RESPIRATORY CHAIN ENERGY CONSERVATION IN THE METHYLOTROPHIC BACTERIUM *METHYLOPHILUS METHYLOTROPHUS*

This thesis was submitted for the degree of Doctor of Philosophy at the University of Leicester

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January 1982

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ABSTRACT

RESPIRATORY CHAIN ENERGY CONSERVATION IN THE METHYLOTROPHIC BACTERIUM

METHYLOPHILUS METHYLOTROPHUS

Michael J. Dawson

M.methylotrophus is an obligately aerobic, Gram negative methylotrophic bacterium which grows preferentially on methanol as the carbon and energy source, and uses the ribulose monophosphate pathway for carbon assimilation. This organism is used for single-cell protein production in the I.C.I. 'PRUTEEN' process. The composition and sequential organisation of the respiratory chain of M.methylotrophus have been studied, and both kinetic ($\rightarrow H^+/O$, $\rightarrow K^+/O$ quotients) and thermodynamic ($\Delta\bar{\mu}\ H^+$, ΔG_p) parameters of energy conservation have been determined. In addition, the effect of the growth conditions on some of these parameters has been investigated.

The respiratory chain of M.methylotrophus was found to branch at the level of cytochrome c to two terminal oxidases, cytochromes aa_3 and o. Methanol is oxidised via a methanol dehydrogenase which donates reducing equivalents to the respiratory chain at the level of cytochrome c, as in other methylotrophs. Proton and charge translocation stoicheiometries indicate the presence of three energy conserving sites between NADH and oxygen, each of which translocates two charges; only the third coupling site, which appears to function by a redox arm mechanism, is involved in respiration from methanol.

M.methylotrophus was found to sustain a ΔG_p, during respiration from methanol, of approximately -45 kJ/mol, but the $\Delta\bar{\mu}H^+$ varied with the reaction conditions such that apparent values of the $\rightarrow H^+/ATP$ quotient ranging from 2.6 to 4.1 g-ion H^+/mol ATP were obtained. It was concluded that the proton current, in this organism, is at least partially localised, and theoretical growth calculations suggest that the true value of the $\rightarrow H^+/ATP$ quotient is probably 2 g-ion H^+/mol ATP. On this basis, the ATP/O quotients for respiration from NADH and methanol are likely to be 3 and 1 mol ATP/g-atom O, respectively.

There was no evidence that the low growth yields of methanol-excess cultures could be explained by a reduced efficiency of respiratory chain energy conservation.

ACKNOWLEDGEMENTS

Above all I would like to thank my supervisor, Dr Colin W. Jones, for his invaluable help and advice throughout the course of this work. Thanks are also due to Prof. William J. Brammar for extending to me the facilities of the Biochemistry Department at Leicester, to my colleagues in the laboratory, Dr Alan M. McKay, Miss Jacquie A. Quilter, Mrs Eleanor Chicken, Miss Sarah E. Cooke and Mr Steven J. Farrand, for their friendship and encouragement, to Mrs Sheila Mackley for typing this thesis, and to the S.R.C. for the provision of a C.A.S.E. award. I am much indebted to I.C.I. for financial support, and to the staff of the protein group at I.C.I. Agricultural Division, particularly Dr Glyn M. Tonge, for their willing help and advice. Last but not least, I am very grateful to my parents and friends for their patient support and encouragement.

DECLARATION

This thesis, submitted for the degree of Doctor of Philosophy, entitled 'Respiratory Chain Energy Conservation in the Methylotrophic Bacterium *Methylophilus methylotrophus*' is based on work conducted by the author in the Department of Biochemistry of the University of Leicester mainly during the period between October 1978 and October 1981.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other university.

CONTENTS

Acknowledgements

Declaration

CHAPTER ONE - INTRODUCTION p.1

CHAPTER TWO - MATERIALS AND METHODS p.61

CHAPTER THREE - RESPIRATORY CHAIN OF METHANOL-LIMITED METHYLOPHILUS METHYLOTROPHUS p.92

CHAPTER FOUR - RESPIRATION-LINKED PROTON TRANSLOCATION IN METHANOL-LIMITED METHYLOPHILUS METHYLOTROPHUS p.121

CHAPTER FIVE - RESPIRATORY CHAIN COMPOSITION AND ENERGY CONSERVATION IN OXYGEN- AND AMMONIUM-LIMITED METHYLOPHILUS METHYLOTROPHUS p.168

CHAPTER SIX - MAGNITUDES OF THE PROTONMOTIVE FORCE ($\Delta\mu H^+$) AND PHOSPHORYLATION POTENTIAL (ΔG_p) IN METHANOL-LIMITED METHYLOPHILUS METHYLOTROPHUS p.198

CHAPTER SEVEN - SUMMARY AND DISCUSSION p.244

References p.261

CHAPTER 1

INTRODUCTION

1.1 Microbiology of the methylotrophs

1.1.1 Obligate methylotrophs

1.1.2 Facultative methylotrophs

1.2 Carbon assimilation pathways of the methylotrophs

1.2.1 Ribulose monophosphate (RMP) pathway

1.2.2 Serine pathway

1.2.3 Relative energetics of the C_1 assimilation pathways

1.3 Carbon dissimilation pathways of the methylotrophs

1.3.1 Methane oxidation

1.3.2 Methanol oxidation

1.3.3 Formaldehyde oxidation

1.3.4 Formate oxidation

1.4 Redox centres of the aerobic respiratory chain

1.5 Mechanism of respiratory chain energy conservation

1.5.1 Chemiosmotic hypothesis

1.5.2 Localised Proton hypothesis

1.6 Mechanism of respiration-linked proton translocation

1.7 Measurement of the stoicheiometries of respiration-linked proton and charge translocation

1.7.1 Oxygen-pulse method

1.7.2 Initial-rate method

1.7.3 Steady-state method

1.8 Variability of bacterial respiratory chains

1.8.1 Species variability

1.8.2 Phenotypic and genotypic variability

1.9 Respiratory chain composition and respiration-linked proton translocation in bacteria

1.9.1 Site 0, nicotinamide nucleotide transhydrogenase

1.9.2 Site I, NADH:quinone oxidoreductase

1.9.3 Site II, quinol:cytochrome c oxidoreductase; quinol oxidase

1.9.4 Site III, cytochrome c oxidase

1.10 ATP synthesis in bacteria

1.10.1 Direct measurement of ATP/0 quotients

1.10.2 ATP/0 quotients from a comparison of the $\rightarrow H^+/0$ and $\rightarrow H^+/ATP$ quotients

1.10.3 ATP/0 quotients from growth studies

1.11 Single-cell protein (SCP)

1.11.1 Choice of substrate for SCP production

1.11.2 Choice of organism for SCP production

1.11.3 I.C.I. SCP process

1.12 Objectives

CHAPTER 1

INTRODUCTION

The intensity of research into all aspects of methylotrophy has escalated in the last decade, largely due to interest in the commercial exploitation of methylotrophic microorganisms. A number of industrial single-cell protein production processes - using both methylotrophic bacteria and yeasts - are now in full commercial operation, and several others have been developed to the pilot-plant stage. Furthermore, as well as for single-cell protein production, the methylotrophs are potentially useful for the catalysis of chemical interconversions (particularly by exploiting the loose specificity of methane monooxygenase), for the production of various cell components such as poly-β-hydroxybutyrate (Yanchinski, 1981) and ubiquinone-10 (Urakami, 1981), and as hosts for the expression of eukaryotic genes (Brammar, 1981).

The fundamental study of the microbiology, biochemistry, growth physiology and more recently genetics, of the methylotrophs has kept pace admirably with the technological advances. Indeed, largely due to the work of Quayle and his colleagues at Sheffield, the basic pathways of assimilation of C1-compounds are now quite well understood. Furthermore, the catabolic sequences of methylotrophic bacteria have been successfully investigated, and a number of novel and interesting features have been brought to light. In contrast, respiratory chain energy conservation in the methylotrophs has been the subject of a comparatively few recent investigations, and several questions remain to be answered in this sphere.

This thesis describes an investigation of respiratory chain energy conservation in Methylophilus methylotrophus - an obligately aerobic, Gram negative methylotrophic bacterium which uses methanol as its preferred carbon and energy source, and which utilises the ribulose monophosphate pathway for carbon assimilation. A study of this bacterium is particularly apposite as it is the organism used by I.C.I. Agricultural Division at Billingham, Cleveland for the large-scale commercial production of single-cell protein.

