan, 1969) were corrected to pH 7.0 using the appropriate pKa values as described by O'Sullivan (1969).

dehydrogenase activity by EDTA, suggests that EDTA, like sonication, causes the functional dislocation of the membrane-associated methanol dehydrogenase.

The mechanism by which divalent metal ions may facilitate the association of methanol dehydrogenase with the membrane bound respiratory chain is unknown; however, divalent metal ions may bridge anionic residues on the enzyme and on various membrane components such as phospholipid or c-type cytochromes.

CHAPTER 4

RESPIRATION-LINKED ATP SYNTHESIS IN *Methylophilus methylotrophus*

4.1 Introduction

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CHAPTER 4

RESPIRATION-LINKED ATP SYNTHESIS IN *Methylophilus methylotrophus*

4.1 Introduction

According to the chemiosmotic theory, aerobic respiration conserves energy by coupling electron transfer within the membrane to the translocation of protons across the osmotic barrier of the membrane, thus generating a trans- or intra-membrane electrochemical potential difference of H^+ (the so-called protonmotive force, $\Delta\bar{\mu}_{H^+}$ or Δp). The latter is subsequently used to drive various energy-requiring membrane processes such as ATP synthesis (see Section 1.8). The efficiency with which the respiratory chain conserves energy in the form of ATP may be expressed as the number of molecules of ATP synthesised from ADP and inorganic phosphate for each atom of oxygen consumed (the ATP/O quotient). The value of the ATP/O quotient reflects the efficiency with which the respiratory chain translocates protons during respiration to generate a trans-membrane electrochemical potential (the $\rightarrow H^+/O$ quotient) and the number of protons required to drive the synthesis of each molecule of ATP (the $\rightarrow H^+/ATP$ quotient). Determination of the ATP/O quotient by comparison of the $\rightarrow H^+/O$ quotient and the $\rightarrow H^+/ATP$ quotient, or by measurement of growth yields, is discussed in section 1.6.

Direct measurement of ATP synthesis (and phosphate utilisation) during respiration have been carried out using both whole cells (Table 4.1) and inside-out membrane vesicles. Early work was almost completely restricted to membrane preparations and the measured P/O quotients were generally much lower than obtained later with whole cells (see Harold, 1972; Gel'man *et al.*, 1975; Jones, 1977). Heterogeneous populations of vesicles with right-side out and inside-out orientations, and hence with poor coupling of ATP synthesis to respiration, may lead to low estimates

Table 4.1 Direct measurements of the efficiency of oxidative phosphorylation in intact bacteria

Organism	Substrate	Acceptor	$P/2e^-$ (mol/g-atom)	$\rightarrow H^+/2e^-$ (g-ion/g-atom)	References
Escherichia coli	endogenous	O_2	3	3.9	Hempfling, 1970; Lawford & Haddock, 1973
"	"	O_2	0.9 - 1.7		Gadkari & Stolp, 1976
Azotobacter vinelandii	endogenous	O_2	3	3.9	Baak & Postma, 1971; Downs & Jones, 1975
"	"	O_2	2		Knowles & Smith, 1970
"	"	O_2	1		van der Beek & Stouthamer, 1973
Proteus mirabilis	endogenous	O_2	1		van der Beek & Stouthamer, 1973
"	"	NO_3	0.37		
Bdellovibrio bacteriovorans	endogenous	O_2	1.4 - 1.8		Gadkari & Stolp, 1976
Beneckea natriegens	succinate	O_2	0.38	2.5 - 3.6	Niven *et al.*, 1977
Paracoccus denitrificans	endogenous	O_2	1	8	Van Verseveld & Stouthamer, 1976; Scholes & Mitchell, 1970
Nitrobacter winogradsky	endogenous	O_2	0.31 - 0.57		Eigener & Bock, 1975
Vibrio succinogenes	formate	fumarate	0.91	2	Krüger & Winkler, 1981 Krüger *et al.*, 1980

of the ATP/O quotient. Exceptionally however, an ATP/O quotient of approximately 3 for NADH oxidation has been obtained with the particularly well-coupled vesicles prepared from various chemolithotrophs (eg. Kiesow, 1964).

Hempfling (1970) measured the formation of ATP and NAD^+, and the concomitant disappearance of AMP and NADH, upon the addition of an oxygen pulse to an anaerobic suspension of intact E.coli in order to determine whether low P/O quotients exhibited by membrane vesicles were due to a genuinely low efficiency of oxidative phosphorylation in the parent organism or to damage incurred by preparation. However, the experiments yielded a P/NADH quotient of 3, and it was therefore concluded that E.coli possessed three sites of oxidative phosphorylation and that ATP synthesis in bacteria was as efficient as in mitochondria. Later evidence however showed that the observed changes in the concentration of NAD(H) were not representative of the amount of oxygen consumed, and it was concluded that despite previous starvation of the cells other substrates were oxidised in addition to NADH and presumably effected a re-reduction of NAD^+ (van der Beek and Stouthamer, 1973). Later studies measured the utilisation of oxygen upon the addition of an oxygen pulse to anaerobic suspensions of various species of bacteria; the increase in the concentration of energy-rich phosphate anhydride bonds in adenine nucleotides ($\Delta \sim P \equiv 2\Delta ATP + \Delta ADP$) gave a ~P/O quotient of 1.75 nmol ~P/ng-atom O in the first second of respiration of endogenous substrates in E.coli (Gadkari and Stolp, 1976). The respiration of endogenous substrates has been shown to be coupled to the translocation of 3.9 g-ion H^+/g-atom O (Lawford and Haddock, 1973) indicating a stoichiometry of proton translocation during ATP synthesis of 2.2 ng-ion H^+/nmol ~P.

The measurement of ATP/O quotients in bacteria has classically

involved the addition of oxygen pulses to anaerobic suspensions of cells, with the subsequent quenching of the reaction and assay of the cell extracts for adenine nucleotides (eg. Gadkari and Stolp, 1976; Baak and Postma, 1971; Knowles and Smith, 1970; Niven et al., 1977). This method is analogous to the oxygen pulse technique for measuring the stoichiometry of respiration-linked proton translocation and, as with this technique, there is possibly some under-estimate of the ATP/O quotient due to the uptake of inorganic phosphate, in symport with protons, following the efflux of phosphate from the cells under the initially anaerobic conditions of the experiment (see Section 1.6). Furthermore, this technique poses problems for the quenching of cells at the precise moment of oxygen depletion. Few workers have attempted to obtain a more complete picture of intracellular energisation by measuring both inorganic phosphate utilisation and ATP synthesis, and few measurements of ATP turnover have been made.

The measurements reported in the literature show a wide variation in the efficiency of oxidative phosphorylation in bacteria with the majority of direct measurements suggesting a low stoichiometry of proton translocation during ATP synthesis (ie. an $\rightarrow H^+$/ATP quotient greater than 2; see Table 4.1). The aim of the work described in this chapter was to measure simultaneously ATP synthesis, inorganic phosphate utilisation and oxygen consumption upon the addition of a respiratory substrate to an aerobic suspension of M.methylotrophus and thus to determine the ~P/O and P_i/O quotient during respiration. The turnover of ATP and inorganic phosphate upon inhibition of ATP synthesis was then determined in order to correct the P_i/O quotients for ATP hydrolysis.

4.2 Results

4.2.1 The efficiency of quenching of cell suspensions with perchloric acid

For the accurate measurement of ATP synthesis during the initial few seconds of respiration, rapid quenching of cell suspensions was required in order that respiration, ATP synthesis and ATP turnover were stopped simultaneously. Quenching was achieved by rapidly sampling the cell suspension into 25% (v/v) perchloric acid. In order to determine whether this quenching procedure was sufficiently rapid, an anaerobic suspension of cells was incubated in the presence of methanol and sampled by means of the rapid sampling apparatus (see Section 2.8) either into 25% (v/v) perchloric acid rendered anoxic by sparging with oxygen-free nitrogen or into aerobic 25% (v/v) perchloric acid. The subsequent determination of the ATP and inorganic phosphate concentrations showed no significant differences between the two experiments (Table 4.2), indicating that the cells were quenched before significant methanol oxidation (and hence ATP synthesis with consequent phosphate utilisation) could occur.

4.2.2 ATP synthesis during methanol oxidation

The addition of methanol to an aerobic suspension of M.methylotrophus resulted in the rapid consumption of oxygen and a linear increase in the ATP concentration over a period of 8 to 9 seconds. During this period the ATP concentration increased from 0.45 to 1.35 nmol/mg cells (Δ[ATP] = 0.9 nmol/mg cells) in a typical experiment, and was matched by a linear decrease in the concentration of AMP from 2.05 to 1.00 nmol/mg cells (Δ[AMP] = -1.05 nmol/mg cells; Fig. 4.1a). The ADP concentration remained essentially unchanged at approximately 1.65 nmol/mg cells. The results thus suggest that the ADP consumed during respiratory chain phosphorylation was subsequently

Table 4.2 The efficiency of quenching of M.methylotrophus by perchloric acid (PCA)

Quenching conditions	[Inorganic phosphate] (nmol/mg cells)
Anaerobic PCA	13.3 ± 0.4 (4)
Aerobic PCA	13.4 ± 1.0 (3)

Washed whole cells (approx. 60 mg dry weight) were incubated in 20 ml 20 mM glycylglycine + 140 mM KCl, pH 7.0 which had previously been rendered anoxic by sparging with oxygen-free nitrogen for 2 minutes prior to the introduction of anoxic methanol (1 mM). Samples (1.5 ml) were injected into either aerobic 25% (w/v) perchloric acid or into 25% (v/v) perchloric acid which had also previously been rendered anoxic by sparging with oxygen-free nitrogen. Samples were subsequently prepared and assayed for inorganic phosphate.

reformed from ATP and AMP under the action of adenylate kinase such that ATP synthesis occurred at the net expense of AMP and two molecules of inorganic phosphate. However, although the concentration of inorganic phosphate decreased during the experiment and exhibited the expected linear kinetics before reaching a steady-state concentration, the extent of this decrease ($\Delta[P_i]$) was considerably greater than the net increase in adenine nucleotide phosphorylation (Δ[~P], calculated as 2Δ[ATP] + Δ[ADP]), ie. 5.0 cf. 2.05 nmol/mg cells (Fig. 4.1b).

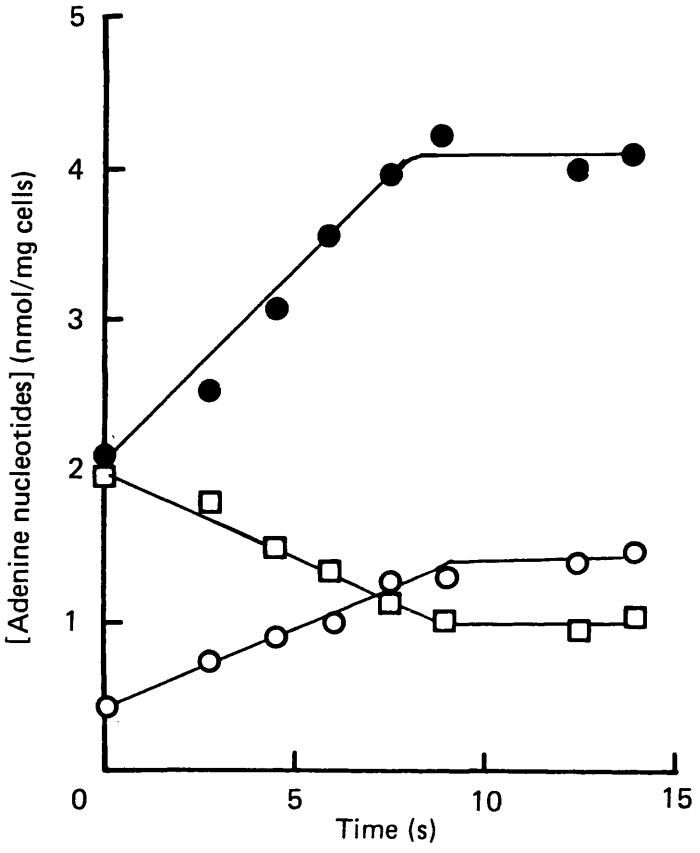
During the first 7.5 seconds of this experiment, Δ[~P] and $\Delta[P_i]$ were 1.91 and 4.5 nmol/mg cells respectively; since Δ[O] during this period was 17.0 ng-atom O/mg cells, the ~P/O and P_i/O quotients for methanol oxidation were 0.11 and 0.26 nmol/ng-atom O respectively.

Oxygen electrode traces showed that the rate of oxygen consumption by whole cells oxidising methanol did not decrease when the concentrations of ATP, AMP and P_i levelled off after 8 to 9 seconds, thus showing that the cells did not exhibit respiratory control under these conditions; the adenine nucleotides and inorganic phosphate thus attained steady-state rather than equilibrium concentrations. It is likely, therefore, that the protonmotive force generated by respiration is continuously dissipated in synthesising ATP to replace the ATP which is used to drive various energy-dependent, intracellular functions. Since ATP utilisation would affect the ~P/O and P_i/O quotients, the rates of turnover of the adenine nucleotides and inorganic phosphate were determined.

4.2.3 Turnover of ATP in M.methylotrophus

The turnover of ATP was determined by measuring the change in concentrations of ATP and P_i upon addition of an uncoupling agent (FCCP) to respiring cells. It was important in these experiments to ensure that the uncoupling action of FCCP was rapid. In order to

a)


[Adenine nucleotides] (nmol/mg cells)
5
4
3
2
1
0
5
10
15
Time (s)

b)

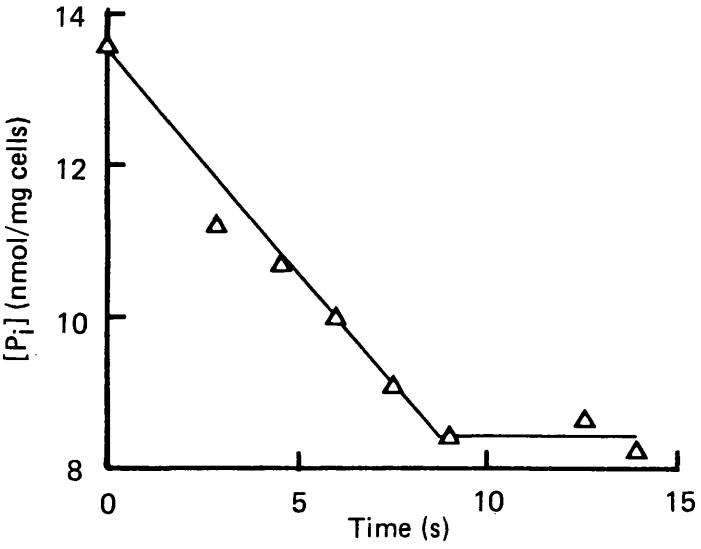
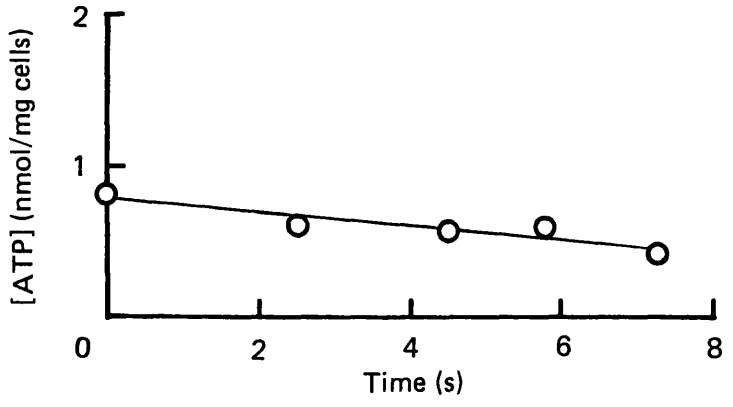

[Pi] (nmol/mg cells)
14
12
10
8
0
5
10
15
Time (s)

Fig. 4.1 Energisation during methanol oxidation

Washed cells (79.4mg dry weight) were incubated in 25ml 20mM glycylglycine + 140mM KCl pH 7.0 at 40°C for 2 minutes prior to the introduction of methanol (10mM). Cells were sampled into 25% (v/v) perchloric acid at regular intervals, and subsequently prepared and assayed for (a) adenine nucleotides and (b) inorganic phosphate. ATP (○), AMP (□), ~P (●), inorganic phosphate (△).

a)


[ATP] (nmol/mg cells)
2
1
0
2
4
6
8
Time (s)

b)

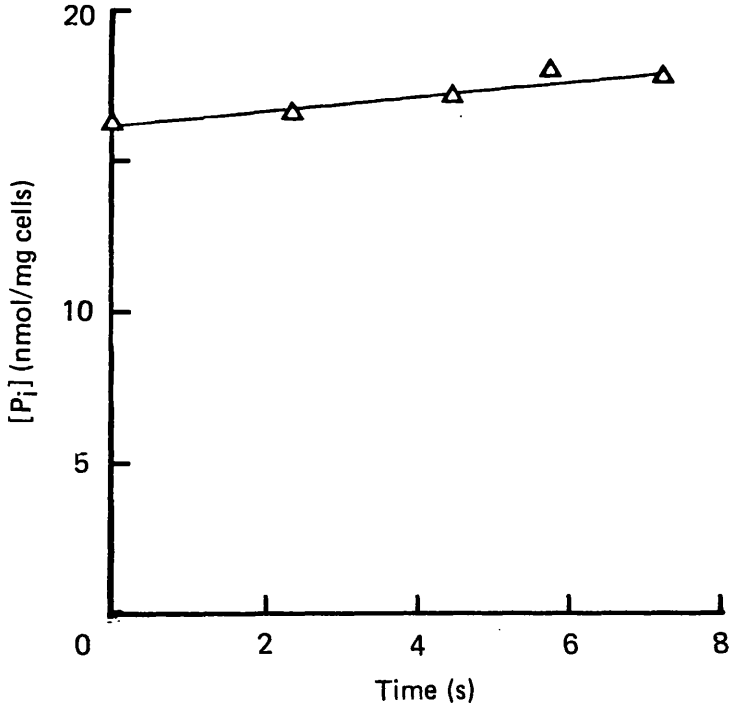
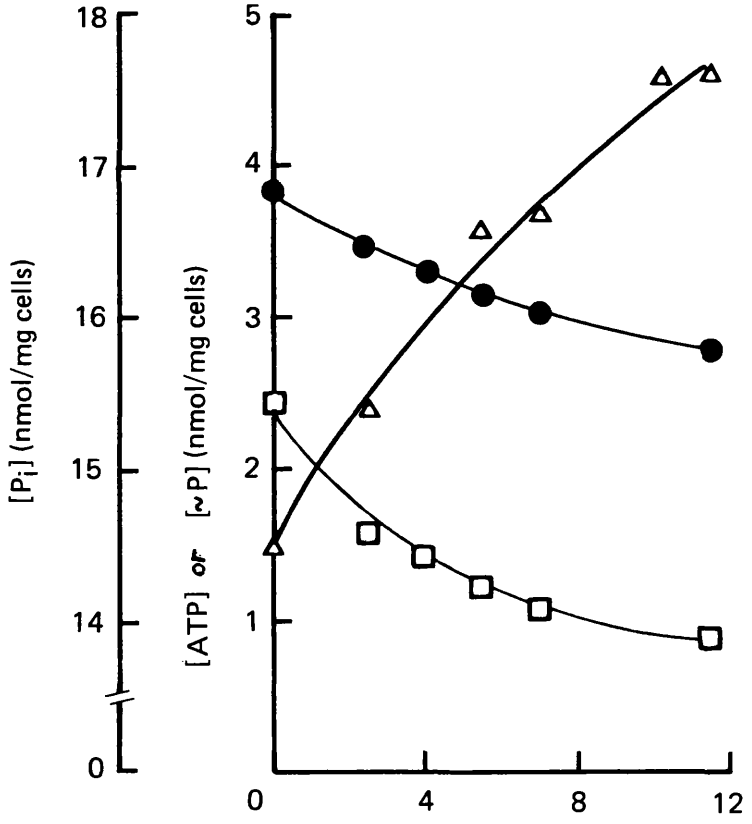