1.1 Microbiology of the methylotrophs

Colby & Zatman (1972) defined the methylotrophs as those organisms which are able to grow non-autotrophically at the expense of carbon compounds containing one or more carbon atoms, but containing no carbon-carbon bonds. The range of microorganisms which are capable of utilising such compounds as their carbon and energy source includes yeasts as well as bacteria, but the following discussion is confined to the latter. The above definition would exclude those organisms such as Paracoccus denitrificans (Cox & Quayle, 1975), Rhodopseudomonas acidophila (Sahm et al., 1976) and Pseudomonas oxalaticus (Quayle & Keech, 1959), which oxidise reduced C1 compounds to carbon dioxide before assimilation (the 'pseudo-methylotrophs'; Zatman, 1981). The relationship between methylotrophy and autotrophy, however, is a very close one, and some authors would group together all organisms which must synthesise all their carbon-carbon bonds de novo as autotrophs (see Whittenbury & Kelly, 1977).

The range of C1 substrates which will support the growth of methylotrophs includes methane, methanol, methylamine, formate, formamide, carbon

monoxide, dimethyl ether, dimethylamine, trimethylamine, trimethylamine N-oxide, and various tetramethylammonium and trimethylsulphonium salts (see Quayle, 1972; Anthony, 1975b; Colby et al., 1979; Wolfe & Higgins, 1979). Methylotrophic bacteria may usefully be divided into two groups - obligate methylotrophs, which are able to grow only on the above substrates, and facultative methylotrophs, which can also utilise other organic compounds for growth. Moreover, this division can be justified taxonomically as these two groups differ in terms of their morphology, lipid content, DNA base composition, and response to biochemical tests (see Byrom, 1981).

1.1.1 Obligate methylotrophs

This group is generally taken to include those organisms, such as M.methylotrophus (Byrom & Ousby, 1975), which can grow albeit slowly on a very limited range of heterotrophic substrates, as well as the truly obligate methylotrophs. All 'obligate' methylotrophs isolated so far are Gram negative, obligate aerobes. The largest group of obligate methylotrophs, the methane-utilisers (methanotrophs), has been further divided by Whittenbury and colleagues into two groups largely on the basis of internal membrane structure and carbon assimilation pathway (see Colby et al., 1979). The Type I methanotrophs assimilate carbon via the ribulose monophosphate pathway, and have their internal membranes arranged as bundles of vesicular discs, whereas the Type II methanotrophs utilise the serine pathway for carbon assimilation, and have paired membranes around the cell periphery.

Those obligate methylotrophs which do not grow on methane have been classified into a large number of genera (e.g. Pseudomonas, Methylomonas,

Methylophilus, Achromobacter), but a more useful classification is made difficult by the general inertness of these organisms to classical taxonomic tests (see Byrom, 1981). These organisms do not have complex internal membrane structures (Rokem et al., 1978), and they all appear to use the ribulose monophosphate pathway for carbon assimilation (see Zatman, 1981).

1.1.2. Facultative methylotrophs

A small number of facultative methanotrophs have recently been isolated (Patt et al., 1974; Patel et al., 1978), the most studied example being Methylobacterium organophilum which utilises the serine pathway for carbon assimilation. There is some evidence that the ability of this organism to utilise methane is conferred by a plasmid (O'Connor, 1981).

In contrast, there is a great deal of diversity amongst the non-methanotrophic facultative methylotrophs, and a large number of genera (e.g. Pseudomonas, Bacillus, Hyphomicrobium) are represented. These organisms utilise either the serine pathway or the ribulose monophosphate pathway for carbon assimilation.

1.2 Carbon assimilation pathways of the methylotrophs

The methylotrophs, like the autotrophs, have special biochemical pathways for the synthesis of C3 compounds from their C1 substrates. Thereafter, however, their intermediary metabolism appears to involve essentially similar pathways to that of the heterotrophs.

Those bacteria which are able to grow on reduced C1 substrates utilise one (or possibly more; see Colby et al., 1979; Whittenbury, 1981) of three basic pathways for the conversion of C1 compounds to C3 compounds. The 'true' methylotrophs (sensu Colby & Zatman, 1972) assimilate carbon at the

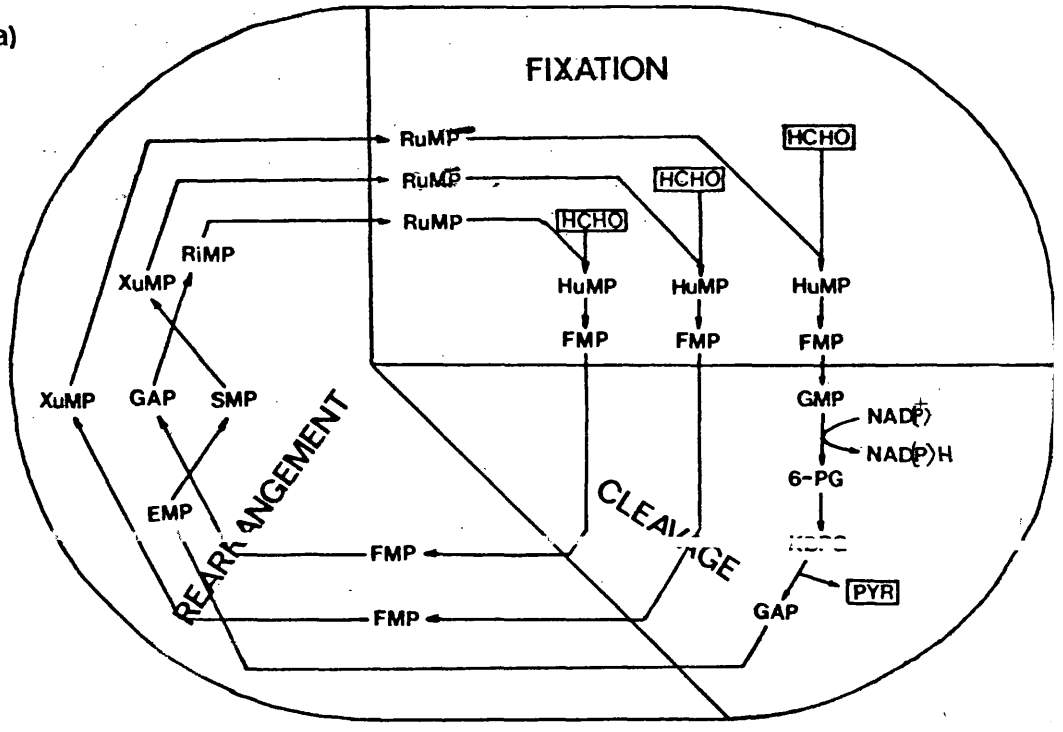
level of formaldehyde using either the ribulose monophosphate (RMP) pathway or the serine pathway, whereas the 'pseudo-methylotrophs' (Zatman, 1981) assimilate carbon at the level of carbon dioxide via the ribulose diphosphate (RDP) pathway. Each of these pathways is characterised by one or more key enzymes which are not involved in either of the alternative pathways, or elsewhere in intermediary metabolism. The RMP and serine pathways are discussed below, but the reader is referred to the reviews of McFadden (1973) and Anthony (1975b) for further consideration of the RDP pathway.

1.2.1 Ribulose monophosphate (RMP) pathway

The first key enzyme of the RMP pathway, hexulose phosphate synthase, catalyses the condensation of formaldehyde with ribulose-5-phosphate to yield D-arabino-3-hexulose-6-phosphate, and this compound is then isomerised to fructose-6-phosphate via the second key enzyme, hexulose phosphate isomerase. The remainder of the cycle functions to regenerate the C1 acceptor, ribulose-5-phosphate, and overall one molecule of triose phosphate, or pyruvate, is formed from three molecules of formaldehyde (Fig. 1.1ab).