[P_i] (nmol/mg cells)
20
10
5
0
2
4
6
8
Time (s)

Fig. 4.2 The efficiency of uncoupling of FCCP

Washed cells (63.3mg dry weight) were incubated in 25ml 20mM glycylglycine + 140mM KCl pH 7.0 at 40°C for two minutes prior to the addition of FCCP in methanol (final concentrations 10μM and 1mM respectively with 1% (v/v) DMF). Cells were sampled into 25% (v/v) perchloric acid at regular intevals, and subsequently prepared and assayed for (a) ATP and (b) inorganic phosphate.

a)


18
17
16
15
14
0
5
4
3
2
1
0
4
8
12
[Pi] (nmol/mg cells)
[ATP] or [~P] (nmol/mg cells)

b)

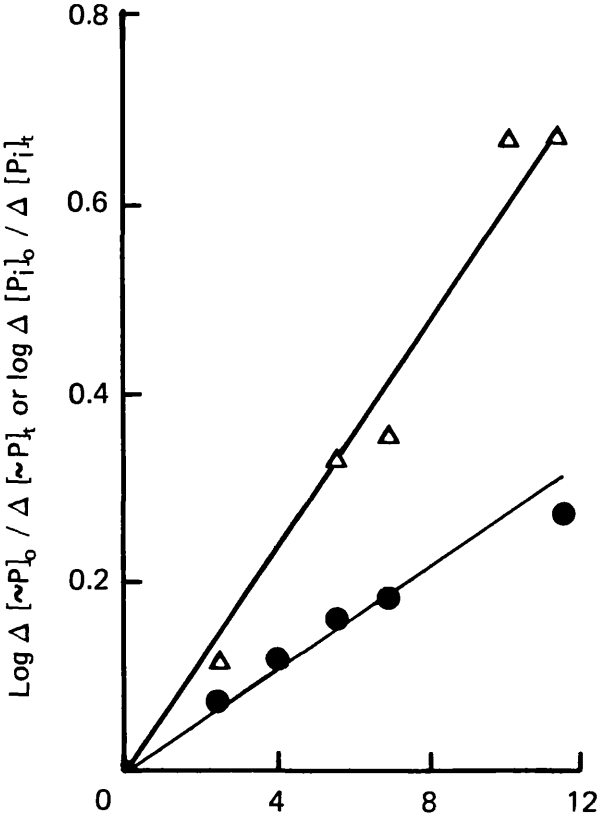

0.8
0.6
0.4
0.2
0
4
8
12
Log Δ [~P]o / Δ [~P]t or log Δ [Pi]o / Δ [Pi]t

Fig. 4.3 Turnover of ATP and inorganic phosphate upon addition of FCCP to cells respiring methanol

Washed cells (approx. 75mg dry weight) were incubated for 2 minutes in 25ml glycylglycine + 140mM KCl pH 7.0 at 40°C prior to the introduction of methanol (10mM). Cells were allowed to respire for 15 seconds before addition of FCCP (10μM) at zero time. Cells were sampled into 25% (v/v) perchloric acid at regular intervals and subsequently prepared and assayed for ATP (□), ~P (●) and P_i (Δ).

a) Absolute concentrations

b) Changes in concentration. $\Delta[\sim P]_o$ is the change in the concentration of ~P between zero time and complete de-energisation, and $\Delta[\sim P]_t$ is the change in concentration between time t and complete de-energisation; $\Delta[P_i]_o$ and $\Delta[P_i]_t$ are the corresponding values for inorganic phosphate.

determine whether there was any significant time lag in the action of FCCP, a mixture of FCCP and methanol was injected into an aerobic suspension of cells and the cell suspension was then sampled into perchloric acid in the normal way (see Section 2.10). Subsequent analysis of the neutralised extracts showed a small decrease in ATP concentration and a small increase in inorganic phosphate concentration (Fig. 4.2). Thus there was no significant synthesis of ATP concomitant with methanol oxidation over the time period of the first sample (2.5 seconds). It was concluded that there was no significant time lapse in the uncoupling action of FCCP.

Upon the addition of FCCP to cells respiring methanol, there was a rapid non-linear decrease in the concentration of ATP which was accompanied by an increase in the concentration of inorganic phosphate (Fig. 4.3a). Kinetic plots of these data indicated pseudo-first order kinetics with typical rate constants for ~P turnover and inorganic phosphate release of 3.8 min^{-1} and 8.1 min^{-1} respectively (Fig. 4.3b).

It has been shown above that the measurement of ATP synthesis and inorganic phosphate utilisation during the first 7 seconds of respiration revealed a significant discrepancy between the values of the ~P/O and P_i/O quotients. A significant difference was also observed between the rate constants of ~P turnover and inorganic phosphate release. To attempt to explain this difference, a search was made for the possible existence of alternative products of phosphate utilisation, such as pyrophosphate and non-adenine nucleoside triphosphates.

4.2.4 Measurement of intracellular pyrophosphate

The measurement of pyrophosphate concentration in cell extracts prepared from cells respiring endogenous substrates or methanol showed no significant difference (Table 4.3). A control experiment indicated that recovery of pyrophosphate following perchloric acid

Table 4.3 The concentration of inorganic pyrophosphate in M.methylotrophus

Substrate	P_i (nmol/mg cells)	PP_i (nmol/mg cells)
Endogenous	15.2	1.6
+ Methanol	8.3	1.4

Washed cells (approx. 25 mg dry weight) were incubated in 5 ml 20 mM glycylglycine + 140 mM KCl pH 7.0 at 40°C for 2 minutes and were then allowed either to continue to oxidise endogenous substrates or to oxidise added methanol (10 mM) for 7 seconds before quenching with 1.6 ml 25% (v/v) perchloric acid. The cell extracts were neutralised and assayed for inorganic phosphate and pyrophosphate.

Table 4.4 Recovery of inorganic pyrophosphate after perchloric acid precipitation

Standard PP_i (μM)	Measured PP_i (μM)	% Recovery
0	0	
1.3	1.4	108
2.7	2.4	78
5.3	4.1	77
10.7	10.5	98

1.5 ml of a standard solution of tetra-sodium pyrophosphate was injected into 0.48 ml of 25% (v/v) perchloric acid. The solution was neutralised with 0.34 ml of neutralising solution (2M Tris, saturated KOH, water in the proportions 30:60:10) and the resultant precipitate was removed by centrifugation. The supernatant was assayed for pyrophosphate as described in Section 2.11.

precipitation was virtually complete, with an average recovery of approximately 90% (Table 4.4).

4.2.5 Measurement of nucleoside triphosphates

Analysis of cell extracts prepared from cells respiring endogenous substrates or methanol by fast protein liquid chromatography (FPLC) indicated a substantial increase in the concentrations of GTP, UTP and CTP in addition to ATP in cells respiring methanol (Fig. 4.4). The increase in the concentrations of GTP, UTP and CTP were sufficient to account for the observed discrepancy between $\Delta[\sim P]$ and $\Delta[P_i]$ and hence between the ~P/O and P_i/O quotients (Table 4.5). Thus nascent ATP appears to be rapidly used to synthesise other nucleoside triphosphates, presumably under the action of nucleoside diphosphate kinases. The rate constant for ~P turnover upon the addition of FCCP to energised cells will therefore be underestimated because much of the ADP formed by ATP hydrolysis will be rapidly rephosphorylated at the expense of non-adenine nucleoside triphosphates. As the reactions catalysed by nucleoside diphosphate kinases involve only the nucleotides and do not affect the concentration of inorganic phosphate, it follows that the P_i/O quotient is the most accurate indicator of the stoichiometry of respiratory chain phosphorylation in whole cells of M.methylotrophus. With this in mind, the investigation of the stoichiometry of respiratory chain phosphorylation with other respiratory substrates was directed towards measurement of the P_i/O quotient rather than the ATP/O or ~P/O quotients.

4.2.6 ATP synthesis and phosphate utilisation during the oxidation of ethanol or acetaldehyde

The oxidation of methanol by M.methylotrophus generates formaldehyde which may be further oxidised via the cyclic or linear routes eventually to yield NAD(P)H (see section 1.3). Since the

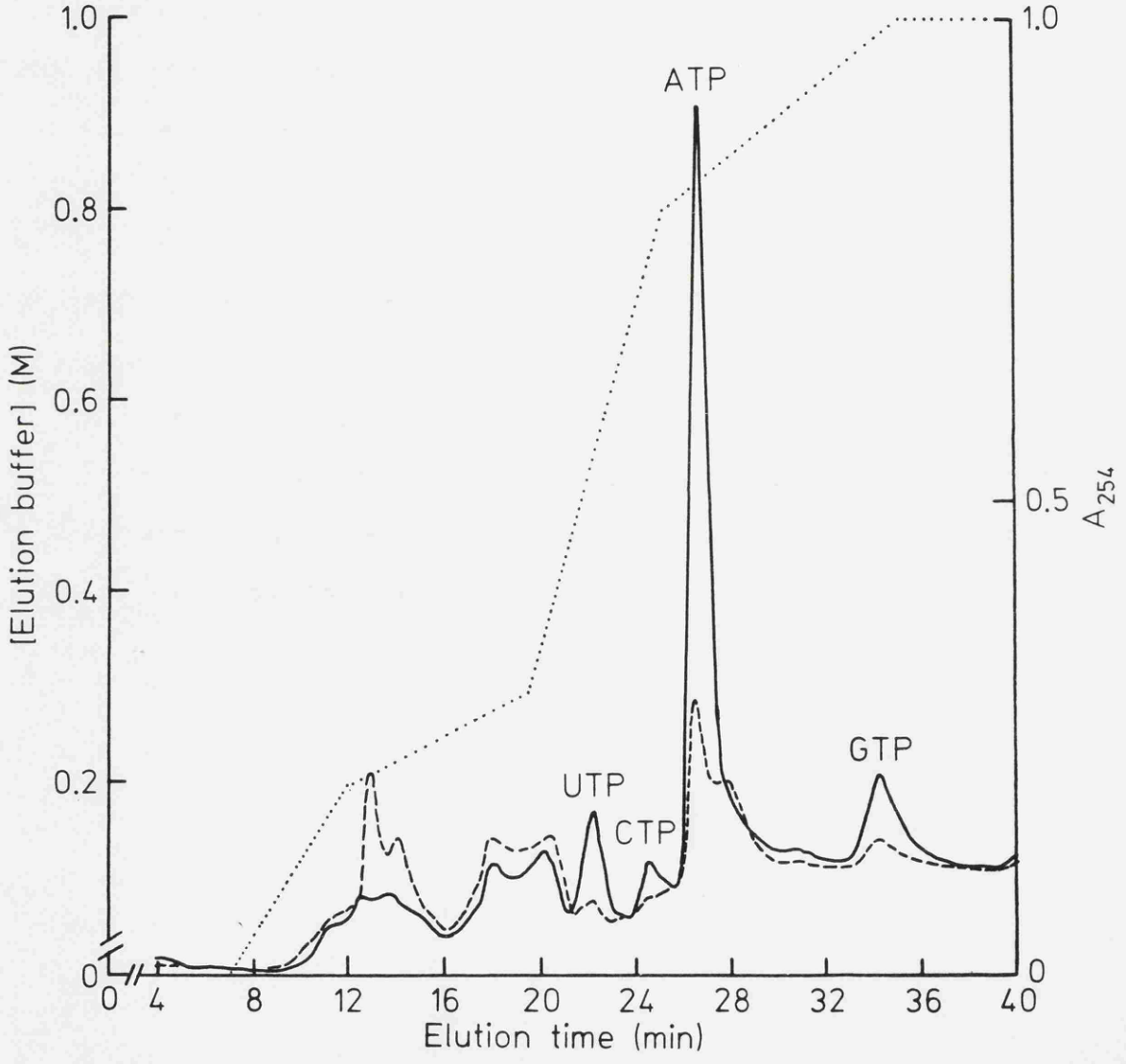

1.0
0.8
0.6
0.4
0.2
0
[Elution buffer] (M)
ATP
UTP
CTP
GTP
0
4
8
12
16
20
24
28
32
36
40
Elution time (min)
1.0
0.5
0
A_{254}

Fig. 4.4 The identification of non-adenine nucleoside triphosphates in M.methylotrophus

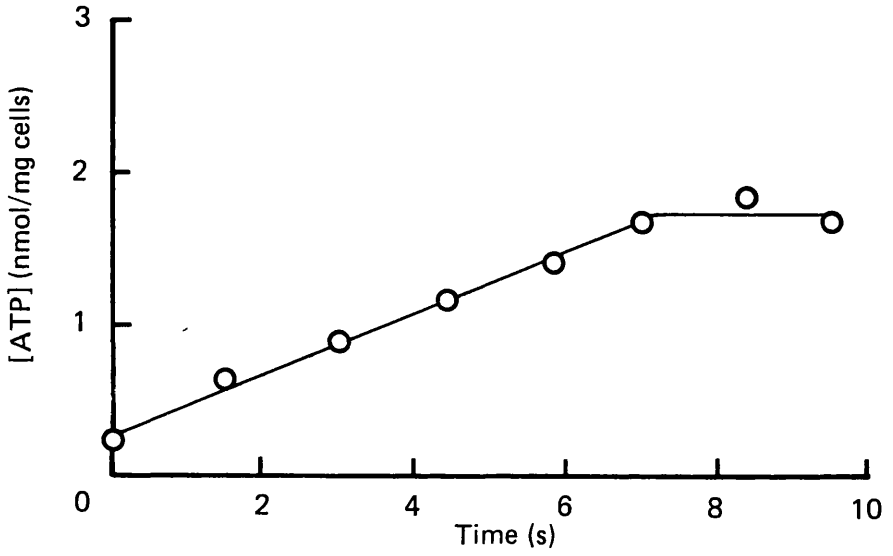
Washed cells (approx. 15mg dry weight) were incubated in 5ml 20mM glycylglycine + 140mM KCl pH 7.0 at 40°C for 2 minutes and were then allowed either to continue oxidising endogenous substrates or to oxidise added methanol (1mM) for 7 seconds prior to quenching with 1.6ml 25% (v/v) perchloric acid. The neutralised cell extracts were subjected to anion exchange chromatography on a polyanion 'Fast protein liquid chromatography' column. Nucleoside triphosphates were eluted with a gradient of 0.02 - 1.0M phosphate buffer pH 7.0 and detected from their absorbance at 254nm. Identification of peaks was by comparison, and co-elution, with standards. The initial eluent contained a large amount of 254nm absorbing material, including nucleoside monophosphates which were not adsorbed under the conditions employed. Endogenous substrates (- - -), plus methanol (——), concentration of elution buffer (·····).

Table 4.5 The concentration of nucleoside triphosphates and inorganic phosphate in M.methylotrophus

Substrate	[ATP]	[GTP]	[UTP]	[CTP]	[Total XTP]	[P_i]
			(nmol/mg cells)			
Endogenous	0.51	0.13	0.08	0.05	0.77	8.5
+ Methanol	2.09	0.41	0.43	0.25	3.18	3.7

The measurement of nucleoside triphosphates was carried out as described in the legend of Figure 4.4. The concentrations of nucleoside triphosphates were determined from their peak area following calibration of the FPLC column with known amounts of each nucleotide. $2\Delta[XTP] = 4.82$ nmol/mg cells, $\Delta[P_i] = 4.8$ nmol/mg cells.

a)


3
2
1
0
[ATP] (nmol/mg cells)
2
4
6
8
10
Time (s)

b)

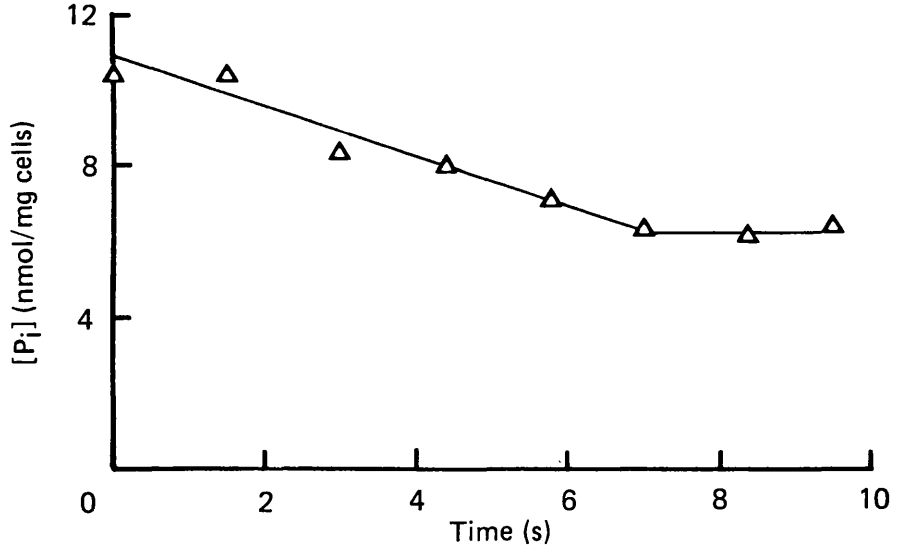
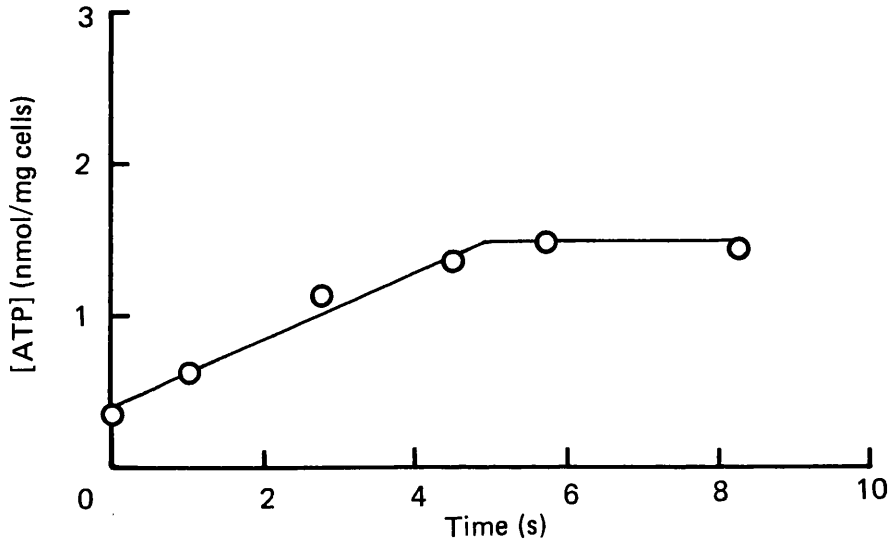

12
8
4
0
[Pi] (nmol/mg cells)
2
4
6
8
10
Time (s)

Fig. 4.5 ATP synthesis and inorganic phosphate utilisation during ethanol oxidation

Washed cells (approx. 75mg dry weight) were incubated in 25ml 20mM glycylglycine + 140mM KCl pH 7.0 at 40°C prior to the addition of ethanol (10mM). Cells were sampled into 25% (v/v) perchloric acid at regular intervals and subsequently prepared and assayed for (a) ATP and (b) P_i.

a)


[ATP] (nmol/mg cells)
3
2
1
0
2
4
6
8
10
Time (s)

b)

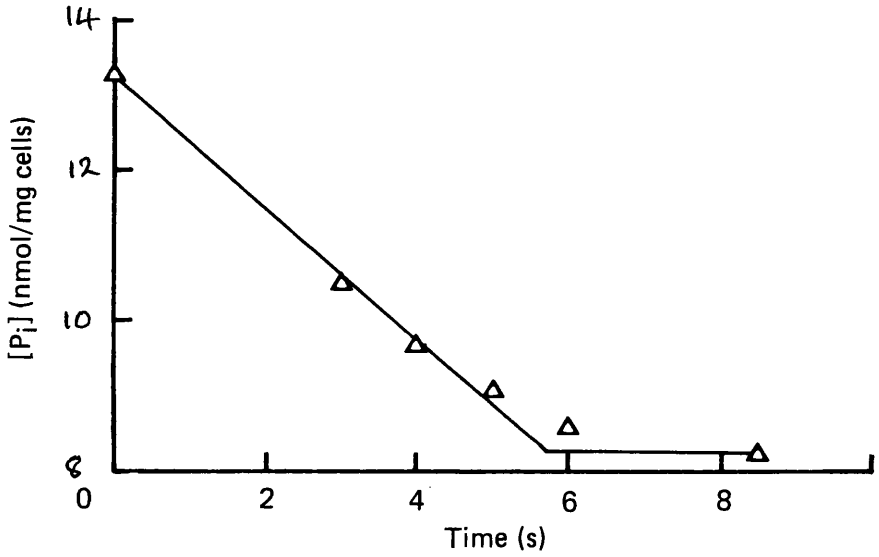

[Pi] (nmol/mg cells)
14
12
10
8
0
2
4
6
8
Time (s)

Fig. 4.6 ATP synthesis and inorganic phosphate utilisation during acetaldehyde oxidation

Washed cells (approx. 75mg dry weight) were incubated in 25ml 20mM glycylglycine + 140mM KCl pH 7.0 prior to the addition of acetaldehyde (10mM) at zero time. Cells were sampled into 25% (v/v) perchloric acid at regular intervals and subsequently prepared and assayed for (a) ATP and (b) P_i.

oxidation of NADH by the respiratory chain-linked NADH dehydrogenase results in energy conservation at sites I, II and III, the use of methanol as a substrate could therefore result in an over-estimation of the stoichiometry of oxidative phosphorylation at site III.

The enzyme methanol dehydrogenase has a low degree of substrate specificity and is able to oxidise various primary alcohols and hydrated aldehydes, including ethanol and acetaldehyde. The end product of these oxidations, acetic acid, is not further oxidised and hence does not appear to be capable of reducing $NAD(P)^+$ (see Section 3.2.7); ethanol and acetaldehyde can therefore be used as control substrates to check that the P_i/O quotient for the oxidation of methanol is not over-estimated due to the formation and subsequent oxidation of NAD(P)H.

The addition of either ethanol or acetaldehyde to whole cells of M.methylotrophus resulted in linear changes in ATP and inorganic phosphate concentrations over the initial 5 to 7 second period until steady-state levels were achieved (Fig. 4.5 and 4.6) which were essentially the same as those observed with methanol (Section 4.3). Oxidation of ethanol typically gave a Δ[ATP] of 1.0 nmol/mg cells and a $\Delta[P_i]$ of 3.4 nmol/mg cells over the initial five seconds of respiration. Comparison with the oxygen consumption which occurred during this period (Δ[O] = 9.1 ng-atom O/mg cells) yielded a ~P/O quotient of 0.22 and a P_i/O quotient of 0.37 nmol/ng-atom O. Similarly, the oxidation of acetaldehyde yielded a ~P/O quotient of 0.18 and a P_i/O quotient of 0.38 nmol/ng-atom O (Fig. 4.6). Thus the ~P/O and P_i/O quotients for the oxidation of ethanol and acetaldehyde were not grossly dissimilar to those of methanol oxidation.

4.2.7 ATP synthesis and phosphate utilisation during the oxidation of duroquinol

The artificial electron donor duroquinol donates electrons

to the respiratory chain at the level of the quinone pool and/or cytochrome b with energy conservation at sites II and III. A higher efficiency of ATP synthesis would therefore be predicted for duroquinol oxidation than was obtained with methanol, ethanol or acetaldehyde.

Upon the addition of duroquinol to an aerobic suspension of cells there was a linear change in the concentrations of ATP and inorganic phosphate over the initial six second period until steady-state concentrations were achieved (Fig. 4.7). The oxidation of duroquinol typically gave a Δ[ATP] and a $\Delta[P_i]$ of 1.25 and 6.7 nmol/mg cells respectively. Comparison of concentration changes over the initial five seconds with the oxygen consumed (Δ[O] = 16.3 ng-atom O/mg dry weight cells) gave a ~P/O quotient of 0.15 and a P_i/O quotient of 0.41 nmol/ng-atom O.

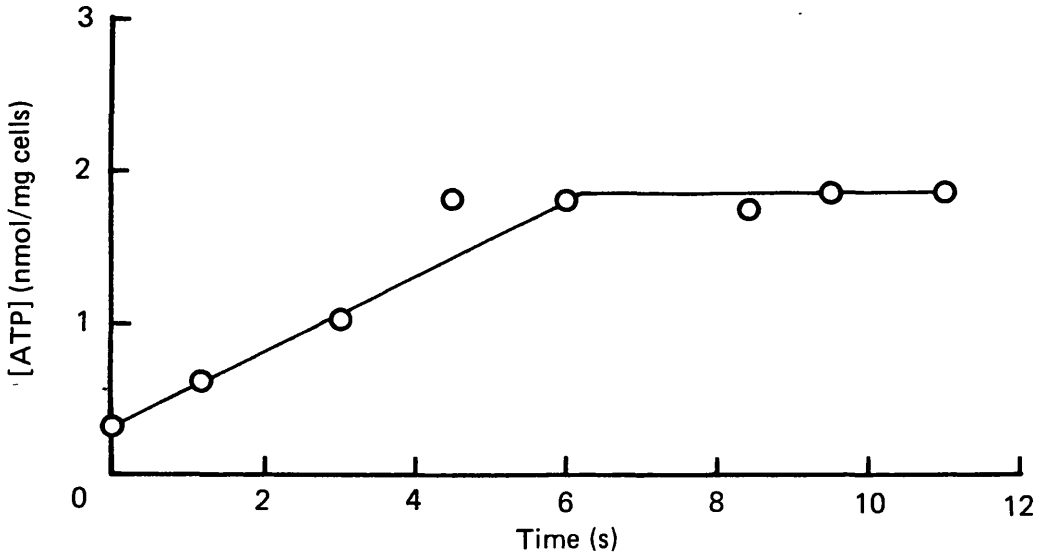
4.2.8 ATP synthesis and phosphate utilisation during the oxidation of formate

In M.methylotrophus formate is oxidised by an NAD^+-linked formate dehydrogenase; thus formate oxidation supplies NADH for the respiratory chain-linked NADH dehydrogenase. Oxidation of formate should therefore result in oxidative phosphorylation at all three sites of energy conservation. Addition of formate to an aerobic suspension of whole cells resulted in linear changes in the concentrations of ATP and inorganic phosphate (Δ[ATP] = 2.2 nmol/mg cells and $\Delta[P_i]$ = 5.45 nmol/mg cells over the initial 4 seconds; Fig. 4.8). Comparison with the oxygen consumed over the same time period (Δ[O] = 4.4 ng-atom O/mg cells) typically gave a ~P/O quotient of 1.0 and a P_i/O quotient of 1.24 nmol/ng-atom O.

4.2.9 The turnover of inorganic phosphate upon the addition of FCCP to respiring cells

The oxidation of ethanol, acetaldehyde, duroquinol and

a)


[ATP] (nmol/mg cells)
3
2
1
0
2
4
6
8
10
12
Time (s)

b)

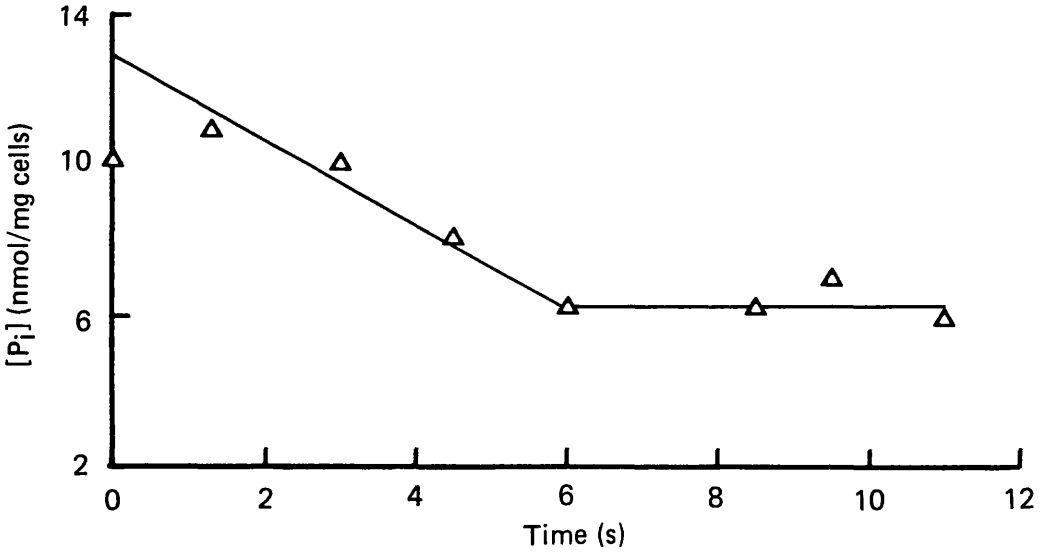
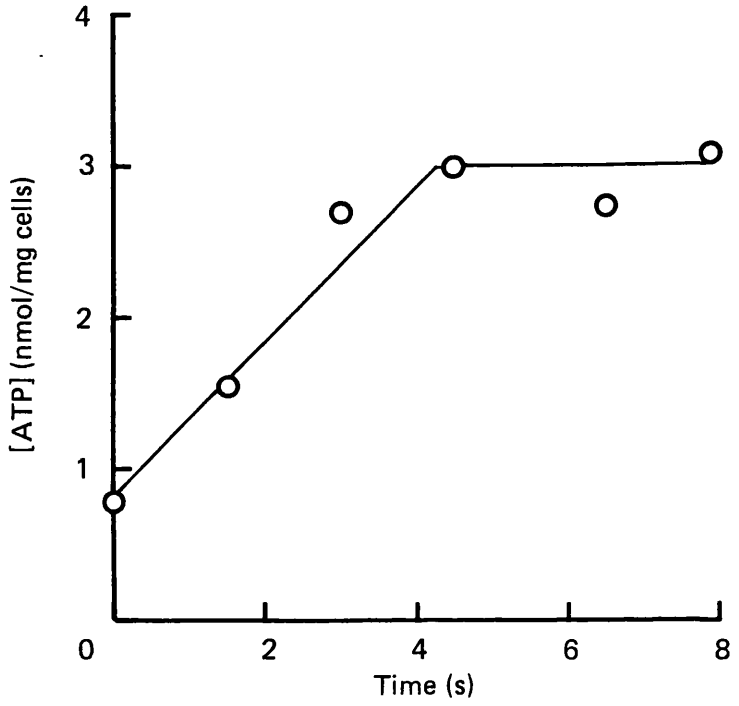

[Pi] (nmol/mg cells)
14
10
6
2
0
2
4
6
8
10
12
Time (s)

Fig. 4.7 ATP synthesis and inorganic phosphate utilisation during duroquinol oxidation

Washed cells (approx. 75mg dry weight) were incubated in 25ml 20mM glycylglycine + 140mM KCl pH 7.0 at 40°C for 2 minutes prior to the addition of duroquinol (1mM) at zero time. Cells were sampled into 25% (v/v) perchloric acid at regular intervals and subsequently prepared and assayed for (a) ATP and (b) inorganic phosphate.

a)


4
3
2
1
0
2
4
6
8
[ATP] (nmol/mg cells)
Time (s)

b)

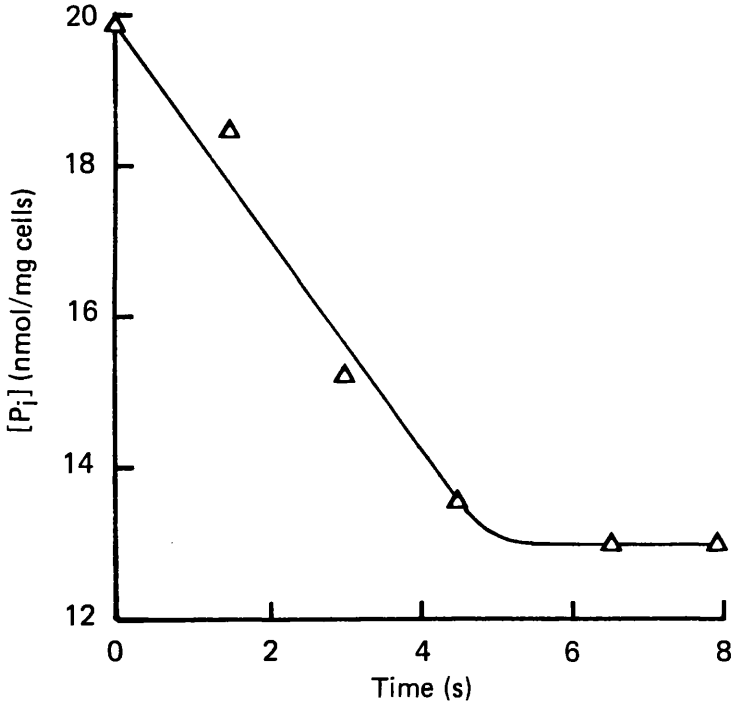

20
18
16
14
12
0
2
4
6
8
[Pi] (nmol/mg cells)
Time (s)

Fig. 4.8 ATP synthesis and inorganic phosphate utilisation during formate oxidation

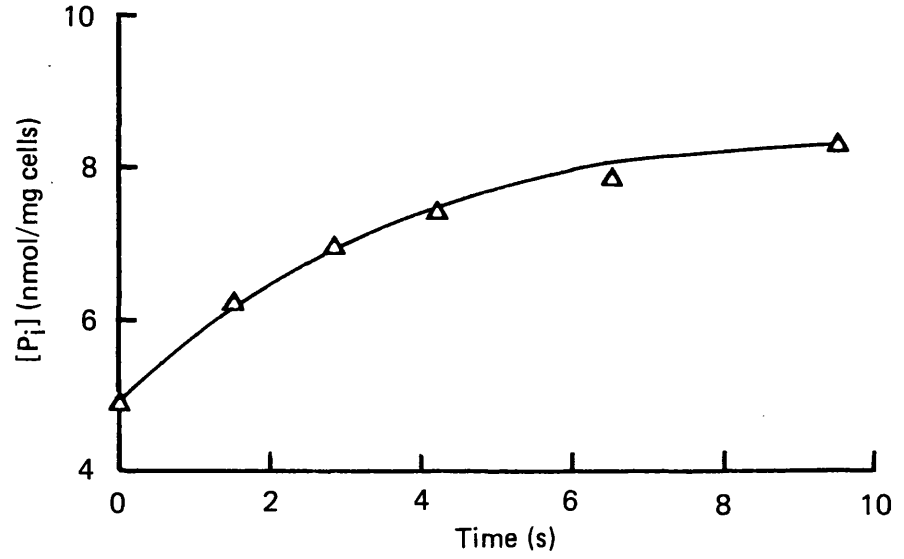
Washed cells (approx. 75mg dry weight) were incubated in 20mM glycylglycine + 140mM KCl pH 7.0 at 40°C for 2 minutes prior to the addition of formate (50mM) at zero time. Cells were sampled into 25% (v/v) perchloric acid at regular intervals and subsequently prepared and assayed for (a) ATP and (b) inorganic phosphate.

formate yields products which, in contrast to formaldehyde, cannot be further metabolised by M.methylotrophus and hence cannot be used as growth substrates. It is possible therefore that the oxidation of these substrates may be associated with significantly different turnover constants for ATP and inorganic phosphate compared with methanol oxidation. In order to correct the measured P_i/O quotients with these substrates for turnover, the turnover constant for inorganic phosphate was measured with each substrate used. The increase in inorganic phosphate concentration upon the addition of FCCP to cells respiring ethanol, acetaldehyde, duroquinol or formate indicated pseudo-first order kinetics of phosphate release (Figs. 4.9, 4.10, 4.11 and 4.12 respectively). No significant differences were observed between the turnover constants for any of the substrates used in these studies (Table 4.6).

The turnover constant (k) was used to correct the measured P_i/O quotient to yield a corrected value for the oxidation of methanol, ethanol, acetaldehyde, duroquinol and formate (Table 4.6). It can be seen that there is no significant difference between the P_i/O quotients for methanol, ethanol and acetaldehyde suggesting that formaldehyde oxidation via NAD(P)H did not occur to a significant extent over the period of the experiment. Electron transfer via site III was therefore associated with a P_i/O quotient of 0.46 - 0.54 nmol/ng-atom O. Oxidation of duroquinol and formate gave corrected P_i/O quotients of 0.77 and 1.37 respectively, these values being consistent with energy conservation at two sites (II and III) and all three sites (I, II and III) respectively.