Variants of the RMP pathway occur both in the mode of cleavage of the fructose-6-phosphate and at the level of the sugar phosphate rearrangements. Fructose-6-phosphate may be cleaved either via the glycolytic pathway (fdp^+) or via the Entner-Doudoroff pathway (edd^+); and the rearrangement of sugar phosphates can also occur by one of two pathways involving either transaldolase (ta^+) or sedoheptulose diphosphatase ($sdhp^+$). Of the four possible combinations of these variants, only two, edd^+ ta^+ (Fig. 1.1a) and fdp^+ $sdhp^+$ (Fig. 1.1b), seem to be

(a)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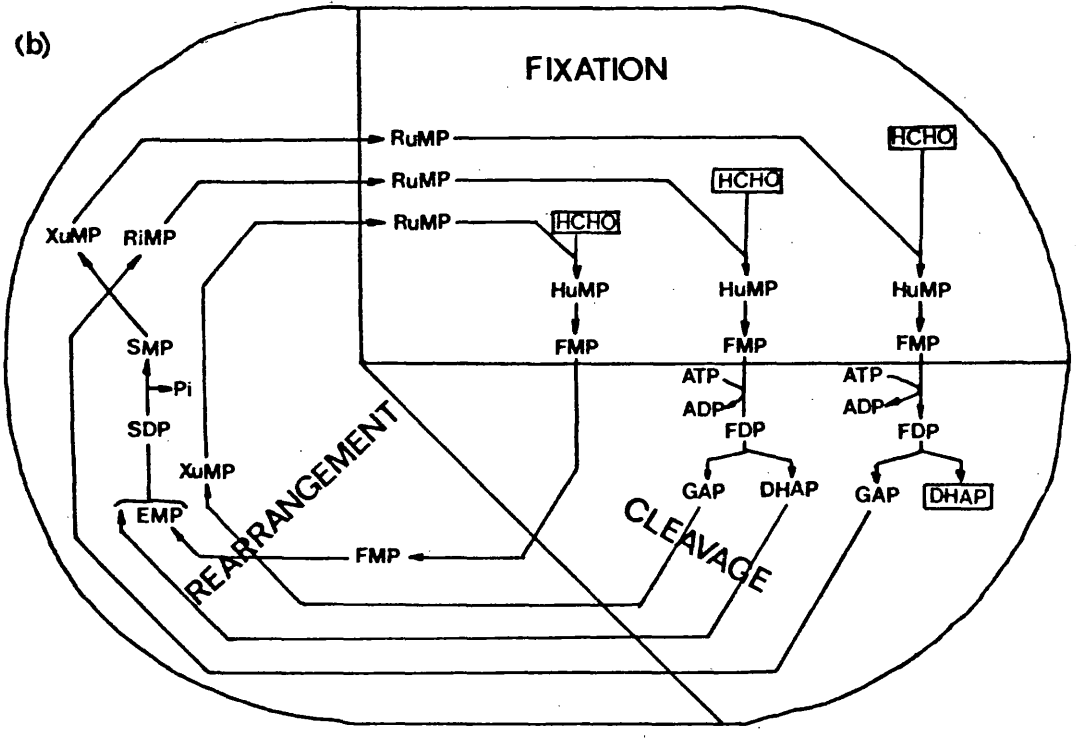
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XuMP
GAP
DHAP
GAP
DHAP
EMP
REARRANGEMENT
CLEAVAGE
FMP

Fig. 1.1 Ribulose monophosphate pathway. (a) edd^+ta^+ variant; (b) fdp^+sdhp^+ variant, after Quayle & Ferenci (1978).

Abbreviations: HuMP, D-arabino-3-hexulose-6-phosphate; FMP, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; GMP, glucose-6-phosphate; 6-PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; PYR, pyruvate; EMP, erythrose-4-phosphate; SMP, sedoheptulose-7-phosphate; SDP, sedoheptulose-1,7-diphosphate; XuMP, xylulose-5-phosphate; RiMP, ribose-5-phosphate; RuMP, ribulose-5-phosphate. H^+ and H_2O have been omitted for simplicity.

physiologically relevant. The former is found primarily in the obligate methylotrophs - including M.methylotrophus (Taylor, 1977; Beardsmore & Quayle, 1978) - and the latter is prevalent in the facultative methylotrophs (Zatman, 1981). The non-occurrence of the edd^+ $sdhp^+$ variant is not surprising as it would be energetically inefficient, but the hypothetical fdp^+ ta^+ variant is potentially the most efficient of all the C1-assimilation pathways (see Table 1.1), and the lack of methylotrophs using this variant of the pathway is puzzling.

Although the RMP pathway has been depicted here as yielding net synthesis of either pyruvate (edd^+ ta^+), or triose phosphate (fdp^+ $sdhp^+$), pathways must also exist for the synthesis of C4, C5 and C6 skeletons as precursors of cell wall compounds, amino acids, nucleic acids and polysaccharides. These precursors may be produced both by the recycling of triose phosphate into the RMP pathway (see Quayle & Ferenci, 1978), and via the tricarboxylic acid (TCA) cycle which is incomplete in the RMP-pathway methylotrophs, and used only for biosynthetic purposes. When the latter route is used, the TCA cycle intermediates must be replenished via the anaplerotic pathways, and net carbon dioxide fixation thus occurs at this level (Loginova & Trotsenko, 1979; Romanovskaya et al., 1980).

1.2.2 Serine pathway

In contrast to the RMP pathway, formaldehyde is incorporated into the serine pathway as N-^{5,10}-methylene-tetrahydrofolate, and one molecule of carbon dioxide is also incorporated for every two molecules of formaldehyde fixed (Fig. 1.2). Two variants of the serine pathway occur with respect to the conversion of acetyl-CoA to glyoxylate. In bacteria containing isocitrate lyase (icl^+), this conversion may be catalysed by the enzymes

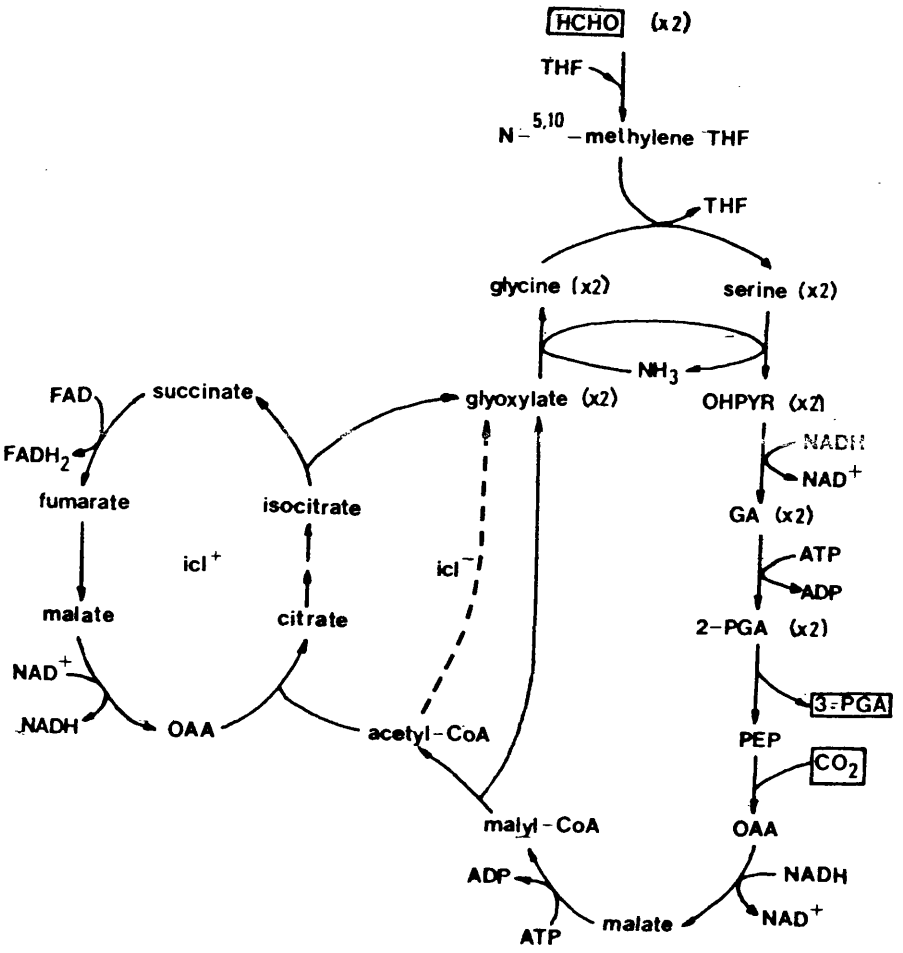


Fig. 1.2 Serine pathway. After Colby et al. (1979). Abbreviations: THF, tetrahydrofolate; OHPYR, hydroxypyruvate; GA, glycerate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolypyruvate; OAA, oxaloacetate. H^+, H_2O and HS-CoA have been omitted for simplicity.

of the TCA and glyoxylate cycles (Fig. 1.2), but when isocitrate lyase is absent (icl^-), an alternative pathway for the regeneration of glyoxylate must operate. The homoisocitrate lyase pathway has recently been proposed to fulfil this role in bacteria containing the icl^- serine pathway (Korstee, 1980, 1981).

1.2.3 Relative energetics of the C1 assimilation pathways

The energy and reductant budgets of the various C1- assimilation pathways, normalised for pyruvate production, are shown in Table 1.1. For a fair comparison of these pathways, it is also necessary to normalise the reactant to formaldehyde. In doing this it has been assumed that the oxidation of formaldehyde to carbon dioxide yields two molecules of NAD(P)H, and that NADH and NAD(P)H are energetically equivalent.