It is pertinent to note that the P_i/O quotient would be under-estimated if the protonmotive force generated by respiration was used significantly to drive energy-dependent reactions, such as reversed

a)


10
8
6
4
[Pi] (nmol/mg cells)
0
2
4
6
8
10
Time (s)

b)

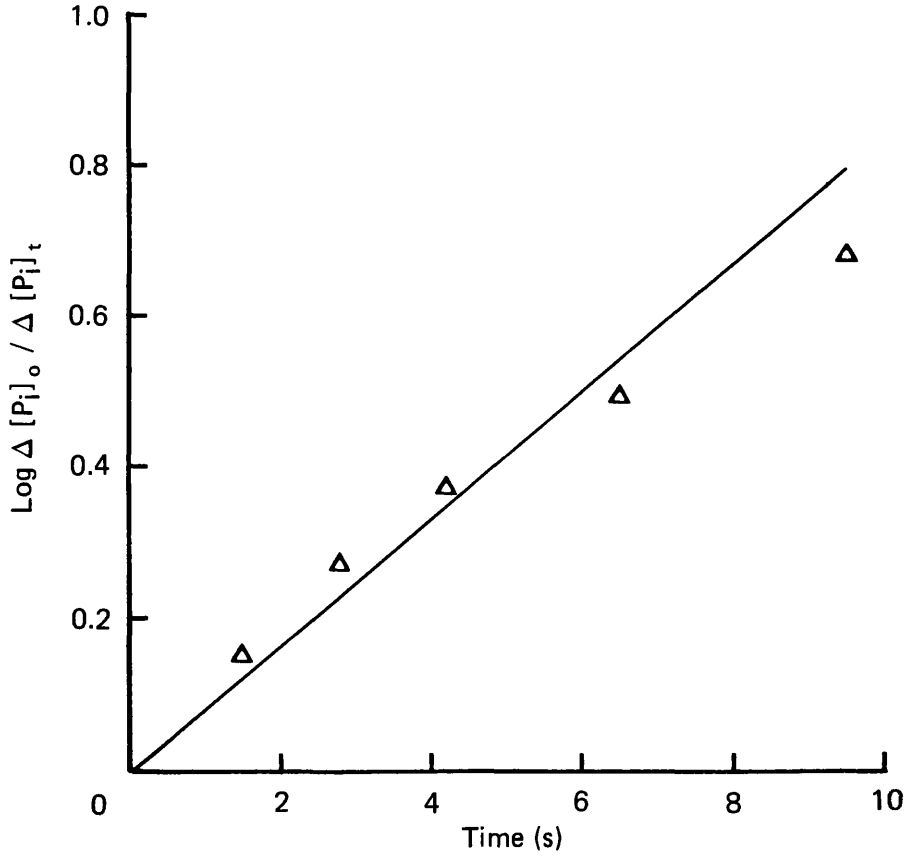
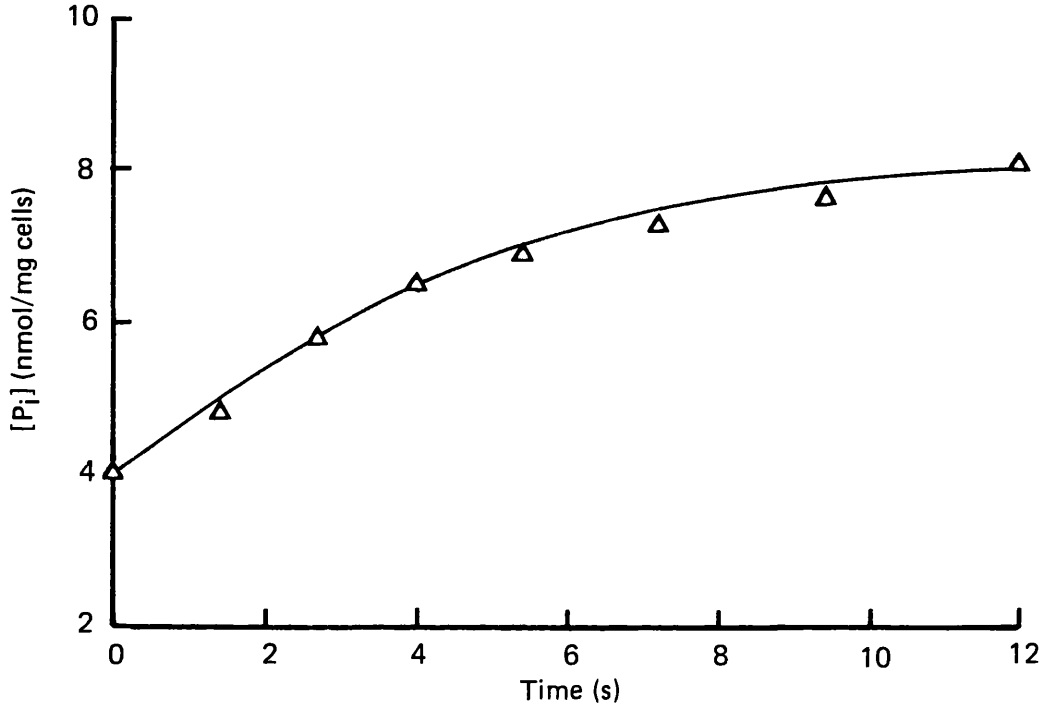

1.0
0.8
0.6
0.4
0.2
0
Log Δ [Pi]o / Δ [Pi]t
2
4
6
8
10
Time (s)

Fig. 4.9 Turnover of inorganic phosphate upon the addition of FCCP to cells respiring ethanol

Washed cells (approx. 75mg dry weight) were incubated for 2 minutes in 25ml 20mM glycylglycine + 140mM KCl pH 7.0 at 40°C prior to the introduction of ethanol (10mM). Cells were allowed to respire for 15 seconds prior to the addition of FCCP (10μM) at zero time. Cells were sampled into 25% (v/v) perchloric acid at regular intervals and subsequently assayed for inorganic phosphate. (a) Absolute concentration. (b) Changes in concentration. $\Delta[P_i]_o$ is the change in concentration of inorganic phosphate between zero time and complete de-energisation and $\Delta[P_i]_t$ is the change in concentration between time t and complete de-energisation.

a)


10
8
6
4
2
[Pi] (nmol/mg cells)
0
2
4
6
8
10
12
Time (s)

b)

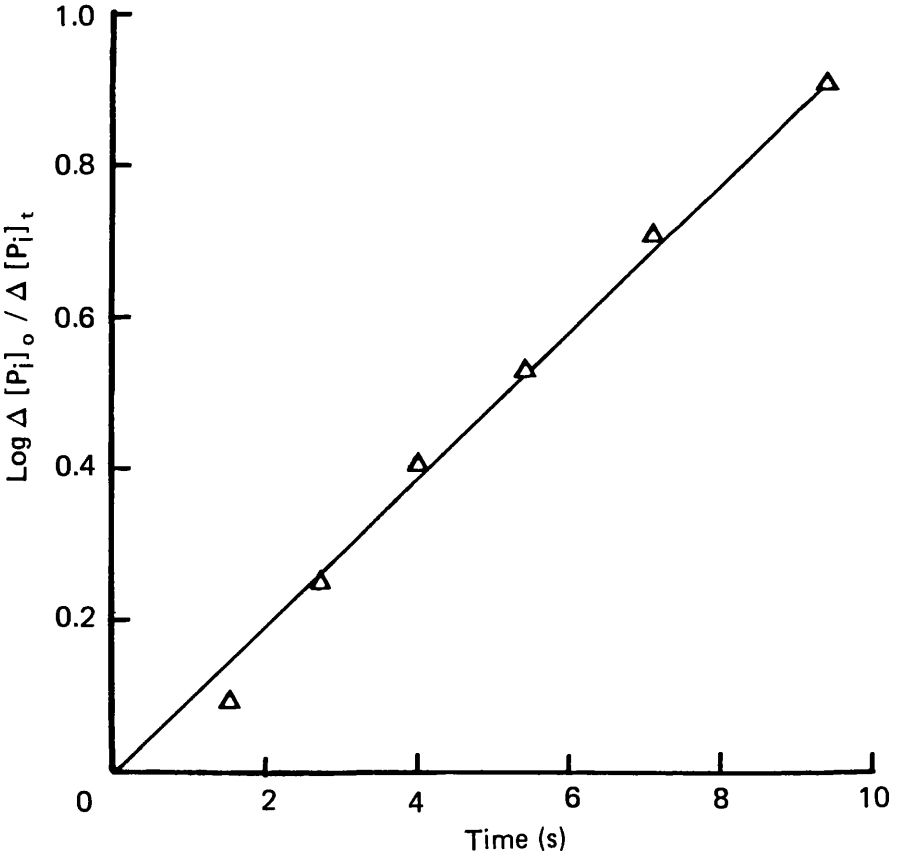
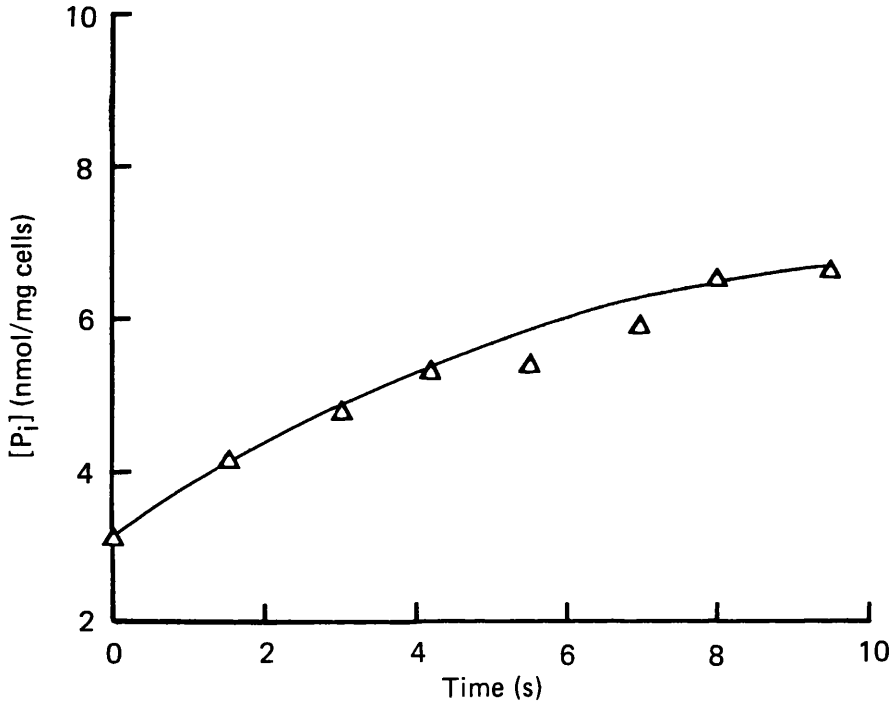

1.0
0.8
0.6
0.4
0.2
0
Log Δ [Pi]o / Δ [Pi]t
2
4
6
8
10
Time (s)

Fig. 4.10 Turnover of inorganic phosphate upon addition of FCCP to cells respiring acetaldehyde

Washed cells (approx. 75mg dry weight) were incubated for 2 minutes at 40°C in 25ml (20mM) glycylglycine + 140mM KCl pH 7.0 prior to the addition of acetaldehyde (10mM). The cells were allowed to respire acetaldehyde for 15 seconds prior to the addition of FCCP (10μM) at zero time. Cells were sampled into 25% (v/v) perchloric acid at regular intervals and subsequently assayed for inorganic phosphate. (a) Absolute concentration. (b) Changes in concentration. $\Delta[P_i]_o$ is the change in concentration of inorganic phosphate between zero time and complete de-energisation and $\Delta[P_i]_t$ is the change in concentration between time t and complete de-energisation.

a)


10
8
6
4
2
$[P_i]$ (nmol/mg cells)
0
2
4
6
8
10
Time (s)

b)

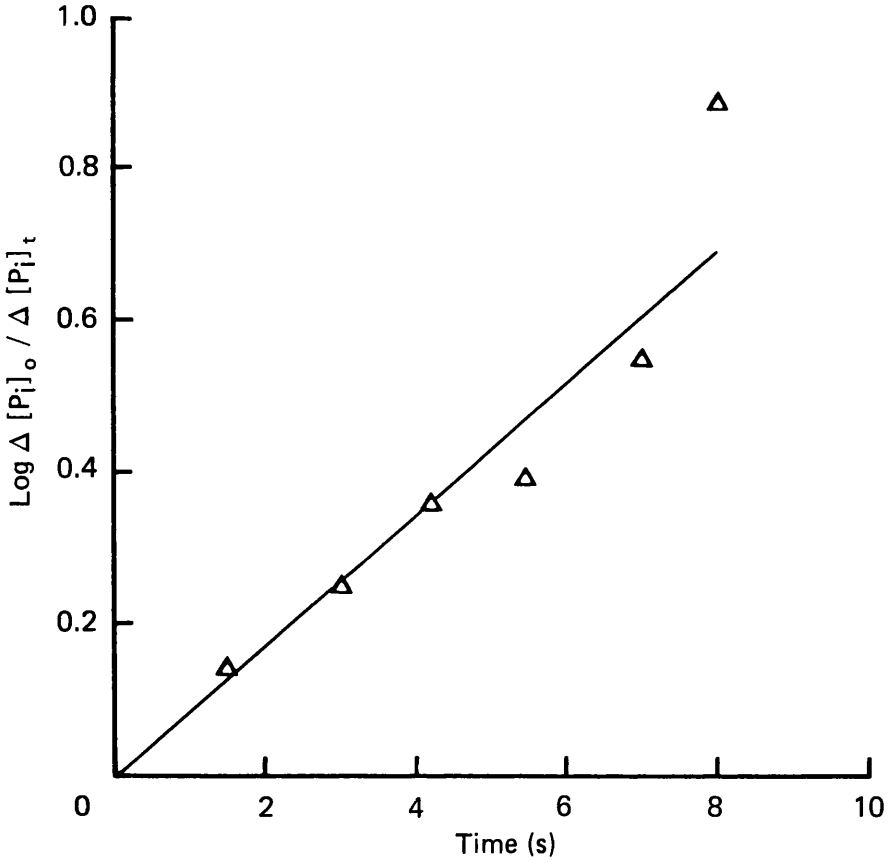
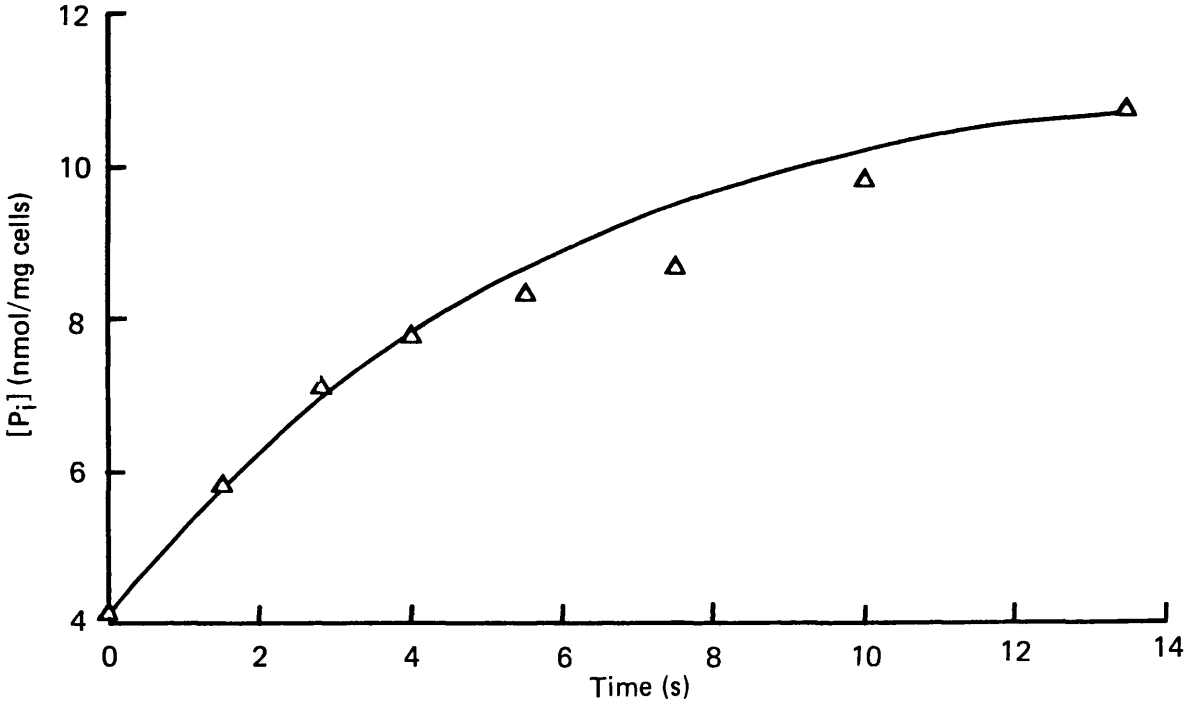

1.0
0.8
0.6
0.4
0.2
0
Log $\Delta [P_i]_o / \Delta [P_i]_t$
2
4
6
8
10
Time (s)

Fig. 4.11 Turnover of inorganic phosphate upon addition of FCCP to cells respiring duroquinol

Washed cells (approx. 75mg dry weight) were incubated for 2 minutes at 40°C in 25ml 20mM glycylglycine + 140mM KCl pH 7.0 prior to the addition of duroquinol (1mM). The cells were allowed to respire duroquinol for 15 seconds prior to the addition of FCCP (10μM) at zero time. Cells were sampled into 25% (v/v) perchloric acid at regular intervals and subsequently assayed for inorganic phosphate. (a) Absolute concentration. (b) Changes in concentration. $\Delta[P_i]_o$ is the change in concentration of inorganic phosphate between zero time and complete de-energisation and $\Delta[P_i]_t$ is the change in concentration between time t and complete de-energisation.

a)


12
10
8
6
4
[Pi] (nmol/mg cells)
0
2
4
6
8
10
12
14
Time (s)

b)

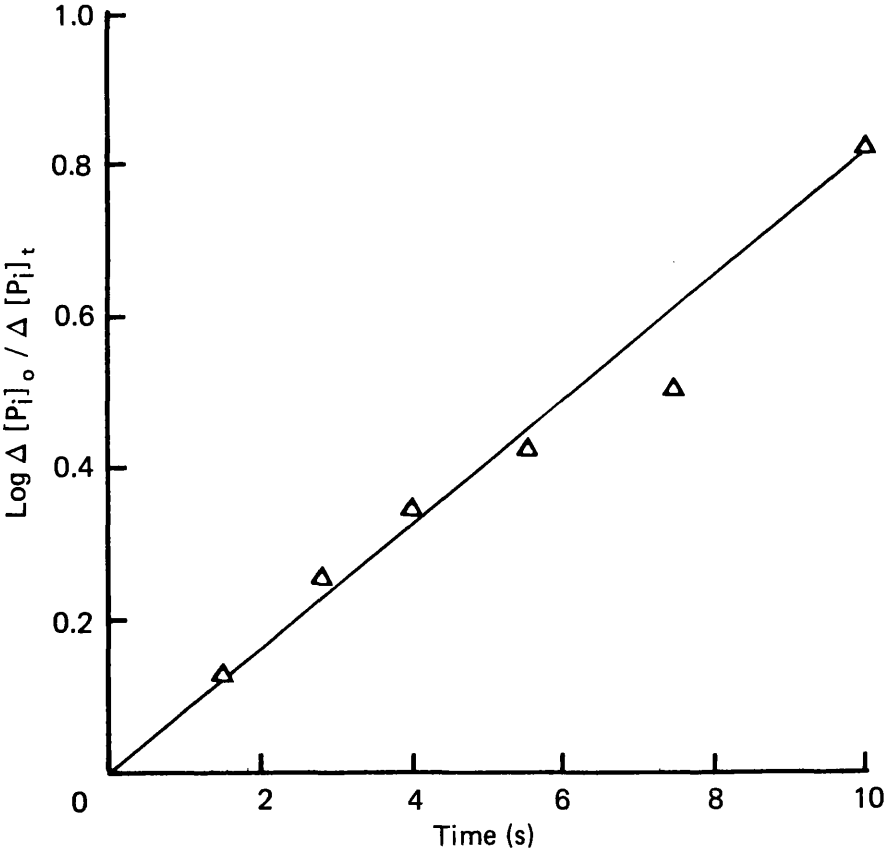

1.0
0.8
0.6
0.4
0.2
0
Log Δ [Pi]o / Δ [Pi]t
2
4
6
8
10
Time (s)

Fig. 4.12 Turnover of inorganic phosphate upon addition of FCCP to cells respiring formate

Washed cells (approx. 75mg dry weight) were incubated for 2 minutes at 40°C in 25ml $^{20}/_{\lambda}$ glycylglycine + 140mM KCl pH 7.0 prior to the addition of formate (50mM). Cells were allowed to respire formate for 15 seconds prior to the addition of FCCP (10μM) at zero time. Cells were sampled into 25% (v/v) perchloric acid nd subsequently assayed for inorganic phosphate.

(a) Absolute concentration. (b) Changes in concentration. $\Delta[P_i]_o$ is the change in concentration of inorganic phosphate between zero time and complete de-energisation and $\Delta[P_i]_t$ is the change in concentration between time t and complete de-energisation.

Table 4.6 P_i/O quotients for respiration in M.methylotrophus

Substrate	Uncorrected P_i/O (nmol P_i/ng-atom O)	k (min^{-1})	Corrected P_i/O (nmol Pi/ng-atom O)
Methanol	0.33 ± 0.05 (4)	9.7 ± 1.4 (4)	0.46 ± 0.05 (4)
Ethanol	0.34 ± 0.02 (5)	9.5 ± 1.0 (5)	0.46 ± 0.01 (5)
Acetaldehyde	0.38 ± 0.03 (5)	11.3 ± 1.9 (4)	0.54 ± 0.06 (5)
Duroquinol	0.59 ± 0.03 (4)	10.9 ± 0.4 (3)	0.77 ± 0.04 (4)
Formate	1.02 ± 0.05 (3)	12.5 ± 0.5 (8)	1.37 ± 0.03 (3)

Summary of directly determined P_i/O quotients and inorganic phosphate turnover constants during respiration of various substrates. The turnover constant (k) was subsequently used to obtain the value of the P_i/O quotient corrected for turnover.

electron transfer, which are not accompanied by the disappearance of inorganic phosphate. The possibility of reversed electron transfer during methanol oxidation was therefore investigated by measuring the uncoupler-sensitive reduction of NAD^+.

4.2.10 Reversed electron transfer in M.methylotrophus during methanol oxidation

To investigate the possibility of reversed electron transfer in M.methylotrophus during methanol oxidation, a search was made for evidence of uncoupler sensitive disappearance of NAD^+. An initial control experiment indicated that recovery of NAD^+ following perchloric acid precipitation was good, with an average recovery of 96% (Table 4.7). The concentrations of NAD^+ in cells respiring endogenous or added substrates in the presence or absence of FCCP were measured (Table 4.8). Cells respiring endogenous substrates contained 1.18 nmol NAD^+/mg dry weight cells, but after oxidising methanol for seven seconds this had decreased to 0.89 nmol/mg dry weight cells, thus indicating the formation of 0.29 nmol NADH/mg dry weight cells due presumably to the dissimilation of formaldehyde and/or reversed electron transfer from methanol to NAD^+. In the presence of the uncoupling agent FCCP, which dissipates the protonmotive force and hence abolishes reversed electron-transfer, methanol oxidation was still accompanied by the disappearance of 0.24 nmol NAD^+/mg dry weight cells. Thus, assuming that the disappearance of NAD^+ represents the formation of NADH, then 0.05 nmol NADH/mg cells was formed in an uncoupler sensitive manner. If this NADH formation is due to reversed electron transfer, and assuming that reversed electron transfer from methanol results in the hydrolysis of 2 mol ATP/mol NAD^+ reduced, the P_i/O quotient for methanol oxidation would increase from 0.46 to 0.56 mol P_i/g-atom O. These results suggest that energy consumption for reversed electron transfer is extremely

Table 4.7 Recovery of NAD^+ from perchloric acid precipitation

Experiment	Sample NAD^+ (nmol)	Assay NAD^+ (nmol)	% Recovery
1	10.2	9.3	91
2	10.2	10.4	102
3	10.2	9.7	95

5 ml 20 mM glycylglycine buffer pH 7.0 + 140 mM KCl containing 10.2 nmol NAD^+ was quenched by the addition of 1.6 ml 25% (v/v) perchloric acid. The solution was neutralised to pH 7.0 by the addition of neutralising solution (2M Tris : saturated KOH : water in the proportions 30:60:10) and the precipitate was removed by centrifugation. The supernatant was assayed for NAD^+ spectrophotometrically as described in Section 2.13.

Table 4.8 NAD^+ concentration in M.methylotrophus

Substrate	Conditions	$[NAD^+]$ (nmol/mg cells)	Δ[NADH] (nmol/mg cells)
Endogenous		1.18	0
Methanol		0.89	0.29
Methanol	FCCP	0.95	0.24
Formate		0.91	0.27
Formate	anaerobic	0.81	0.37

Washed cells (approx 15 mg dry weight) were incubated in 5 ml 20 mM glycylglycine + 140 mM KCl pH 7.0 at 40°C in a rapidly stirred open top 25 ml beaker for 2 minutes prior to the addition of methanol (10 mM) or formate
(10 mM) with or without FCCP (10 μM). Respiration was allowed for 7 seconds prior to quenching with 1.6 ml 25% (v/v) perchloric acid. In the anaerobic experiment the buffer and formate were sparged with oxygen free nitrogen before the experiment; sparging was continued during the experiment, this being performed in a sealed bottle with inlet and outlet parts. The neutralised extracts were assayed for NAD^+.

small. Furthermore, the presence of FCCP may be expected to stimulate formaldehyde dissimilation at the expense of assimilation, in an attempt to maintain the intracellular phosphorylation potential. As this would inevitably lead to an increase in the concentration of NADH, formation of the latter by reversed electron transfer may be over-estimated.

The oxidation of formate by whole cells resulted in an increase in NADH concentration of 0.27 nmol/mg dry weight cells. Since formate is oxidised by an NAD^+/linked formate dehydrogenase, a significant increase in NADH concentration is to be expected. When formate was added to cells maintained under anaerobic conditions, the NADH concentration increased by 0.37 nmol/mg dry weight cells. The smaller increase which was observed under aerobic conditions thus suggests that NADH was being concomitantly re-oxidised via the respiratory-chain linked NADH dehydrogenase. Thus the estimate of 0.05 nmol NADH/mg dry weight cells formed by reversed electron transfer during the first seven seconds of methanol oxidation could be underestimated due to the subsequent oxidation of NADH.

In addition to NAD^+, M.methylotrophus possesses no energy linked transhydrogenase, thus NAD^+ and $NADP^+$ will be in equilibrium via the soluble energy-independent transhydrogenase. Determination of NAD^+ and $NADP^+$ concentrations in extracts from cells respiring endogenous substrates showed that these were present at 0.87 nmol/mg dry weight cells and 0.32 nmol/mg cells respectively (Table 4.9). As the $NADP^+$ pool appears to be only 37% of the size of the NAD^+ pool, it follows the NADPH concentration may only be expected to increase by 0.0085 nmol/mg cells (0.05 x 0.37) and hence will make only an extremely small difference to the amount of reversed electron transfer.

Table 4.9 Concentrations of NAD^+ and $NADP^+$ in M.methylotrophus

Substrate	$[NAD^+]$ (nmol/mg cells)	$[NADP^+]$ (nmol/mg cells)
Endogenous	0.87	0.32
+ Methanol	0.74	0.25

Washed cells (approx. 15 mg dry weight) were incubated with rapid stirring in 5ml 20 mM glycylglycine + 140 mM KCl pH 7.0 at 40°C in open top beakers for 2 minutes. Cells were allowed to respire endogenous substrates or methanol (10 mM) for a further 7 seconds then quenched with 1.6 ml 25% (v/v) perchloric acid. The neutralised cell extracts were assayed for NAD^+ and $NADP^+$ as described in Section 2.13.

4.3 Discussion

The results described in this chapter show that respiration-linked ATP synthesis in M.methylotrophus occurs at the net expense of AMP and inorganic phosphate. This observation is in agreement with results obtained previously using several other species of bacteria (Baak and Postma, 1971; Hempfling, 1970; Gadkari and Stolp, 1976; but see also van der Beek and Stouthamer, 1973; Van Verseveld and Stouthamer, 1976). The ADP utilised during ATP synthesis is presumably replenished from ATP and AMP by the action of adenylate kinase:

$$2\ ADP + 2P_i \longrightarrow 2\ ATP + 2\ H_2O$$

$$ATP + AMP \longrightarrow 2\ ADP$$

overall giving

$$AMP + 2P_i \longrightarrow ATP + 2\ H_2O$$

Experimentally a discrepancy was observed between the phosphate incorporated into ATP ($\Delta[\sim P]$) and the disappearance of inorganic phosphate ($\Delta[P_i]$), and a similar observation was also made with E.coli by Hempfling (1970). The results described in this chapter show that some of the ATP formed is presumably used for the synthesis of other nucleoside triphosphates, presumably due to the presence of nucleoside diphosphate kinase, thus accounting for the discrepancy between changes in the concentrations of ~P and inorganic phosphate. Measurement of ATP utilisation and phosphate release upon addition of FCCP to respiring cells showed a significant turnover of ATP and inorganic phosphate, which is commensurate with the observation that M.methylotrophus failed to exhibit respiratory control. Typical pseudo-first order rate constants for the turnover of ~P and inorganic phosphate of 3.8 min^{-1} and 8.1 min^{-1} respectively were obtained during methanol oxidation (see Section 4.3). This difference was presumably due to the action of nucleoside diphosphate kinase causing re-phosphorylation of ADP at the

expense of other nucleoside triphosphates. It was concluded that the value of the P_i/O quotient, corrected for the turnover of inorganic phosphate, was the most accurate indicator of the efficiency of oxidative phosphorylation.

An average pseudo-first order rate constant for inorganic phosphate turnover of 11 min^{-1} was obtained. The nature of the respiratory substrate appeared to make no significant difference to the turnover of inorganic phosphate, indicating that the production of formaldehyde during methanol oxidation did not result in a subsequent stimulation of ATP hydrolysis for assimilation. It is possible that the turnover rates may have been over-estimated if the ATP synthase is reversible and is able to hydrolyse ATP under conditions where the protonmotive force is abolished by the addition of FCCP. However, it has been reported that many obligately aerobic bacteria appear to possess an essentially irreversible ATP synthase (Ferguson, 1977; Chernyak and Kozlov, 1986), although the irreversibility of the ATP synthase of M.methylotrophus has not been confirmed experimentally.

The P_i/O quotients for the oxidation of methanol, ethanol, acetaldehyde, duroquinol and formate were in reasonably constant proportion to the measured or assumed $\rightarrow H^+/O$ or $\rightarrow K^+/O$ quotients for each substrate (Dawson and Jones, 1981a,b,c; see Table 4.10). However, even when corrected for turnover, the P_i/O quotients for the oxidation of these substrates were relatively low, and were commensurate with an average $\rightarrow H^+/ATP$ quotient of just over 4 g-ion H^+/mol ATP. Comparably low efficiencies of ATP synthesis have also been reported for the oxidation of methanol and NADH by membrane vesicles of the methylotrophic bacterium Pseudomonas AM1 (Netrusov and Anthony, 1979) and for the oxidation of various substrates by non-methylotrophic bacteria (Eigener and Bock, 1975; van Verseveld and Stouthamer, 1976;

Table 4.10 Energy conservation in M.methylotrophus

Substrate	P_i/O (nmol/ng-atom O)	$\rightarrow H^+/O$ or $\rightarrow K^+/O$ (ng-ion/ng-atom O)	$\rightarrow H^+/ATP$ (ng-ion/nmol)
Methanol	0.46	2.0	4.3
Ethanol	0.46	(2.0)	4.3
Acetaldehyde	0.54	(2.0)	3.7
Duroquinol	0.77	3.5	4.5
Formate	1.37	6.3	4.6

P_i/O quotients corrected for turnover were taken from Table 4.6. $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients were taken from Dawson and Jones (1981a); the values shown in brackets were assumed to be the same as those for methanol. The $\rightarrow H^+/ATP$ quotient is assumed to be numerically identical to the $\rightarrow H^+/P_i$ quotient.

Niven et al., 1977). However, substantially higher efficiencies have also been reported (Knowles and Smith, 1970; Gadkari and Stolp, 1976; Kröger and Winkler, 1981). It is possible that the P_i/O quotients obtained by direct measurement of the type described in this chapter were underestimated due to dissipation of the protonmotive force by other protonmotive force dependent reactions in addition to ATP synthesis. These include the synthesis of inorganic pyrophosphate, reversed electron transfer, solute transport, motility and proton leakage. The synthesis of pyrophosphate was not detected in M.methylotrophus. However, it should be noted that the assay system employed was not particularly sensitive and could have failed to detect small changes in the concentration of pyrophosphate. The elimination of reversed electron transfer from methanol to NAD^+ by the use of specific respiratory chain inhibitors was not possible as rotenone and Antimycin A are ineffective in whole cells of M.methylotrophus, whilst HQNO may possess significant uncoupling activity (C.W. Jones, personal communication). The uncoupler-sensitive disappearance of NAD^+ which was detected in whole cells during methanol oxidation was too small to make an appreciable difference to the P_i/O quotient. It is possible, however, that the energy-dependent formation of pyrophosphate and NADH could have occurred at a significant rate but have produced only very small changes in concentration due to the high substrate affinities and catalytic activities of subsequent hydrolysis/oxidation reactions. No energy should be consumed in the transport of methanol, ethanol or acetaldehyde since methanol dehydrogenase is located on the periplasmic surface of the respiratory membrane, and both duroquinol and oxygen can diffuse to their site of action. Formate, however, may be taken up in symport with H^+, and this could lead to a slight underestimate of the P_i/O quotient during formate oxidation. Furthermore, some uptake of

inorganic phosphate may occur in the first few seconds of respiration with any of the substrates used, but the pre-incubation of cells under aerobic conditions should minimise the leakage of phosphate from the cells prior to the addition of substrate. An energy-dependent K^+ uptake or an energy-dissipating cycling of K^+ would appear to be unlikely since the extent of intracellular energisation appears to be maximal at high concentrations of K^+ (Dawson and Jones, 1982).

Under steady-state conditions, the extent of intracellular energisation is equal to the rate of energisation divided by the rate constant for its decay. Since the rate of energisation is equal to the rate of respiration multiplied by the stoichiometry of respiratory chain phosphorylation, then the latter is proportional to the steady-state energisation. Thus the relative efficiencies of respiration-linked phosphorylation with different substrates may be determined at fixed respiration rates. The steady-state energisation achieved after 1 minute of respiration with each substrate at a given respiration rate was in the order methanol > duroquinol > acetaldehyde (Patchett et al., 1985). At a respiration rate of 200 ng-atom O/min/mg cells the increases in the [ATP]/[AMP] ratio over the uncoupled value were 4.3, 3.0 and 0.9 for the oxidation of methanol, duroquinol and acetaldehyde respectively, whilst the decreases in the concentration of inorganic phosphate were respectively 5.9, 4.4 and 2.3 nmol/mg cells. Since the oxidation of duroquinol yields an $\rightarrow H^+/O$ quotient of 3.5 g-ion H^+/g-atom O (Dawson and Jones, 1981a), these results were commensurate with steady-state $\rightarrow H^+/O$ quotients of 5.0 and 4.7 for the oxidation of methanol and 1.1 and 1.8 for the oxidation of acetaldehyde. The relative efficiencies of respiration-linked phosphorylation during the oxidation of duroquinol and acetaldehyde are in the same order as the P_i/O quotients obtained during the initial rate experiments described in

this chapter. The low value of the steady-state $\rightarrow H^+/O$ quotient obtained during the oxidation of acetaldehyde suggests that the high concentrations of acetate produced during these experiments had some uncoupling activity at high concentrations (>0.8 mM acetate was produced by the end of the steady-state experiment compared with <0.08 mM during the initial rate experiments described in this chapter). The steady-state $\rightarrow H^+/O$ quotients of 5.0 and 4.7 for the oxidation of methanol are close to the $\rightarrow H^+/O$ quotient of 4.67 which can be calculated for the complete oxidation of methanol via methanol dehydrogenase and the dissimilatory ribulose monophosphate cycle (one $PQQH_2$ and 2NAD(P)H, ie. [2+6+6]/3 = 4.67).

The protonmotive force for a two electron transfer reaction is predicted by the equation

$$\Delta p = \frac{2\ \Delta E_h}{\rightarrow H^+/O}$$

where ΔE_h is the difference in redox potential between the reducing and oxidising couples. The predicted Δp of all the respiratory substrates used is therefore in excess of 400 mV (see Table 4.11) although the measured transmembrane electrochemical potential ($\Delta\bar{\mu}_{H^+}$) is no more than 165 mV (Dawson and Jones, 1982). Assuming that the measured $\Delta\bar{\mu}_{H^+}$ is not a massive underestimate of the actual Δp, then there is a large discrepancy between the predicted and the actual values of Δp. This discrepancy could reflect a high rate of proton leakage, particularly at high values of Δp (see Clarke et al., 1983). Interestingly, measurement of the half-life of decay of a proton gradient formed by synchronously growing cells of Alcaligenes eutrophus has shown that cells at different stages of the life cycle show different degrees of membrane permeability (Edwards et al., 1978). It was suggested that at those stages of the life cycle where the proton permeability is greatest, the BF_o subunit

Table 4.11 Standard redox potentials and redox potential differences for respiratory substrates

Substrate couple	Eo' (V)	ΔEo' (V)	$\rightarrow H^+/O$ or $\rightarrow K^+/O$ (ng-ion/ng-atom O)	Δp (V)
formaldehyde/methanol	-0.19	-1.01	2	1.01
acetaldehyde/ethanol	-0.163	-0.98		
acetate/acetaldehyde	-0.581	-1.40		
carbon dioxide/formate	-0.42	-1.24		
$NAD^+/NADH + H^+$	-0.32	1.14	6.3	0.36
duroquinone/duroquinol	0.06	0.88	3.5	0.50

Standard redox potentials at pH 7.0 (Eo') were taken from Segel (1970), Sober (1968) and Kroger and Klingenberg (1973). $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients were obtained from Dawson and Jones (1981a). The protonmotive force (Δp) was calculated from

$$\Delta p = \frac{-2\Delta E'o}{\rightarrow H^+/O}$$

may act as a route for proton leakage due to the lack of an associated BF_1 subunit. In an asynchronous culture this would lead to a basal level of proton leakage through the membrane.

The reported discrepancies between experimental and theoretical values of Y_{ATP}^{max} (see Stouthamer, 1979) have indicated that uncoupling between energy input and biomass formation in micro-organisms is fairly common (see Section 1.6). To account for this it has been suggested that biological energy converters have evolved to work under conditions of maximum rate of energy output and consequently function at a reduced efficiency (Harder et al., 1981; Westerhoff et al., 1983). Stucki (1980) suggested that the efficiency of energy transduction in biological systems drops rapidly when the degree of coupling of the system deviates from unity, such that when the degree of coupling is 0.94 the efficiency has dropped to 50%.

An $\rightarrow H^+/ATP$ quotient $\geq$ 3 g-ion H^+/mol ATP has been determined for the ATP synthase of Paracoccus denitrificans based on thermodynamic data with membrane vesicles (McCarthy and Ferguson, 1983). Thermodynamic data from whole cells of M.methylotrophus has also indicated an $\rightarrow H^+/ATP$ quotient of approximately 3 g-ion H^+/mol ATP (Dawson and Jones, 1982). This value, together with a significant leakage of protons through the respiratory membrane, would thus be compatible with the average $\rightarrow H^+/ATP$ quotient of just over 4 g-ion H^+/mol ATP calculated from the kinetic data described in this chapter.

CHAPTER 5

THE EFFECT OF TEMPERATURE ON ENERGY CONSERVATION IN *Methylophilus methylotrophus*

5.1 Introduction

5.2 Results

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CHAPTER 5

THE EFFECT OF TEMPERATURE ON ENERGY CONSERVATION IN *Methylophilus methylotrophus*

5.1 Introduction

A number of early reports have described the effect of temperature on the efficiency of bacterial growth (Senez, 1962; Ng, 1969; Coultate and Sundaram, 1975). However, these experiments were carried out using batch cultures and thus failed to take account of the effect of maintenance energy (Pirt, 1965; Stouthamer and Bettenhausen, 1973). The variation in growth rate with temperature therefore makes interpretation of these results difficult; however, they generally indicated a decreased cell yield as the temperature was increased above a threshold value. This problem is largely overcome by using continuous cultures where it is of course possible to maintain a fixed specific growth rate (≡ dilution rate). Thus lowering the growth temperature has been shown to cause a small increase in the growth yield of *Klebsiella aerogenes* in continuous culture (Topiwala and Sinclair, 1971). In a more detailed study, Mainzer and Hempfling (1976) investigated the effect of temperature on the yield and maintenance energy requirements of a glucose-limited continuous culture of *E.coli*. During anaerobic growth Y_{ATP}^{max} increased from 10.3 to 12.7 g cells/mol ATP as the growth temperature was lowered from 37°C to 25°C. It was suggested that the small increase in Y_{ATP}^{max} at lower temperatures reflected an increased efficiency of ATP utilisation. During aerobic growth below 32°C, Y_{ATP}^{max} was more than double that observed during growth above 35°C. It was suggested that an increased efficiency of ATP utilisation was not sufficient to account for the large increase in Y_{ATP}^{max}, and that the latter reflected an increased efficiency of oxidative phosphorylation.