Taking into account the above assumptions, it is clear that the relative bioenergetic efficiencies of the C1 assimilation pathways are in the order RMP pathway > serine pathway > RDP pathway, and furthermore these theoretical predictions are borne out in practice (e.g. Goldberg et al., 1976; Goldberg, 1977). As relatively little reductant is formed during the dissimilation of methane (which may be oxidised by an NAD(P)H-requiring monooxygenase), or during that of highly oxidised substrates such as formate, growth on these substrates may well be limited by the NAD(P)H supply, as well as by the ATP supply (Anthony, 1978). This is particularly true if the serine pathway is used for carbon assimilation, or if the oxidation of formaldehyde to carbon dioxide yields less than two molecules of NAD(P)H. The fixation of carbon dioxide via the anaplerotic pathways will also be an extra burden on the reductant supply, unless the two molecules of NAD(P)H required for carbon dioxide fixation are produced during the oxidation of formaldehyde to carbon dioxide.

Pathway	Variant	Reactants	Product	Energy/reductant change		
				ΔNAD(P)H	ΔFPH_2	ΔATP
RMP	* fdp^+ta^+	3HCHO	Pyruvate	+1	0	+1
	fdp^+sdhp^+			+1	0	0
	edd^+ta^+			+1	0	0
	* edd^+sdhp^+			+1	0	-3
Serine	icl^+	2HCHO + CO_2 (3HCHO)		-2(0)	+1	-2
RDP	$sdhp^+$	$3CO_2$ (3HCHO)		-5(+1)	0	-7

Table 1.1 Energy and reductant budgets for the C_1-assimilation pathways, normalised for pyruvate production.

Adapted from Quayle & Ferenci (1978). The figures in parenthesis have also been normalised for HCHO as reactant. The assumptions made are discussed in the text. *Denotes a hypothetical variant.

Detailed theoretical studies of the predicted growth yields of methylotrophs have been made by van Dijken & Harder (1975) and by Anthony (1978).

1.3 Carbon dissimilation pathways of the methylotrophs

1.3.1 Methane oxidation

The enzymology of methane oxidation has been studied in some detail (see Wolfe & Higgins, 1979; Colby *et al.*, 1979; Dalton, 1981; Higgins *et al.*, 1981) but only a few brief comments will be included here. Methane is oxidised via a broad specificity monooxygenase of which there would appear to be two basic types: one type is particulate and uses both NADH and ascorbate as electron donors, whereas the other type is soluble and can use only NADH as an electron donor. Despite the apparently reductant-consuming monooxygenase reaction, the molar growth yields of bacteria grown on methane and methanol appear to be similar (Linton & Vokes, 1978), and it seems likely, therefore, that this highly exothermic reaction is linked, in some unknown way, to energy conservation.

1.3.2 Methanol oxidation

In methylotrophic bacteria (including *M.methylotrophus*; Ghosh & Quayle, 1978), methanol is oxidised via a soluble, or readily-solubilised, dehydrogenase which was first described by Anthony & Zatman (1964). The methanol dehydrogenases which have been purified so far have a subunit size of 60,000-75,000D, and are either monomers or dimers (Bamforth & Quayle, 1978; Colby *et al.*, 1979). Methanol dehydrogenase is $NAD(P)^+$-

independent, and has optimum activity when linked to PMS as an artificial electron acceptor. The purified enzyme has a broad substrate specificity, which includes the product formaldehyde (the structure of which in the hydrated form resembles that of methanol; Sperl et al., 1974), and a high pH optimum for activity. The K_M for methanol is very low - typically less than 10μM. In extracts, methanol dehydrogenase requires ammonium or primary amines for activity, but in vivo, or after purification under anaerobic conditions (Duine et al., 1979a), there is no such requirement.

The prosthetic group of methanol dehydrogenase was originally thought to be a pteridine (Anthony & Zatman, 1967), however it has now been purified and shown to be a nitrogen-containing orthoquinone (Salisbury et al., 1979; Duine & Frank, 1980; Duine et al., 1980), the structure of which is shown in Fig. 1.3. This coenzyme has been given the trivial name

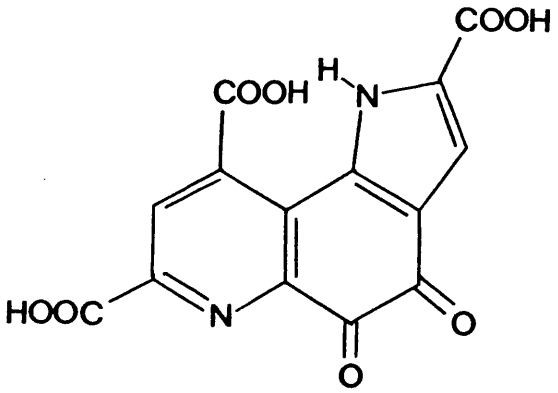


Fig. 1.3 The structure of the methanol dehydrogenase coenzyme (PQQ or methoxatin)

'methoxatin' (Salisbury et al., 1979), or the semi-systematic name 'pyrrolo-quinoline quinone' (PQQ; Duine et al., 1980); and interestingly its presence has now been demonstrated in a wide range of dehydrogenases (many from non-methylotrophs) which have been collectively termed 'quinoproteins' (Duine et al., 1979b; Duine & Frank, 1981a; Ameyama et al., 1981).

The redox potential ($E_{m,7}$) of the $PQQ/PQQH_2$ couple is +120mV (Duine & Frank, 1981b) which is consistent with the proposed coupling of methanol dehydrogenase to the respiratory chain at the level of cytochrome c (see section 3.1). This coupling, however, is very readily destroyed (probably by oxygen) even under mild cell breakage conditions, and functional coupling of methanol oxidation to cytochrome c reduction has only been demonstrated in enzyme preparations made under anaerobic conditions (Duine et al., 1979a).

The reaction mechanism of methanol dehydrogenase appears to be very complex. Several interesting model reactions between PQQ and various substrates have been demonstrated but - beyond the fact that each approximately 120,000 MW unit contains two molecules of PQQ which may shuttle between either the redox - forms $2PQQ/2PQQ^{\bullet}$ or $2PQQ^{\bullet}/2PQQH_2$ - little is known of the in vivo reaction mechanism (Duine & Frank, 1981b). Indeed, in view of the substantial differences in properties of the in vivo and in vitro forms of the enzyme, study of the latter may be of limited value.

A mechanism for the reduction of cytochrome c by methanol dehydrogenase, involving the autoreduction properties of the cytochrome, has been proposed by O'Keefe & Anthony (1980a).

1.3.3 Formaldehyde oxidation

The complete oxidation of formaldehyde to carbon dioxide, in methylotrophic bacteria, may occur via one or more of four possible routes:

(1) Dissimilatory RMP cycle.

(2) Linear oxidation via formate.

(3) Linear oxidation via tetrahydrofolate derivatives.

(4) Serine pathway plus the TCA cycle.

(1) Dissimilatory RMP cycle

The reactions of the dissimilatory RMP cycle are shown in Fig. 1.4. This cycle involves the key enzyme 6-phosphogluconate dehydrogenase in addition to the enzymes of the RMP pathway — two molecules of NAD(P)H are formed during the complete oxidation of formaldehyde to carbon dioxide. This appears to be the major route of formaldehyde oxidation in the non-methane-utilising RMP-pathway methylotrophs (Zatman, 1981).

(2) Linear oxidation via formate

At least seven types of enzyme capable of oxidising formaldehyde to formate have been identified in methylotrophic bacteria (see Stirling & Dalton, 1978; Higgins, 1980). These enzymes fall into three basic groups:

(i) Methanol dehydrogenase. As mentioned in section 1.3.2, methanol dehydrogenase will readily oxidise hydrated formaldehyde *in vitro*; however, there is as yet no clear evidence for formaldehyde oxidation catalysed by methanol dehydrogenase *in vivo* (see Heptinstall & Quayle, 1970).

(ii) Dye-linked aldehyde dehydrogenase. Only low specific activities of the dye-linked aldehyde dehydrogenase are measured in methylotrophs using artificial electron acceptors (e.g. 2,6-dichlorophenolindophenol [DCPIP]), and furthermore, Marison & Attwood (1980) have shown that this enzyme is not usually induced during the growth of facultative methylotrophs on C1 compounds. These authors also found that formaldehyde is not as good a

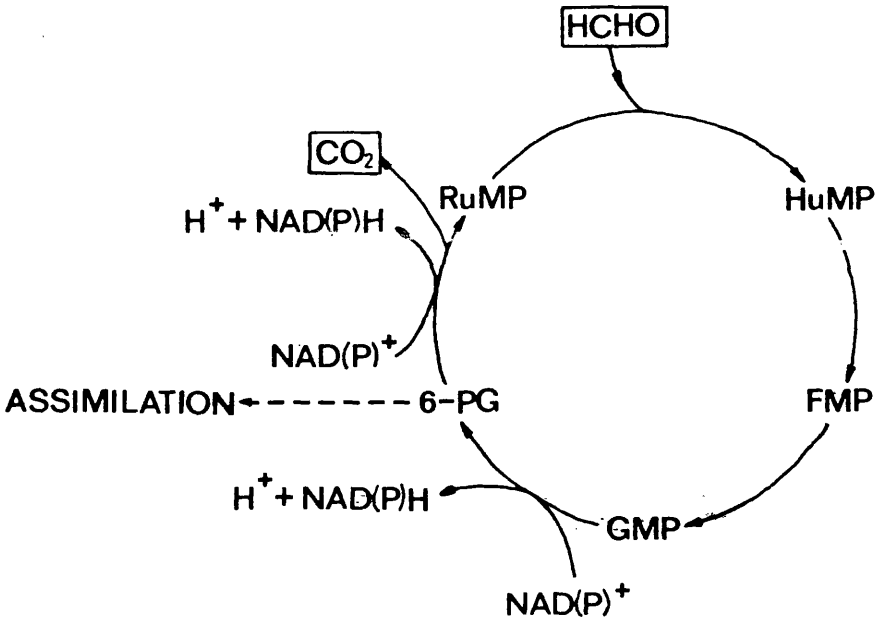


Fig. 1.4 Dissimilatory RMP cycle. After Quayle & Ferenci (1978). Abbreviations: HuMP, D-arabino-3-hexulose-6-phosphate; FMP, fructose-6-phosphate; GMP, glucose-6-phosphate; 6-PG, 6-phosphogluconate; RuMP, ribulose-5-phosphate. The dashed line shows the removal of 6-PG for assimilation purposes.

substrate for the purified enzyme as are higher aldehydes, and they thus concluded that the dye-linked aldehyde dehydrogenase is unlikely to play a major role in the oxidation of formaldehyde during the dissimilation of C1 compounds.

(iii) $NAD(P)^+$-linked formaldehyde dehydrogenase. At least four types of $NAD(P)^+$-linked formaldehyde dehydrogenase have been reported in the methylotrophs. These enzymes differ in terms of their requirement for activators - some require glutathione, some require tetrahydrofolate, and others require no activator - and in their ability to oxidise higher aldehydes (see Stirling & Dalton, 1978).

(3) Linear oxidation via tetrahydrofolate derivatives

Johnson & Quayle (1964) suggested that, in serine-pathway methylotrophs, the linear oxidation of formaldehyde to carbon dioxide might occur via tetrahydrofolate derivatives. The presence of enzymes capable of catalysing this pathway has been demonstrated in methanol-grown Pseudomonas AM1 by Large & Quayle (1963).

(4) Serine pathway plus the TCA cycle

Newaz & Hersh (1975) suggested that the complete oxidation of formaldehyde to carbon dioxide could be brought about by a combination of the serine-pathway and the TCA cycle. Many serine-pathway methylotrophs do indeed have a complete TCA cycle, and evidence for a dissimilatory role has been obtained in Pseudomonas MA (Newaz & Hersh, 1975).

It should be noted that the bioenergetic efficiencies of the different routes for formaldehyde oxidation proposed above vary in terms of the production of NAD(P)H and ATP (see Table 1.2). The relative significance *in vivo* of these various routes is difficult to assess, as most methylotrophs have at least two possible routes for formaldehyde oxidation. Indeed,

Formaldehyde oxidation route	Reactant	Product	Energy/reductant change		
			ΔNAD(P)H	Δother reductant	ΔATP
Dissimilatory RMP cycle	HCHO	CO_2	+2	0	0
Methanol dehydrogenase	HCHO	HCOOH (CO_2)	0 (+1)	+1 $PQQH_2$	0
Dye-linked aldehyde dehydrogenase	HCHO	HCOOH (CO_2)	0 (+1)	+1 unknown	0
$NAD(P)^+$-linked formaldehyde dehydrogenase	HCHO	HCOOH (CO_2)	+1 (+2)	0	0
THF-linked pathway	HCHO	HCOOH (CO_2)	+1 (+2)	0	+1
Serine pathway +TCA cycle	HCHO	CO_2	+1	+1 $FADH_2$	0

Table 1.2 Relative energetics of the various routes for formaldehyde oxidation. The figures in parentheses are normalised for carbon dioxide production, assuming that formate dehydrogenase is $NAD(P)^+$-linked.

methanol-grown M.methylotrophus contains the enzymes of the dissimilatory RMP cycle (Taylor, 1977; Beardsmore & Quayle, 1978), plus low levels of $NAD(P)^+$-linked formaldehyde dehydrogenase (Large & Haywood, 1981; but see Taylor, 1977) in addition, of course, to methanol dehydrogenase.

1.3.4 Formate oxidation

Formate is oxidised in all methylotrophs studied so far (including M.methylotrophus; Taylor, 1977) by a soluble NAD^+-linked formate dehydrogenase (see Johnson & Quayle, 1964).

1.4 Redox centres of the aerobic respiratory chain

The aerobic respiratory chain is a membrane-bound complex of respiratory carriers-situated in the mitochondrial inner membrane or the cytoplasmic membrane of bacteria - which catalyses the energy-conserving oxidation of reduced substrates by oxygen. As a diverse range of substrates may be oxidised, there is extensive branching at the level of the primary dehydrogenases. Many substrates (e.g. malate, isocitrate) are oxidised via soluble $NAD(P)^+$-linked enzymes, and the reduced coenzyme is then reoxidised via the membrane-bound NADH dehydrogenase (in conjunction with nicotinamide nucleotide transhydrogenase in the case of NADPH). Other substrates (e.g. succinate, methanol), however, are oxidised via dehydrogenases which interact directly with the respiratory chain.

The dehydrogenases, and other components responsible for the transfer of reducing equivalents (e.g. quinones, cytochromes), and reduction of molecular oxygen (cytochrome oxidases), each contain one or more redox centres. These redox centres are basically of two types i.e. either metallic (iron-sulphur centres, haem), or organic (flavins, quinones).

The metallic and organic centres generally carry reducing equivalents as electrons and hydrogen atoms, respectively, and this has important consequences for the mechanism of energy conservation.

The iron-sulphur centres (see Lovenberg, 1973, 1977) and cytochromes (see Lemberg & Barrett, 1973) both act as one electron carriers and have the property of being able to operate over a wide range of redox potentials. This variation in redox potential depends both on the nature of the redox centre and on the protein environment.

The flavins may carry up to two reducing equivalents (2H), though they probably only carry one equivalent in vivo by oscillating between the oxidised and semiquinone, or the semiquinone and reduced forms. The E_o' values of the free $FMN/FMNH_2$ and $FAD/FADH_2$ couples are -205 mV and -219 mV respectively, but, like the iron-sulphur centres and cytochromes, the flavins are known to operate over a wide range of potentials in vivo (see Hatefi & Stiggal, 1976).

In contrast, the quinones, which are lipophilic hydrogen (2H) carriers, only operate over a narrow range of redox potentials around zero (ubiquinone, +100 mV; menaquinone, -74 mV). The reduction of the quinone to quinol probably occurs via the semiquinone (see Gutman, 1980).

Fig. 1.5 shows the current state of knowledge of the sequential organisation of the more well-characterised redox centres of the mitochondrial respiratory chain. This aspect of the bacterial respiratory chain has been studied in considerably less detail, but many of the features of the mitochondrial respiratory chain appear to be also present in bacteria.