However, although aerobic cultures of E.coli growing on glycerol, a non-fermentable substrate, showed a decrease in Y^{max}_{ATP} as the growth temperature was increased from 30°C to 40°C, the stoichiometry of respiration-linked proton translocation was independent of growth temperature (Farmer and Jones, 1976). It was suggested that the efficiency of utilisation of the energy initially conserved via oxidative phosphorylation, was significantly diminished at the higher temperatures.

Growth studies of several species of thermophilic bacteria have shown that these organisms exhibit diminished cell yields as the growth temperature is increased (McKay et al., 1982; Farrand et al., 1983). In Thermus thermophilus and Bacillus acidocaldarius low growth yields at a given growth rate (Y) have been shown to reflect a mixture of lowered maximum growth yield (Y^{max}) and higher maintenance requirements (McKay et al., 1982; Farrand et al., 1983). Furthermore, it was shown that the half-life of decay of a pH gradient generated by respiration following the addition of a pulse of oxygen, decreased with increasing temperature. It was suggested that in these organisms the low maximum growth yields reflect a high permeability of the cytoplasmic membrane to protons, such that the protonmotive force is rapidly dissipated by leakage rather than by driving ATP synthesis or solute transport. The higher growth yields and the slower rate of decay of the pH gradient which are observed at lower temperatures may therefore reflect a decrease in the passive permeability of the coupling membrane to protons. An alternative explanation may be an increased turnover of ATP at higher temperatures leading to renewed ATP synthesis with consequent dissipation of the pH gradient. In contrast, Y^{max} increased between 50° and 70°C during growth of the thermophile Bacillus caldotenax (Kuhn et al., 1980).

Methylophilus methylotrophus is a mesophilic organism and it was of interest to see if it responded to growth temperature in a similar way to E.coli in spite of its quite different metabolism. The growth yield is an important consideration in the production of single cell protein; higher yields at lower temperatures would reduce the cost of methanol although this would have to be offset against the additional cooling costs incurred by maintaining a large culture at a lower temperature. M.methylotrophus was studied during growth at several different temperatures in order to determine whether the growth temperature affected cell yield and the efficiency of respiration-linked energy conservation.

5.2 Results

5.2.1 The growth of M.methylotrophus at different temperatures

Methylophilus methylotrophus was grown in methanol-limited continuous culture with a constant input methanol concentration of 43.2 mM ($D = 0.18\ h^{-1}$) at temperatures between 26°C and 40°C. Steady-state cultures grown at 26°C showed a higher optical density at 680 nm than cultures grown at 40°C. Determination of culture dry weights by filtration of known volumes of cells grown at 26°C or 40°C showed that the relationship between optical density at 680 nm and dry weight of bacteria was unaffected by the temperature of growth, giving significantly different cell densities at 26°C and 40°C of 0.59 ± 0.01 (4) and 0.53 ± 0.01 (6) $mg_{dry\ wt.}$ cells/ml respectively and thus $Y_{methanol}$ of 13.7 ± 0.23 (4) and 12.3 ± 0.23 (6) g/mol respectively. The cell density was unaffected by growth temperature up to approximately 34°C, then falls as the temperature of growth is raised to 40°C (Fig. 5.1).

The removal of a small aliquot from the culture vessel onto ice followed by rapid filtration yielded a clear cell-free solution of spent growth medium. Addition of a suspension of M.methylotrophus to this

solution in an oxygen electrode chamber did not cause oxygen consumption, indicating the absence of methanol in the culture filtrate. This suggests that cells grown at 26°C or 40°C were genuinely methanol-limited although some methanol may have been oxidised during the sampling procedure. The higher growth yield at 26°C than at 40°C ($Y_{methanol}$ = 13.7 ± 0.23 (4) cf. 12.3 ± 0.23 (6)) thus suggesting a more efficient utilisation of the carbon and energy source at the lower growth temperature.

The significant difference in cell yield between the two growth temperatures was further examined by measuring the culture density following a decrease in growth temperature from 40°C to 26°C. The culture density was constant at 0.56 $mg_{dry\ wt.}/l$ cell/ml prior to altering the growth temperature, and remained so for approximately 3 hours afterwards before decreasing to a minimum of approximately 0.535 $mg_{dry\ wt}/l$ cells/ml after 6 to 7 hours. The cell density then started to increase and reached a new steady-state of 0.61 $mg_{dry\ wt.}/l$ cells/ml within 24 hours (Fig. 5.2a).

In the reverse experiment the growth temperature was increased from 26°C to 40°C. The culture density remained constant at approximately 0.61 $mg_{dry\ wt.}/l$ cells/ml for 3.75 hours. This was followed by a decrease in cell density to approximately 0.54 $mg_{dry\ wt.}/l$ cells/ml after 13 hours. The cell density then increased slightly to a new steady-state value of approximately 0.57 $mg_{dry\ wt.}/l$ cells/ml. (Fig. 5.2b). These fluctuations in cell density were not the result of a slow altertion in temperature since the new growth temperature was achieved in less than 10 minutes of adjustment.

The maximum growth rate of M.methylotrophus was determined by measuring growth rate in batch cultures at 26°C yielding a maximum specific growth rate of 0.24 ± 0.02 (3) h^{-1}. The maximum growth rate at 40°C is 0.55 ± 0.01 (3) h^{-1} (Jones, C.W. unpublished data), thus the

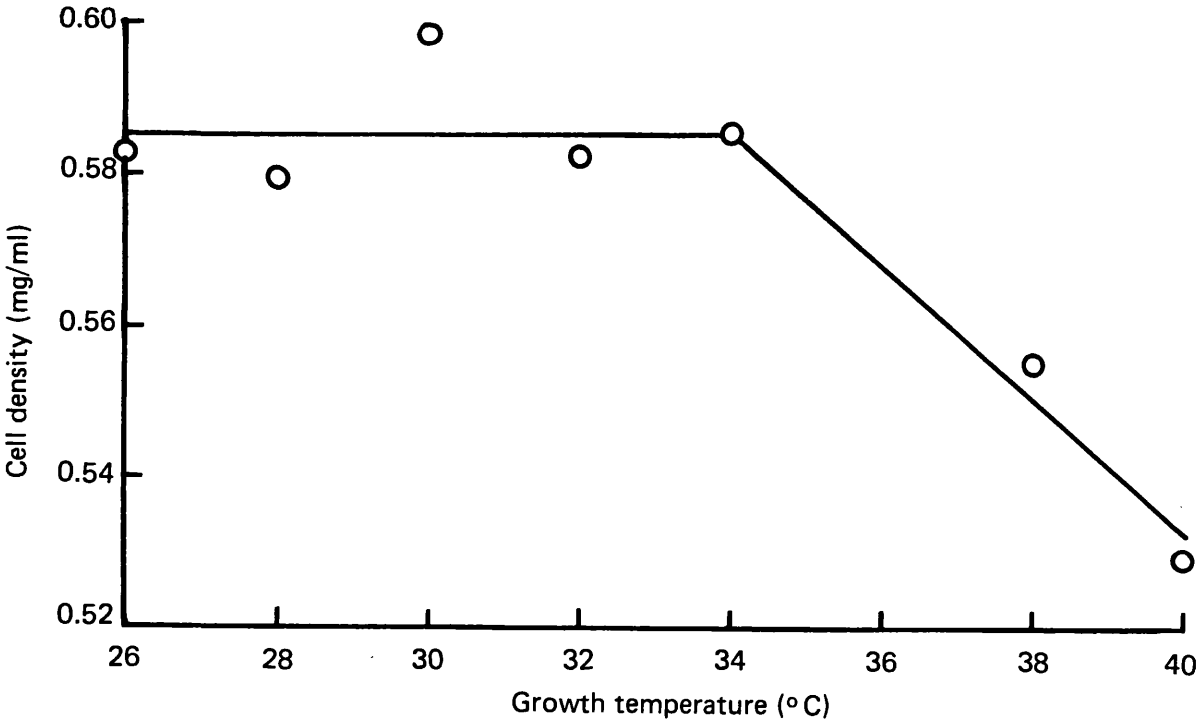
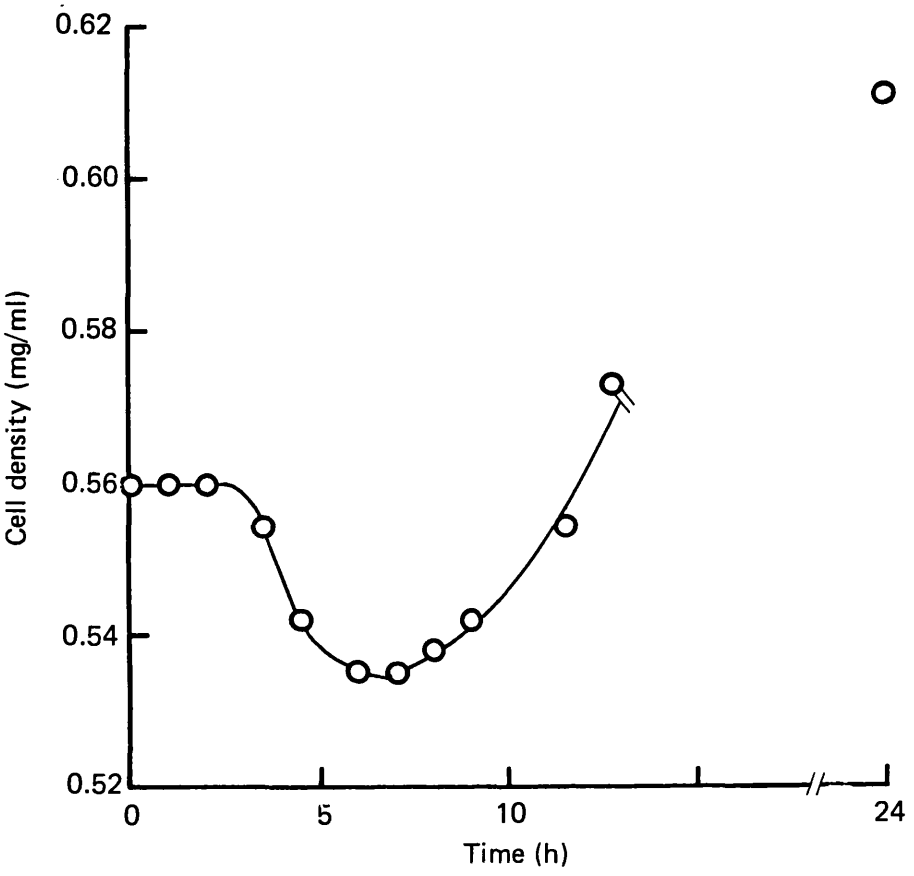


Fig. 5.1 Cell density of M.methylotrophus grown in continuous culture at different temperatures

M.methylotrophus was grown in methanol-limited continuous culture ($D = 0.18h^{-1}$) at a series of temperatures in the range 26°C to 40°C as described in section 2.2. A constant input concentration of 43.2mM methanol was achieved by bulk preparation of media with mixing of separate batches. The culture was allowed at least 24 hours (equivalent to approximately four culture volumes) to attain steady-state conditions at each temperature prior to the removal of a 3ml sample for the determination of the culture density.

a)


0.62
0.60
0.58
0.56
0.54
0.52
Cell density (mg/ml)
0
5
10
24
Time (h)

b)

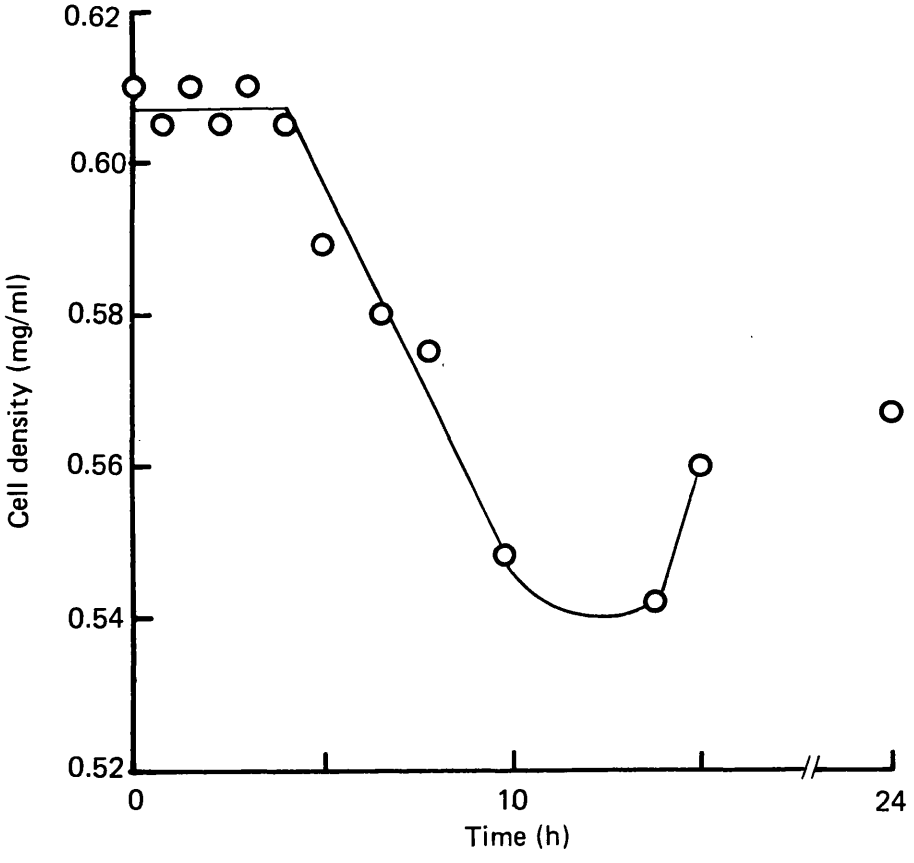

0.62
0.60
0.58
0.56
0.54
0.52
Cell density (mg/ml)
0
10
24
Time (h)

Fig. 5.2 Culture density of M.methylotrophus during alteration in growth temperature

M.methylotrophus was grown in continuous culture ($D = 0.18\ h^{-1}$) as described in section 2.2. At zero time the temperature was altered

(a) from 40°C to 26°C, and

(b) from 26°C to 40°C

3ml Samples were removed for the determination of cell density. Values at zero time and 24 h were confirmed by earlier and later measurements respectively, ie. prior to changeover and after the final steady state had been achieved.

growth rates in the preceding experiments ($D \equiv \mu = 0.18\ h^{-1}$) were within the maximum growth rates at 40°C and at 26°C.

5.2.2 The activities of methanol dehydrogenase and methanol oxidase in M. methylotrophus grown at different temperatures

The methanol oxidase activity of whole cells of M.methylotrophus was assayed at 40°C. As the growth temperature was increased from 26°C to 40°C the methanol oxidase activity decreased by over 30%. In contrast, the methanol dehydrogenase activity remained constant (Fig. 5.3). This may have been due to an increased lability of methanol oxidase at 40°C in cells grown at 26°C; however, Arrhenius plots of methanol oxidase activity in cells grown at 26°C or 40°C showed no significant difference (Fig. 5.4).

Reduced minus oxidised difference spectra of whole cells of M.methylotrophus grown at 40°C (Fig. 5.5) or 26°C showed no significant difference between the concentrations of total c-type or total b-type cytochromes in the two types of cells (Table 5.1). The low concentration of cytochrome aa_3 made accurate comparisons difficult and it is not clear whether there is any significant difference in the concentration of a-type cytochromes in cells grown at 40°C or 26°C.

The ascorbate-TMPD oxidase activity of whole cells of M.methylotrophus showed no significant decrease over the range of growth temperatures employed in these studies (Fig. 5.3). As the artificial electron donor TMPD interacts with the respiratory chain at the level of the c-type cytochromes, including cytochrome oxidase co (Carver and Jones, 1983; Froud and Anthony, 1985), these results suggest that there is no significant decrease in the cytochrome oxidase activities of the respiratory chain. Thus, since the activities and/or concentrations of the major redox components of the methanol oxidase system appear to be

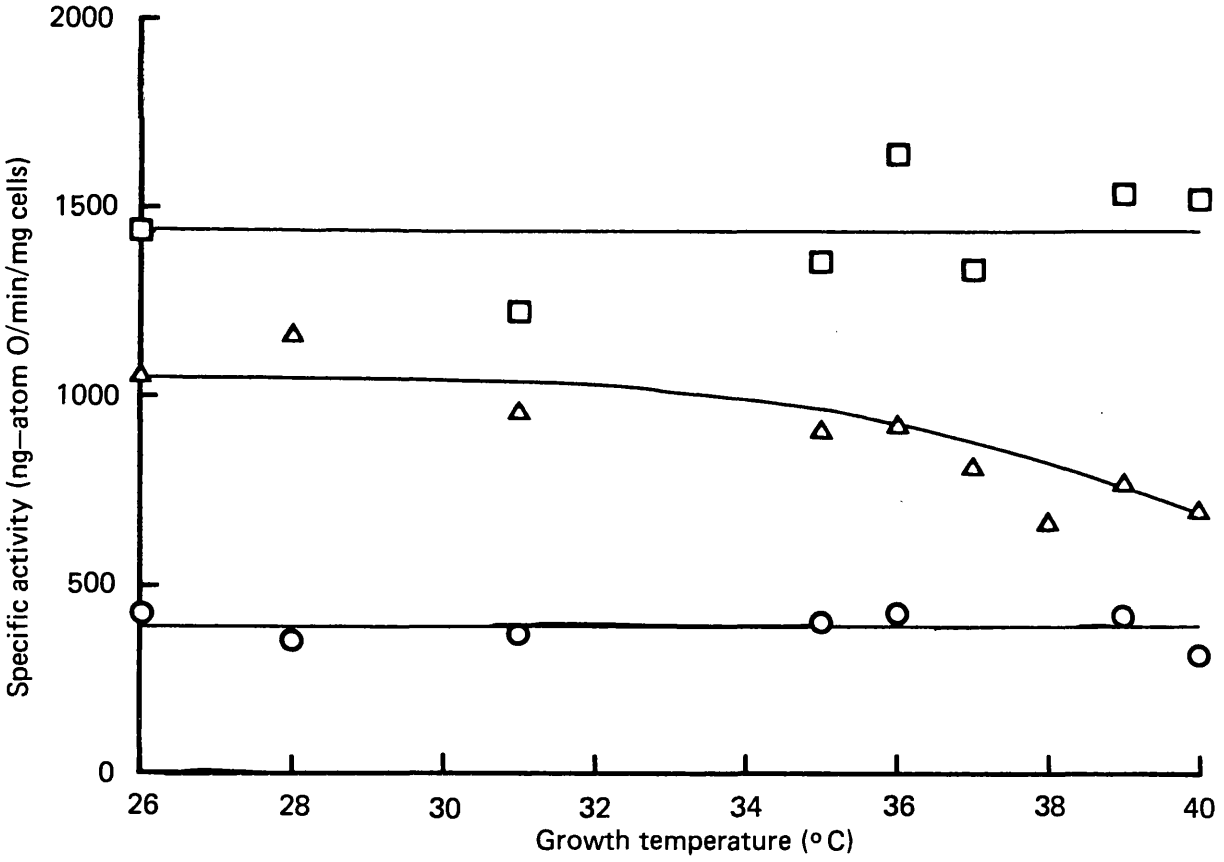


Fig. 5.3 The methanol dehydrogenase, methanol oxidase and ascorbate-TMPD oxidase activities in whole cells of M.methylotrophus grown at different temperatures

M.methylotrophus was grown in methanol-limited continuous culture (D = $0.18h^{-1}$) at different temperatures. The culture was allowed to adapt to each new growth temperature for at least 24 hours before cells were harvested and assayed for methanol dehydrogenase (O), methanol oxidase (Δ) and ascorbate-TMPD oxidase (□) activities as described in sections 2.7 and 2.14.