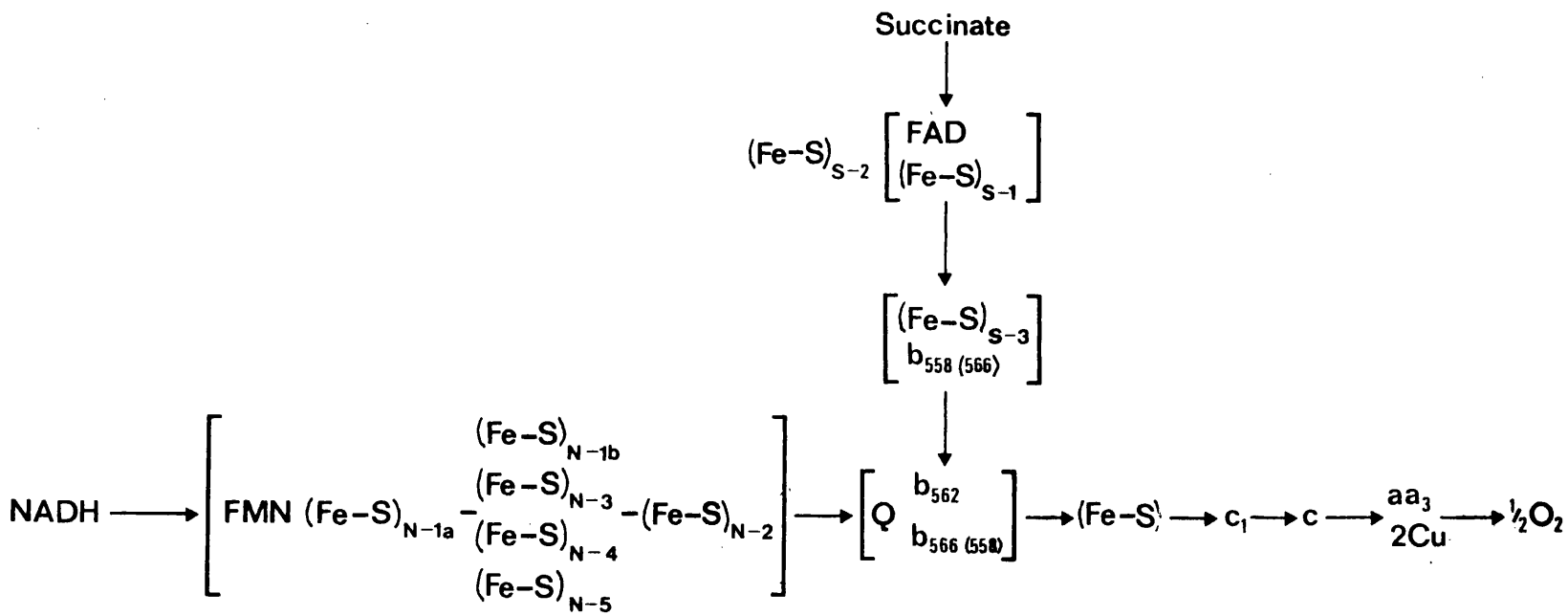


Fig. 1.5 Sequential organisation of redox centres in the mitochondrial respiratory chain

1.5 Mechanism of respiratory chain energy conservation

Early attempts to understand the mechanism of oxidative phosphorylation were based upon the well-understood mechanism of substrate-level phosphorylation, in which oxidation-reduction leads to the formation of ATP via the sequential production of non-phosphorylated and phosphorylated intermediates. Slater (1953) proposed the Chemical hypothesis of oxidative phosphorylation, and thus initiated an intensive effort to isolate and identify modified respiratory carriers. It was the repeated failure to find these postulated intermediates which prompted the proposal of other mechanisms of energy conservation which took more account of the membrane-located nature of the respiratory chain.

One such proposal, the Conformational hypothesis of Boyer (1965), envisaged that respiration would generate protein conformational changes, and this conformational energy would be transmitted to the ATP phosphohydrolase either directly by protein-protein interaction, or indirectly via the formation of covalent intermediates. However, although conformational changes of proteins clearly occur during the transfer of reducing equivalents down the respiratory chain, and are very probably important in energy conservation, the transmission of energy from respiration to ATP synthesis by direct protein-protein interaction is not supportable by current evidence on the structural organisation of the respiratory chain (Boyer _et al._, 1977).

Mitchell (1961) and Williams (1961) independently proposed that the principal coupling intermediate between respiration and ATP synthesis is the 'energised' proton. This has now become the view of the majority of workers in this field (e.g. Boyer _et al._, 1977).

1.5.1 Chemiosmotic hypothesis

The value of this hypothesis, which was proposed and developed by Mitchell (1961, 1966, 1968), is that it makes four essential postulates which are experimentally testable:

(1) The membrane-located respiratory chain reversibly couples the flow of reducing equivalents to the translocation of protons across the coupling membrane.

(2) The membrane-located ATP phosphohydrolase reversibly couples ATP synthesis to the translocation of protons across the coupling membrane.

(3) Substrate-specific exchange-diffusion carrier systems are located in the coupling membrane.

(4) The coupling membrane has a low permeability to protons and other ions.

In both mitochondria and bacteria, the flow of reducing equivalents down the respiratory chain is envisaged to be linked to outward proton translocation, thus setting up a transmembrane proton gradient - or more accurately an electrochemical potential difference of protons - between the inner and outer bulk aqueous phases. This electrochemical potential difference ($\Delta\bar{\mu}H^+$) has both electrical ($\Delta\Psi$) and chemical (ΔpH) components which may be summed as in the following equation:

$$\Delta\bar{\mu}H^+_{(in-out)} = \Delta\Psi_{(in-out)} - 2.303 \frac{RT}{F} \Delta pH_{(in-out)} \qquad (1.1)$$

The ΔpH and $\Delta\Psi$ components are theoretically interchangeable, and their relative contributions to the $\Delta\bar{\mu}H^+$ will depend on the conditions.

The $\Delta\bar{\mu}H^+$ exerts a protonmotive force which may be used to drive ATP synthesis, substrate transport and possibly also flagellar rotation in bacteria (Mitchell, 1972a; Harold, 1977). Energy transduction is thus mediated via a proton current (proticity), which circulates through the insulating membrane and the surrounding bulk aqueous phases.

1.5.2 Localised Proton hypothesis

This hypothesis, which was proposed and developed by Williams (1961, 1978ab), differs fundamentally from the Chemiosmotic hypothesis in that the 'energised state' is envisaged to comprise local proton concentration gradients which do not, under normal conditions, equilibrate rapidly with the adjacent aqueous phases. According to the Localised Proton hypothesis, respiration induces charge separation, and coupling to ATP synthesis occurs via the controlled diffusion of protons through the coupling membrane itself to the ATP phosphohydrolase. Williams (1978a) has advocated that the diffusion of protons occurs primarily through intramembrane channels, but other workers (e.g. Archbold *et al.*, 1976; Kell, 1979) have proposed that the proton current is carried along localised channels on the membrane surface (i.e. in the high electrostatic potential Stern layer).

A brief summary of some of the basic evidence for the Chemiosmotic hypothesis *vis a vis* the Localised Proton hypothesis is presented below.

As predicted by the Chemiosmotic hypothesis:

(1) Most reports suggest that energy conservation is dependent on a vesicular system.

(2) Transmembrane movements of protons do accompany respiration and ATP hydrolysis (Mitchell & Moyle, 1965; Scholes & Mitchell, 1970b).

(3) Proton gradients are formed across coupling membranes (e.g. Rottenberg, 1975, 1979 ; but see Tedeschi, 1981).

(4) An artificially-imposed protonmotive force will drive ATP synthesis (Thayer & Hinkle, 1975; Maloney, 1977).

(5) The growth yields of bacteria appear to vary in accordance with measured proton translocation stoicheiometries (e.g. Jones, 1977).

Furthermore, the Chemiosmotic hypothesis provides simple and appropriate mechanisms for:

(1) The action of uncoupling agents, almost all of which are lipophilic weak acids.

(2) Solute transport.

(3) Energy conservation in some simple bacterial respiratory systems (e.g. Ingledew et al., 1977; Kröger, 1978; Jones, 1980; Jones et al., 1980).

However, none of the above points - with the possible exception of the requirement for a vesicular system - is irreconcible with the Localised Proton hypothesis. It has been suggested that there may be some interaction between the localised protons and the bulk aqueous phases, and that the degree of this interaction is greatly increased under the non-physiological conditions which are used for the observation of respiration-linked proton translocation, and bulk-phase proton gradients (see Kell, 1979).

The following points are more readily explained by the Localised Proton hypothesis than by the Chemiosmotic hypothesis:

(1) There are some claims of energy conservation in non-vesicular systems (Cole & Aleem, 1973; Knobloch, 1978; Storey et al., 1980).

(2) The ratio between the $\Delta\bar{\mu}H^+$ and the phosphorylation potential (ΔG_p) depends on the experimental conditions, and in many cases the $\Delta\bar{\mu}H^+$ is not commensurate with ATP synthesis unless high $\rightarrow H^+$/ATP quotients ($\geqslant 3$) are assumed (e.g. Kell, 1978ab).

(3) In the absence of $\Delta\Psi$ -collapsing agents a $\Delta\Psi$ can be established, and ATP synthesised, without measureable proton translocation (Archbold et al., 1979; Conover & Azzone, 1980).

(4) A chemiosmotic mechanism is difficult to envisage in alkalophiles, or in bacteria containing concentric layers of respiratory membranes.

Due to its high experimental applicability, many of the experiments described in this thesis have been designed in terms of the Chemiosmotic hypothesis, but the possibility that the bulk-phase, transmembrane protonmotive force is not an obligatory intermediate between respiration and ATP synthesis has been considered during the interpretation of experimental results.

1.6 Mechanism of respiration-linked proton translocation

The classical experiments of Lehninger and Lardy in the 1950's (e.g. Copenhaver & Lardy, 1952; Lehninger, 1954) indicated the presence of three energy coupling sites in the mitochondrion-site I associated with NADH : ubiquinone oxidoreductase, site II associated with ubiquinol : cytochrome c oxidoreductase, and site III associated with cytochrome c oxidase. Later experiments demonstrated a fourth site, site 0, at the level of nicotinamide nucleotide transhydrogenase (see Ernster & Lee, 1964) which probably functions *in vivo* to reduce $NADP^+$ by reversed electron transfer. All four sites of energy conservation may also occur in bacteria, though many species appear to lack one or more functional coupling sites (see section 1.8).

In his original formulation of the Chemiosmotic hypothesis, Mitchell envisaged that proton translocation, at each coupling site, would occur via a redox loop mechanism - each loop consisting of the outward transfer of 2H followed by the inward transfer of $2e^-$ (Fig. 1.6a; Mitchell, 1966, 1968). However, as no obvious hydrogen carrier was identified between sites II and III of the respiratory chain, Mitchell later modified his

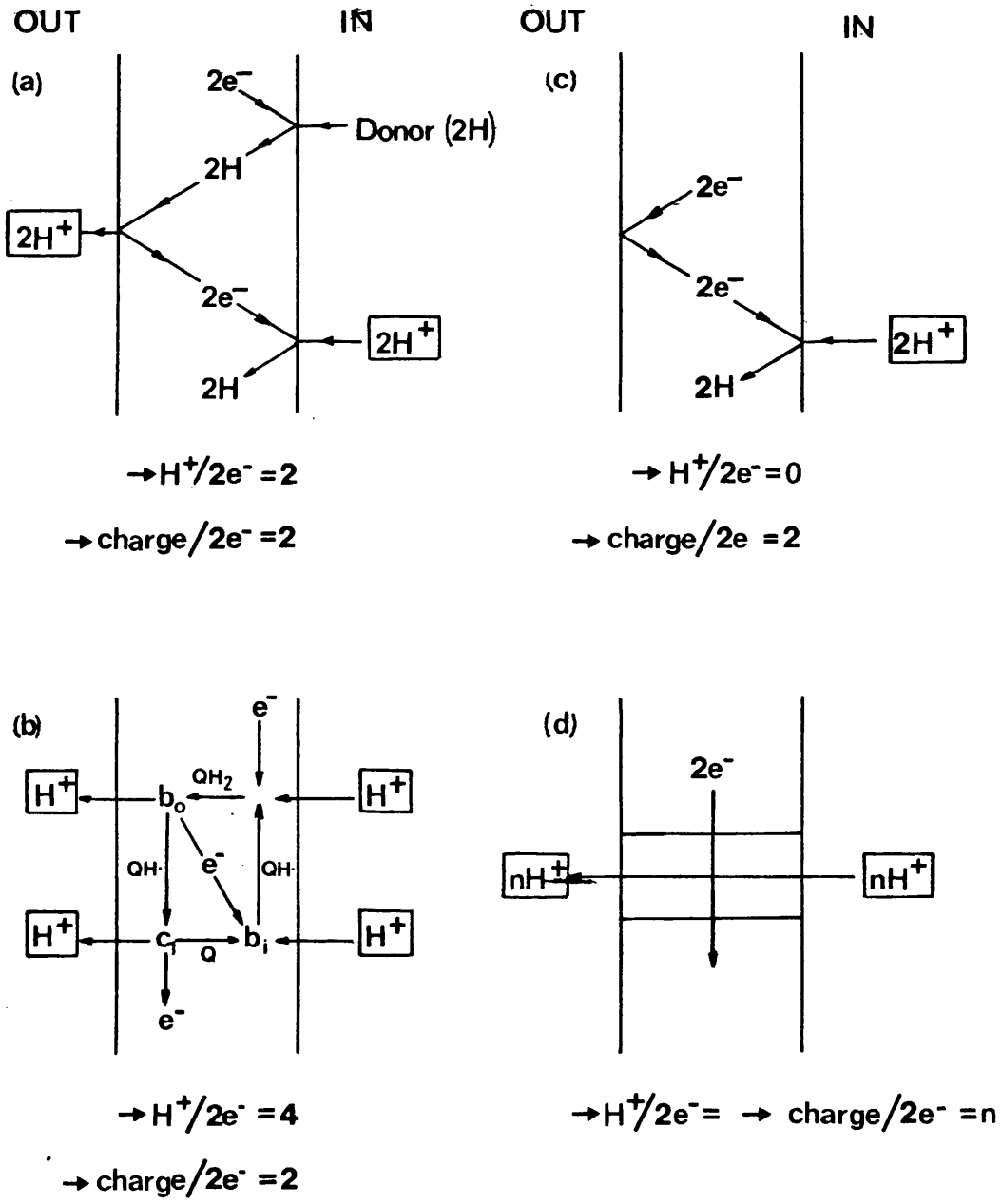


Fig. 1.6 Possible mechanisms of respiration-linked proton translocation. (a), redox loop; (b), protonmotive quinone cycle (Q-cycle); (c), redox arm; (d), redox-linked proton pump.

original scheme such that site II consisted of a protonmotive quinone cycle (Q-cycle) involving ubiquinone both before and after cytochrome b (Fig. 1.6b; Mitchell, 1975ab), and site III was associated only with transmembrane electron flow (redox arm; Fig. 1.6c).

Mitchell's proposed proton translocation mechanisms have not, however, met with unanimous support. Other workers have suggested true proton pump mechanisms in which the species crossing the coupling membrane is H^+ itself, rather than effective H^+ translocation occurring via opposite movements of H and e^- (Fig. 1.6d). Papa (1976) has proposed a Bohr-like mechanism for proton pumping (by analogy with haemoglobin) in which reduction of a metallic redox centre is accompanied by an increase in the pK of an acidic group of the apoprotein at the inner surface of the membrane, thus causing proton uptake; reoxidation causes a decrease in the pK of an acidic group at the outer face of the membrane, and hence proton release into the external phase. Translocation of the proton between the two acidic groups - or of the acidic group between the two positions - is brought about either by the opening of an asymmetric channel, or by conformational change of the protein, respectively. It should be noted that the stoicheiometry of 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. Wikström & Krab (1978) have proposed a rather more direct (and pH-independent) mechanism for proton pumping by cytochromes which involves protonation and deprotonation of the haem, or of a group in its immediate vicinity.

The four proton translocation mechanisms shown in Fig. 1.6 differ in terms of the types of redox centre(s) present, the spatial location of these centres, and the proton and charge translocation stoicheiometries.

These parameters may thus be used diagnostically to obtain information about the mechanism of proton translocation operating in a particular region of the respiratory chain (see section 1.9).

1.7 Measurement of the stoicheiometries of respiration-linked proton and charge translocation

The first method to be used for the determination of proton translocation stoicheiometries was the oxygen-pulse method which was introduced by Mitchell & Moyle (1965, 1967a). In this method, a small pulse of oxygen (as air-saturated KCl) is added to an anaerobic suspension of mitochondria (or bacteria), and the resulting acidification of the external medium is measured using a conventional pH electrode. Due to the low electrical capacitance of the coupling membrane, a permeant anion (e.g. SCN^-) or cation (e.g. K^+/valinomycin, Ca^{2+}) must be present in order to collapse the rapidly-formed $\Delta\Psi$ (by symport or antiport, respectively, with the translocated H^+), and hence allow full expression of the ΔpH.

By the use of various substrates and electron acceptors, in conjunction with specific respiratory inhibitors, coupling sites may be functionally isolated, and the $\rightarrow H^+/2e^-$ quotients for individual sites determined. Using the oxygen-pulse method, Mitchell and others have obtained $\rightarrow H^+/2e^-$ quotients in mitochondria of 2 at sites 0 and I, 4 at site II, and zero at site III (e.g. Mitchell & Moyle, 1965, 1967ab); and furthermore $\rightarrow$ charge/$2e^-$ quotients of 2 have been obtained for sites I to III by measurement of the movements of the counter-ion (K^+ or Ca^{2+}) used to collapse the $\Delta\Psi$ (Mitchell, 1969; Moyle & Mitchell, 1978).

The oxygen-pulse method for the measurement of proton and charge translocation stoicheiometries in mitochondria has, however, been vigorously

criticised, and several groups have obtained higher proton and charge translocation stoicheiometries by other experimental approaches. Some of the advantages and disadvantages of the various methods which have been used for measuring proton and charge translocation stoicheiometries are discussed below.