The ascorbate-TMPD oxidase rate was corrected for auto-oxidation and the rate obtained in the methanol dehydrogenase assay was divided by 2 to correct for the formation of hydrogen peroxide.

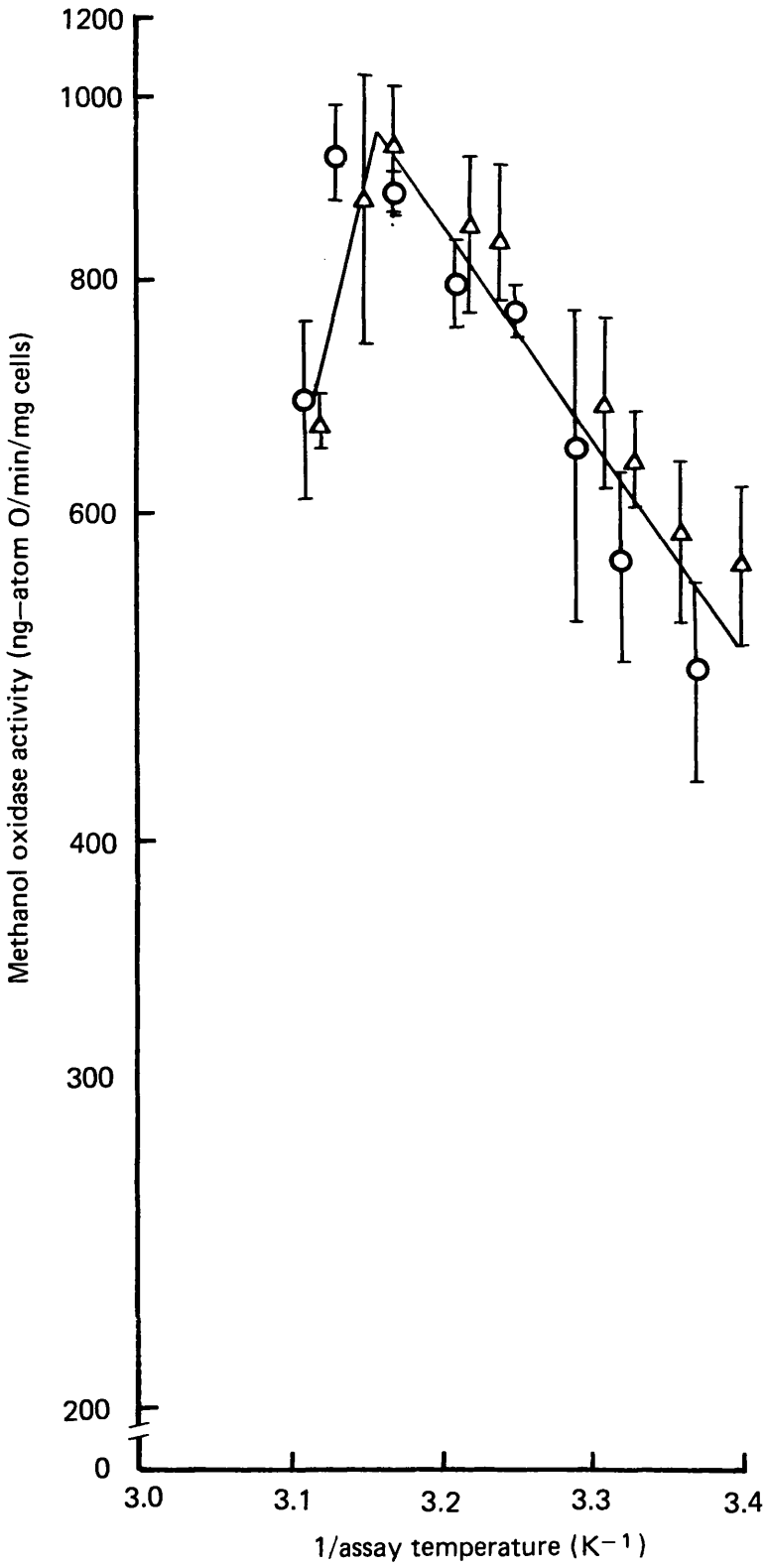

1200
1000
800
600
400
300
200
0
Methanol oxidase activity (ng–atom O/min/mg cells)
3.0
3.1
3.2
3.3
3.4
1/assay temperature (K⁻¹)

Fig. 5.4 Arrhenius plot of methanol oxidase activity in M.methylotrophus grown at 26°C or 40°C

M.methylotrophus was grown in methanol-limited continuous culture ($D = 0.18\ h^{-1}$) at 26°C (○) or 40°C (△) as described in section 2.2. Cells were harvested and assayed for methanol oxidase activity in 20mM glycylglycine/KOH buffer pH 7.0 with 1mM methanol at different temperatures as described in section 2.4. Error bars show the standard error of three independent determinations.

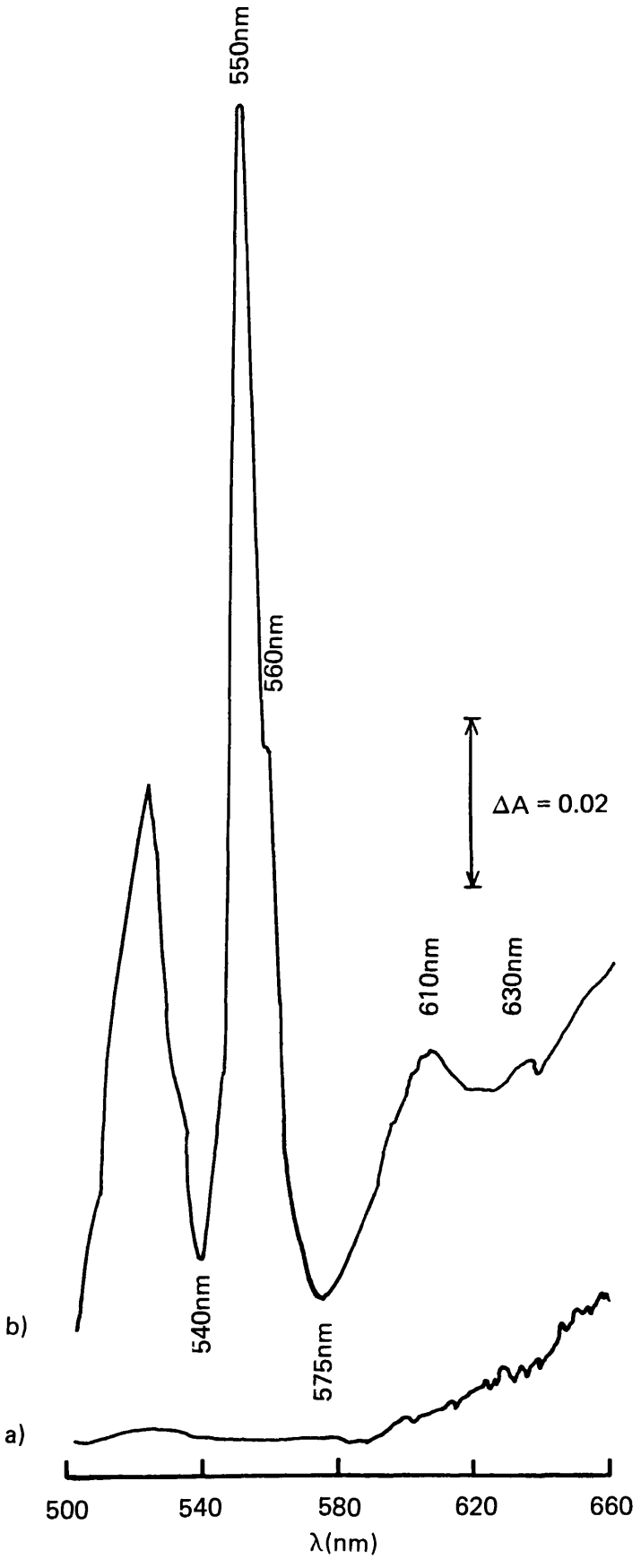

550nm
560nm
ΔA = 0.02
610nm
630nm
540nm
575nm
b)
a)
500
540
580
620
660
λ(nm)

Fig. 5.5 Reduced minus oxidised difference spectrum of whole cells of M.methylotrophus

M.methylotrophus was grown in methanol-limited continuous culture (D = $0.18h^{-1}$) at 40°C. Cells were harvested, resuspended in glycylglycine buffer pH 7.0 and

a) oxidised minus oxidised

b) dithionite reduced minus ferricyanide oxidised different spectra were recorded as described in section 2.4.

Table 5.1 Cytochrome content of whole cells of M.methylotrophus following growth at 40°C or 26°C

Growth temp. (°C)	Cytochromes		
	Total c-type (nmol/mg cells)	Total b-type (nmol/mg cells)	aa_3 (nmol/mg cells)
40	1.37 ± 0.06 (3)	0.42 ± 0.04 (3)	0.051 ± 0.01 (3)
26	1.39 ± 0.07 (3)	0.42 ± 0.04 (3)	0.070 ± 0.01 (3)

M,methylotrophus was grown in methanol-limited continuous culture (D = 0.18 h^{-1}) at 40°C or 26°C. Cells were harvested and assayed for cytochromes via dithionite reduced minus ferricyanide oxidised difference spectra as described in Section 2.16.

unchanged as a function of growth temperature, the decreased activity of the complete methanol oxidase system may simply reflect a decrease in the stability of the association between methanol dehydrogenase and the remainder of the respiratory chain.

5.2.3 Respiration-linked proton translocation by M.methylotrophus grown at 40°C or 26°C

The addition of air-saturated 140 mM KCl to a lightly-buffered suspension of cells resulted in a rapid acidification of the reaction medium. The extent of acidification was maximal in the presence of 1 μg valinomycin/mg cells (Dawson, 1982) and at pH 5.9 (Fig. 5.6). The decay of the resultant ΔpH showed first-order kinetics with respect to proton concentration for at least 2 minutes, but after this period the rate of decay slowed. Cells grown at either 26°C or 40°C exhibited $\rightarrow H^+/O$ quotients for the oxidation of endogenous substrates of approximately 6 ng-ion H^+/ng-atom O when assayed at either 26°C or 40°C, ie. the stoichiometry of respiration-linked proton translocation was not significantly affected by either the growth temperature or the assay temperature (Table 5.2).

The half-life of decay of pH_{out} resulted from an oxygen pulse was determined from plots of log ($pH^t_{out} - pH^o_{out}$) against time, where pH^o_{out} is the pH of the reaction medium at zero time and pH^t_{out} is the pH of the reaction medium at time t after the addition of a small amount of oxygen (Fig. 5.7). When assayed at 40°C, cells grown at 40°C have a $t_{1/2}$ of decay of pH_{out} of 162 seconds compared with 121 seconds for cells grown at 26°C; similarly, when assayed at 26°C, cells g rown at 40°C have a $t_{1/2}$ of decay of pH_{out} of 613 seconds compared with 386 seconds for cells grown at 26°C (Table 5.3). Thus cells grown at the lower temperature are more permeable to H^+ if both types of cells are assayed at the same temperature. However, cells grown at 26°C are less

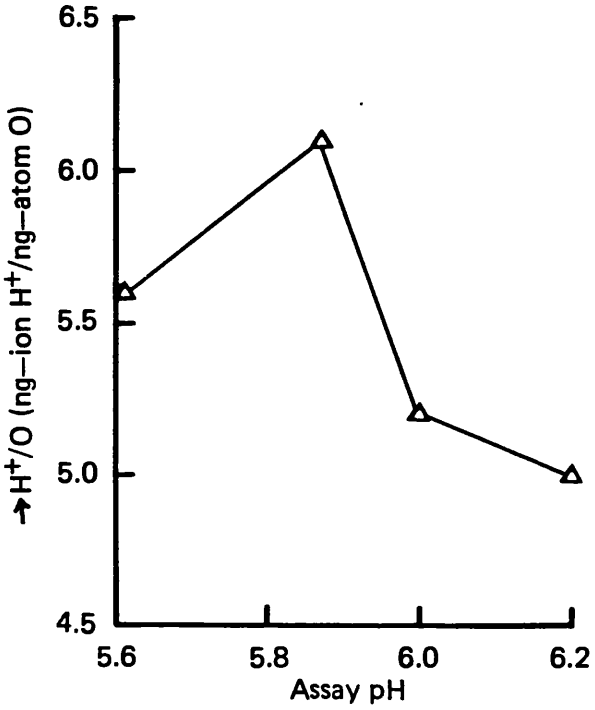


Fig. 5.6 The effect of assay pH on the $\rightarrow H^+/O$ quotient in M.methylotrophus

Washed cells (approx. 25mg dry weight) of M.methylotrophus grown at 40°C in methanol-limited continuous culture ($D = 0.18h^{-1}$=, were incubated for 30 minutes at 40°C in 4ml 1.5mM glycylglycine buffer + 140mM KCl and valinomycin (1µg/mg cells) at a series of pH values between 5.6 and 6.2, prior to the measurement of $\rightarrow H^+/O$ quotients by the oxygen pulse method (Section 2.13).

Table 5.2 The stoichiometry of respiration-linked proton translocation in M.methylotrophus grown and assayed at 26°C and/or 40°C

Growth temp.	$\rightarrow H^+/O$ assay temp.	
	26°C	40°C
(°C)	(ng-ion H^+/ng-atom O)	(ng-ion H^+/ng-atom O)
26	5.9 ± 0.3 (3)	6.4 ± 0.3 (3)
40	5.6 ± 0.2 (5)	5.9 ± 0.3 (5)

M.methylotrophus was grown in methanol-limited continuous culture (D = 0.18 h^{-1}) at 40°C or 26°C. Whole cell $\rightarrow H^+/O$ quotients for the oxidation of endogenous substrates were assayed at either 26°C or 40°C using the oxygen pulse method (Section 2.15).

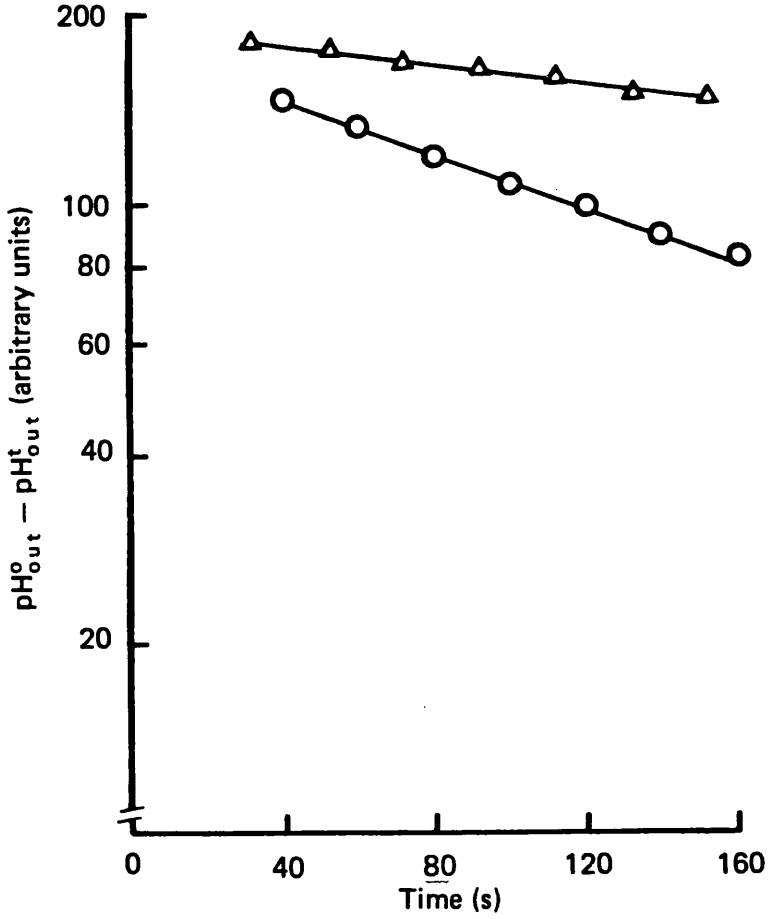


Fig. 5.7 The decay of the proton gradient in M.methylotrophus

M.methylotrophus was grown in methanol-limited continuous culture ($D = 0.18h^{-1}$) at 40°C (O) or 26°C (Δ). Washed cells (approx. 25mg dry weight) were incubated in 4ml anoxic 1.5mM glycylglycine + 140mM KCl pH 5.9 at 40°C or 26°C with valinomycin (1μg/mg cells) for 30 minutes. A 50μl aliquot of 140mM KCl was added and the decay of the resulting pH change was recorded (see section 2.13) and subsequently re-plotted as $pH^o_{out} - pH^t_{out}$ against time, where pH^o_{out} is the extracellular pH at zero time and pH^t_{out} is the extracellular pH at time t.

Table 5.3 The half-life of decay of the proton gradient in M.methylotrophus

Growth temp. (°C)	$t_{1/2}$ assay temp. 26°C (sec)	40°C (sec)
26	386 ± 26 (5)	121 ± 5 (5)
40	613 ± 59 (5)	162 ± 13 (5)

The half-life of decay of the proton gradient generated following the addition of a small amount of oxygen to an anaerobic suspension of M.methylotrophus was obtained from plots of $pH^{t}_{out} - pH^{o}_{out}$ against time (see Fig. 5.8).

permeable to H^+ when assayed at 26°C than are cells grown at 40°C and assayed at 40°C. A lower permeability to protons may result either from a proportional decrease in proton flux through all of the available routes or from a specific decrease in the activity of one route of proton permeability. Assuming that decay of pH_{out} represents either (a) H^+ leakage (either via inherent membrane permeability or via a specific leakage channel eg. in brown adipose tissue; Nicholls, 1977) or (b) ATP turnover resulting in resynthesis of ATP and hence H^+ uptake, then either of these will lead to energy dissipation. A decrease of either route of energy dissipation will lead to a greater efficiency of energy conservation and hence to a higher growth yield. To test this hypothesis the P_i/O quotient for the respiration of methanol at 26°C by cells grown at 26°C was measured.

5.2.4 The P_i/O quotient for respiration of methanol at 26°C

Methylophilus methylotrophus grown at 26°C in methanol-limited continuous culture ($D = 0.18\ h^{-1}$) was incubated in aerobic 20 mM glycylglycine buffer pH 7.0 + 140 mM KCl at 26°C. In a typical experiment (Fig. 5.8) the addition of methanol to the cell suspension caused the concentration of inorganic phosphate to fall from 9.9 nmol/mg cells to 6.1 nmol/mg cells over the initial 10.4 seconds and reached a steady-state concentration of 5.7 nmol/mg cells after approximaely 12 seconds. As 14.3 ng-atom of oxygen were consumed over the initial 10.4 seconds, the cells exhibited a P_i/O quotient of 0.27 nmol P_i/ng-atom O.

The addition of FCCP to cells respiring methanol at 26°C typically resulted in an increase in the concentration of inorganic phosphate from 8.6 to 12.4 nmol/mg cells over a period of 12 seconds (Fig. 5.9a). During this period, phosphate release approximated to pseudo-first order kinetics with a typical rate constant of $11.9\ min^{-1}$

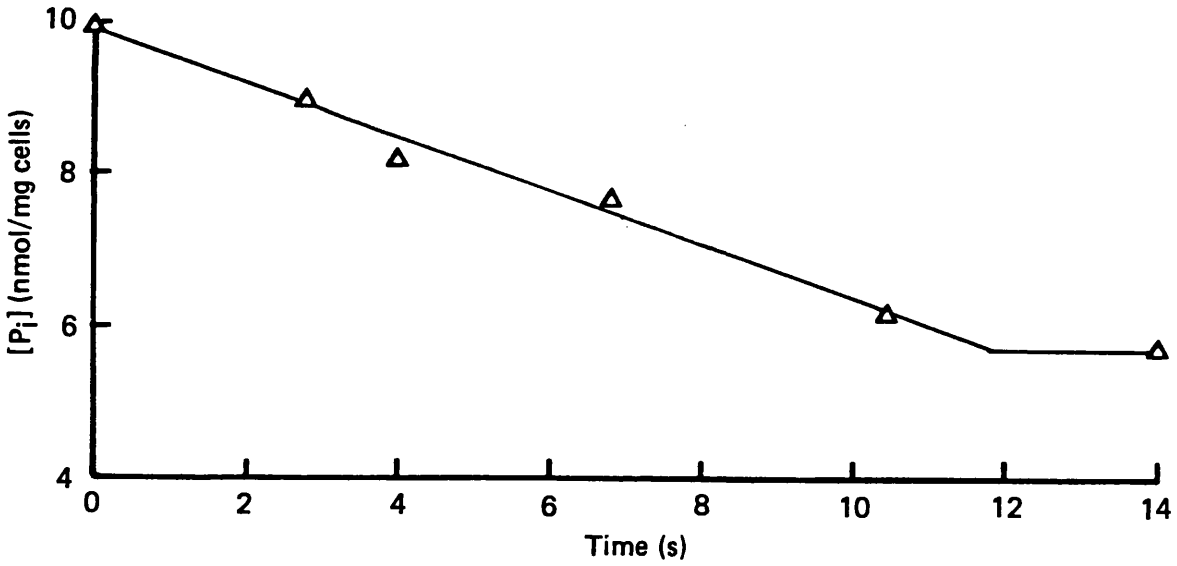
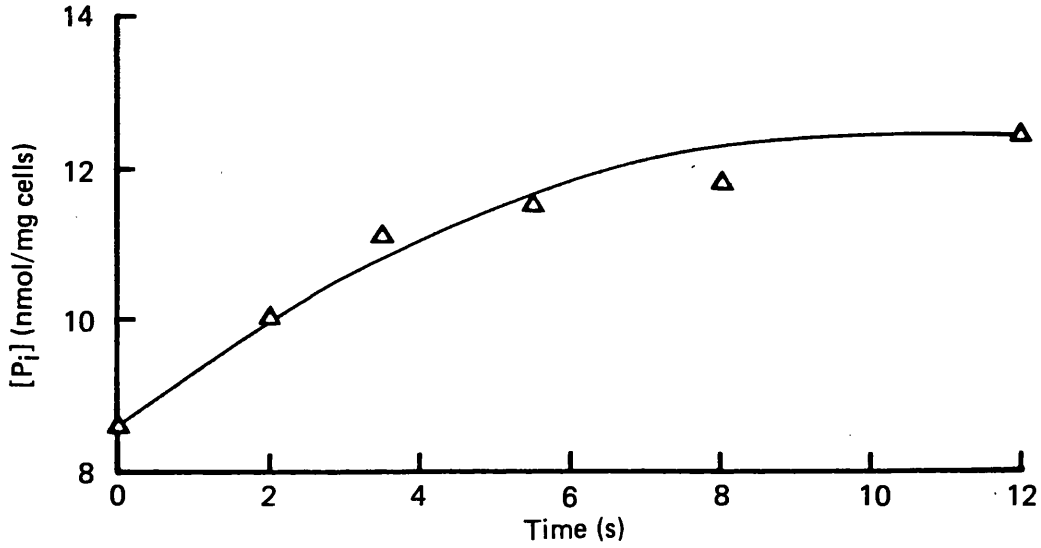


Fig. 5.8 The utilisation of inorganic phosphate during the respiration of methanol by whole cells of M.methylotrophus

Washed cells (approx. 60mg dry weight) prepared from a methanol limited continuous culture of M.methylotrophus ($D = 0.18h^{-1}$, 26°C), were incubated in 25ml 20mM glycylglycine buffer pH 7.0 + 140mM KCl at 26°C for 2 minutes prior to the addition of methanol (10mM) at zero time. Cells were sampled into 25% (v/v) perchloric acid at regular intervals and subsequently prepared and assayed for inorganic phosphate.

a)


14
12
10
8
[Pi] (nmol/mg cells)
0
2
4
6
8
10
12
Time (s)

b)

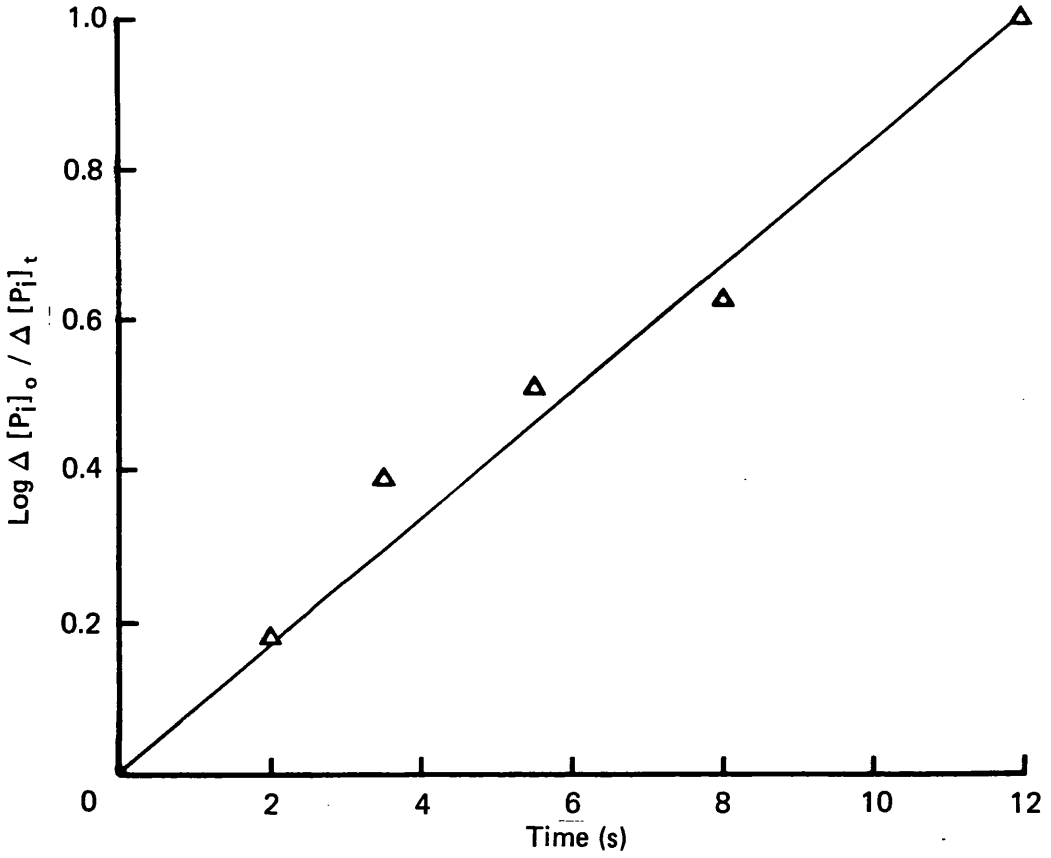

1.0
0.8
0.6
0.4
0.2
0
Log Δ [Pi]o / Δ [Pi]t
2
4
6
8
10
12
Time (s)

Fig. 5.9 Turnover of inorganic phosphate upon addition of FCCP to M.methylotrophus respiring methanol at 26°C

Washed cells (approx. 60mg dry weight) grown at 26°C were incubated for 2 minutes in 25ml 20mM glycylglycine + 140mM KCl pH 7.0 at 26°C prior to the introduction of methanol (10mM). The cells were allowed to respire methanol for approximately 10 seconds prior to the introduction of FCCP (10μM) at zero time. Samples were quenched in 25% (v/v) perchloric acid and assayed for inorganic phosphate as described in section 2.11 (a) Absolute concentration. (b) Changes in concentration. $\Delta[P_i]_o$ is the change in concentration of inorganic phosphate between zero time and complete de-energisation, and $\Delta[P_i]_t$ is the change in concentration between zero time and time t.

Table 5.4 The P_i/O quotient for methanol respiration by whole cells of *M.methylotrophus* at 26°C and 40°C

Temperature (°C)	P_i/O (nmol/ng-atom O)	Turnover, k (min^{-1})	Corrected P_i/O (nmol/ng-atom O)
26	0.31 ± 0.03 (5)	10.5 ± 1.3 (7)	0.50 ± 0.05 (5)
40	0.33 ± 0.05 (4)	9.7 ± 1.4 (4)	0.46 ± 0.05 (4)

P_i/O quotients were determined at 26°C and corrected for the turnover of inorganic phosphate as shown in Fig.s 5.11 and 5.12.

P_i/O quotients and turnover constants at 40°C were taken from Table 4.6.

(Fig. 5.9b).

Average values of the P_i/O quotient and the rate constant for phosphate turnover (k) of 0.31 ± 0.03 (5) nmol P_i/ng-atom O and 10.5 ± 1.3 (7) min^{-1} were obtained respectively, and yielded a P_i/O quotient corrected for turnover of 0.50 nmol P_i/ng-atom O (Table 5.4).

5.3 Discussion

A methanol-limited continuous culture of M.methylotrophus (D = 0.18 h^{-1}) growing in steady-state at different temperatures over the range 26°C to 40°C exhibited its highest cell densities at growth temperatures of 34°C or below. As the input concentration of methanol was constant, these results indicate that the growth yield with respect to methanol ($Y_{methanol}$) is highest at the lower temperatures, although the possibility that the yields are artificially depressed at the higher temperatures as a result of a certain amount of cell lysis cannot be completely ruled out.

During step up (26°C to 40°C) and step down (40°C to 26°C) experiments a lag period of approximately 3 hours was observed prior to the onset of significant changes in culture density. This lag period could not be accounted for by the time taken for the culture to change to the new temperature (less than 10 minutes) and is not easily explained. Interstingly, during the step down experiment (40°C to 26°C) the lag period was followed by a transient (3 hour) decline in cell density before the latter started to rise to its new, and higher, steady-state level. During the step-up experiment (26°C to 40°C) the lag period was followed by a similar, but steeper and more prolonged decline in culture density. Thus it appears that prior to adaptation to the new growth temperature there is always a period of decreased cell yield.

Methanol oxidase activities of cells grown at different

temperatures clearly decreased as a function of increasing growth temperature. Arrhenius plots indicated there was no significant difference in the temperature of inactivation of methanol oxidase in cells grown at 26°C or 40°C suggesting the decreased methanol oxidase activities were a genuine effect of the growth temperature rather than a spurious effect of the 40°C assay temperature.

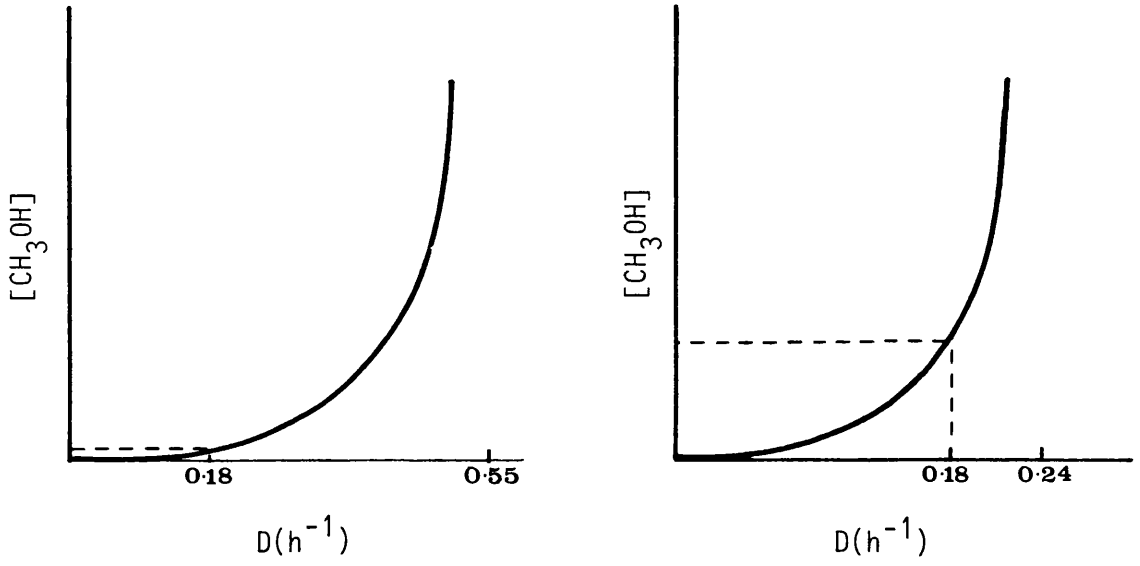
The concentration of c-type cytochromes was not significantly different in cells grown at 26°C or 40°C, and no change in methanol dehydrogenase activity or ascorbate-TMPD oxidase activity was observed. As the activities of the major components of the methanol oxidase system (methanol dehydrogenase and cytochrome c oxidase) appear to be unaffected by growth temperature, the decreased methanol oxidase activity at the higher growth temperatures could be due to an effect of the growth temperature on the interaction of methanol dehydrogenase with the terminal portion of the respiratory chain.

The stoichiometry of proton translocation during the oxidation of endogenous substrates was unaffected by the temperature of growth and assay. Following growth at 26°C a lower permeability to protons was observed, but both the P_i/O quotient and the rate constant for inorganic phosphate turnover were not significantly affected compared with growth at 40°C. Indeed, since the growth rate was maintained constant at the two growth temperatures ($D = 0.18\ h^{-1}$) it might reasonably be expected that the two types of cells utilised ATP at the same rate.

The growth of M.methylotrophus at 26°C and 40°C at a fixed growth rate in the chemostat has implications for the efficiency of utilisation of methanol in the culture medium. The growth rate μ is described by the equation

$$\mu = \frac{\mu_{max}\ [CH_3OH]}{K_m + [CH_3OH]}$$

If K_m is 20 μM, and μ_{max} is 0.55 h^{-1} at 40°C and 0.24 h^{-1} at 26°C, then at μ = 0.18 h^{-1} the concentration of methanol in the growth medium may be expected to be 9.7 μM and 60 μM at 40°C and 26°C respectively.



Alternatively, to maintain the steady-state methanol concentration at 9.7 μM at 26°C, the K_m would have to change to 3.2 μM ie. the K_m would have to be temperature dependent. Although there is no experimental information on this point, such a phenomenon is probably unlikely. The utilisation of methanol by the culture may therefore be less complete during growth at 26°C than at 40°C. Rapid analysis of filtered culture samples failed to reveal significant concentrations of methanol following growth at either 40°C or 26°C, but it should be noted that this procedure would be far too slow to prevent the further metabolism of very low standing concentrations of methanol. A more satisfactory

method of measuring the steady-state concentration of methanol in the culture would be to sample rapidly and directly into perchloric acid with subsequent analysis of the cell extract by gas-liquid chromatography; unfortunately this equipment was not available. The calculated concentrations of methanol in the cultures was too low to make an appreciable difference to the cell yield with respect to methanol.

The cell yield with respect to methanol may be calculated as 12.2 and 13.7 g/mol methanol at 40°C and 26°C respectively, thus decreasing the growth temperature from 40°C to 26°C increased the cell yield by approximately 11%. In order to explain this increase solely in terms of the P_i/O quotient, the latter would have to increase from 0.46 at 40°C to 0.51 at 26°C. A P_i/O quotient of 0.50 nmol P_i/ng-atom O was measured for cells grown at 26°C, suggesting the increased growth yield at 26°C was indeed due to an increased ATP/O quotient. However, such a conclusion should be regarded with caution as this increase in the P_i/O quotient of approximately 9% falls just within the boundaries of experimental error. Alternative explanations for the increased growth efficiency at 26°C should therefore be borne in mind. Thus the pathway of metabolism of the carbon and energy source can be temperature dependent, for example Lactobacillus brevis ferments glucose by the heterolactic pathway at 24°C whilst at 37°C fructose is used as a hydrogen acceptor with the formation of mannitol (DeLey, 1962). By analogy, it is possible that the growth temperature could affect the proportion of formaldehyde which passes through the assimilatory and dissimilatory pathways in M.methylotrophus.

Bacteria are able to turn over many of their macromolecular components such as RNA, protein and cell wall material. However, the rates of turnover are generally slow and probably unable to account for

more than a small portion of the energy dissipated in growth unassociated functions (DeBoer et al., 1981; Pine, 1972). An increase in the turnover rate at higher temperatures could consume more ATP and lead to lower growth yields; however, turnover of inorganic phosphate was not affected by temperature.

Measurement of the half-life of decay of a proton gradient across the cell membrane showed a higher permeability to protons at 40°C than at 26°C. It is possible therefore that the higher growth temperature also leads to an increased permeability to other ions. If this were so, it may be speculated that a lower cell yield at higher temperatures may be due to a higher energy requirement to maintain ionic gradients across the cell membrane.

CHAPTER 6

DISCUSSION

The results described and discussed in chapters 3, 4 and 5 raise several questions which may be worthy of further investigation.

The inability of whole cells of M.methylotrophus to oxidise NADH raises the possibility that the reported ability of H.parainfluenzae to oxidise exogenous NADH (White and Smith, 1964; White and Sinclair, 1971) may be a similar artifact due to the respiration of contaminating ethanol.

Oxygen electrode studies using M.methylotrophus have indicated that ethanol is oxidised to the level of acetic acid and that the latter is not further oxidised. Dawson (1982) attempted to use acetic acid as a probe for the measurement of the ΔpH component of the protonmotive force in this organism, but these studies were unsuccessful due to the failure to achieve complete recovery of the labelled acetic acid. This raises some interesting questions as to the fate of acetic acid in this methylotroph. The incorporation of labelled acetate into cell carbon has been observed in a number of obligate methanotrophs and methylotrophs when grown on methane or methanol supplemented with acetate (see Egli and Harder, 1984). An investigation of whether this occurs in M.methylotrophus, the mechanism of possible incorporation and the effect of the latter on growth yields may be of interest.

The M-protein reported by Anthony's group might play an important role in regulating methanol dehydrogenase and preventing the oxidation of formaldehyde by this enzyme (Ford et al., 1985; Page and Anthony, 1986). It would be interesting to determine to what extent the growth yield may be decreased due to the further oxidation of formaldehyde to formate by methanol dehydrogenase in a mutant which lacks the M-protein.

Growth of M.methylotrophus at 26°C and 40°C revealed differences in the proton permeability of the cell membrane of the two types of cells, indicating a physiological adaptation to the different growth temperatures. A study of the membrane lipid composition at the two growth temperatures may reveal the nature of such an adaptation, and it may be possible to correlate the latter with changes in membrane fluidity.

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