1.7.1 Oxygen-pulse method

Brand *et al*. (1976) obtained evidence that $\rightarrow H^+/O$ quotients determined by the oxygen-pulse method are underestimated due to rapid secondary proton movements in symport with phosphate. If H^+/phosphate symport was prevented by adding the -SH reagents NEM or mersalyl, by depleting the mitochondria of phosphate, or by assaying at lower temperature, increased values of the $\rightarrow H^+/O$ quotient were obtained. Interestingly, it was Mitchell (1972b) who first noted the enhancing effect of NEM, but he suggested that this was due to the inhibition of NAD^+-linked dehydrogenases and succinate dehydrogenase, thus encouraging the participation of the complete respiratory chain from NADPH to oxygen in proton translocation (see Mitchell, 1979). Indeed, Moyle & Mitchell (1978) observed no effect of NEM on the $\rightarrow H^+/O$ or $\rightarrow$ charge/O quotients for site III, as measured by the oxidation of ferrocyanide or $DADH_2$, and in any case electroneutral H^+/phosphate symport would not be expected to affect the $\rightarrow$ charge/O quotient. These latter experiments have, however, been criticised on the grounds that the $\Delta\Psi$ may not be fully collapsed during the measurement of $\rightarrow$ charge/O quotients by the oxygen-pulse method. The very small extent of ion movements measured in these experiments necessitates the presence of very low counter-ion concentrations in order

to allow adequate sensitivity of the ion-selective electrodes; the residual $\Delta\Psi$ may then cause underestimation of the proton and counter-ion movements (Heinz et al., 1981).

1.7.2 Initial-rate method

Reynafarje et al. (1976) introduced a new method for the measurement of $\rightarrow H^+/O$ quotients in mitochondria, in which initial-rates of H^+ ejection and oxygen consumption are measured following the initiation of respiration by the addition of substrate. Lehninger's group were also able to measure $\rightarrow$ charge/O quotients using K^+- and Ca^{2+}-selective electrodes (Reynafarje & Lehninger, 1978; Vercesi et al., 1978). The period of respiration considered, and hence the extent of ion movements, is somewhat greater in the initial-rate method than in the oxygen-pulse method. This allows higher counter-ion concentrations to be used, and the risk of ion-movements being limited by a residual $\Delta\Psi$ is thus less serious.

Using this method, Lehninger's group have obtained an average charge/site quotient of 4 in mitochondria, independent of NEM, and an average $\rightarrow H^+$/site quotient of approximately 3 which increased to 4 in the presence of NEM (see Lehninger et al., 1979). The $\rightarrow H^+$/site quotients of greater than 2, obtained by the initial-rate method, were less dependent on the presence of NEM than in the oxygen-pulse method, probably because less phosphate leaks from the mitochondria under the aerobic conditions of the initial-rate experiments.

Azzone's group have obtained similar values to those from Lehninger's laboratory using the initial-rate method, and also by initiating respiration and proton translocation by collapsing the $\Delta\Psi$ using valinomycin (in the presence of K^+) or Ca^{2+} (Azzone et al., 1979; Pozzan et al., 1979) – the

substrate being present throughout. This latter method has, however, been criticised on the grounds that the high $\Delta\Psi$ of the mitochondria, prior to the addition of the $\Delta\Psi$-collapsing agent, will drive super-stoicheiometric cation uptake once valinomycin or Ca^{2+} is added (see Mitchell, 1972b).

Papa et al. (1980ab) have claimed that the average $\rightarrow H^+$/site stoicheiometry of greater than 2, measured by the initial-rate method, is overestimated due to underestimation of the initial rate of oxygen consumption by the slow-responding polarographic oxygen-electrodes which are normally used in these experiments. By using haemoglobin to monitor oxygen-consumption spectrophotometrically (Barzu, 1978; Capuano et al., 1980), Papa's group have obtained $\rightarrow H^+/O$ quotients, by the initial-rate method, which support Mitchell's original concept of an average $\rightarrow H^+$/site quotient of 2 (Papa et al., 1980ab). It is worth noting, however, that Lehninger et al. (1980) have been able to reproduce an average $\rightarrow H^+$/site quotient of 4 using a special oxygen electrode with a response time ($t_{1/2}$ for dithionite or H_2O_2 addition) of approximately 30 ms.

1.7.3 Steady-state method

Brand et al. (1978) introduced a steady-state method for the measurement of $\rightarrow$ charge/O quotients in mitochondria which appears to avoid many of the drawbacks of the techniques discussed above. This method requires that the coupling membrane should be made slightly more permeable to protons (using a suboptimal concentration of uncoupler) such that the $\Delta\Psi$ formed is proportional to the rate of charge translocation. Unfortunately, only relative $\rightarrow$ charge/O quotients are obtained by this

method, but Brand et al. (1978) have measured → charge/O quotients for sites I, II and III in the ratio 1:1:2, the most probable stoicheiometries being 2:2:4, 3:3:6 or 4:4:8.

It is clear from the above discussion that there is considerable controversy over the true proton and charge translocation stoicheiometries of mitochondrial respiration. In bacteria, → H^+/O quotients have as yet been measured only by the oxygen-pulse method, and no determination of → charge/O quotients has been reported. The → H^+/O quotients so far determined in bacteria have generally supported the Mitchellian concept of an average → H^+/site quotient of 2 (e.g. Jones, 1977; but see Meijer et al., 1977; van Verseveld et al., 1981), but clearly a more rigorous investigation of the proton translocation stoicheiometry of bacterial respiration - using various experimental methods, and the determination of → charge/O quotients in bacteria, is long overdue. Some of the difficulties peculiar to the measurement of the proton and charge translocation stoicheiometries of bacterial respiration are discussed in section 4.1.

1.8 Variability of bacterial respiratory chains

Although, as discussed above, respiration-linked proton translocation has been studied in far less detail in bacteria than in mitochondria, it is clear that bacterial → H^+/O quotients vary substantially, and this variation is reflected in the wide range of respiratory chain compositions and growth yields (Jones, 1977) found amongst bacteria.

1.8.1 Species variability of bacterial respiratory chains

Although the respiratory chains of bacteria and mitochondria contain essentially similar redox centres, only a few species of bacteria (e.g. P.denitrificans, Alcaligenes eutrophus) have respiratory chains that are closely similar to those of mitochondria. Striking differences between bacteria and mitochondria, and between bacterial species, occur in the terminal region of the respiratory chain where the presence of cytochrome c in bacteria is variable, and the single cytochrome oxidase of mitochondria may be replaced by up to three CO-binding cytochromes. Although five bacterial cytochromes (aa_3, o, d, a_1, c_{co}) have been shown to bind CO (see Lemberg & Barrett, 1973; Jurtshuk et al., 1975), as yet rapid kinetic analysis has confirmed oxidase roles only for cytochromes aa_3, o and d (Smith et al., 1970; Haddock et al., 1976; Lawford et al., 1976). The major differences observed amongst the respiratory chains of bacterial species, and their consequences for energy conservation, are listed below (see Jones, 1977):

(1) Site 0 is dependent on a membrane-bound energy-conserving nicotinamide nucleotide transhydrogenase. In some bacteria transhydrogenase is soluble and energy-independent, or absent altogether.

(2) Site I is present in most bacteria, but the NADH:ubiquinone oxidoreductase may be uncoupled under some conditions (see section 1.8.2).

(3) Site II appears to be ubiquitous. The replacement of ubiquinone by menaquinone does not affect energy conservation.

(4) The existence of site III depends on the presence of a high potential membrane-bound cytochrome c plus cytochrome oxidases aa_3 and/or o. Cytochrome aa_3 may act as a proton pump (i.e. $\rightarrow H^+/O$ at site III > 0) in some bacteria (Chicken et al., 1981; van Verseveld et al., 1981).

1.8.2 Phenotypic and genotypic variability of bacterial respiratory chains

Unlike mitochondria, bacteria live in a rapidly changing environment, and many species of bacteria thus have the ability to modify their respiratory chains in response to changing environmental conditions. The widespread effects of varying the dissolved oxygen tension on the respiratory chain composition and proton-translocation stoicheiometry are discussed in section 5.1. Other interesting modifications to the respiratory chain can be induced by growth in iron-limitation which decreases the levels of cytochromes and iron-sulphur proteins (e.g. Rainnie & Bragg, 1973), and by growth in sulphate-limitation which also decreases the level of iron-sulphur proteins, and causes the production of alternative cytochromes (e.g. Poole & Haddock, 1975). Both these modes of growth tend to lead to lower growth yields, and lower proton translocation stoicheiometries; the loss of iron-sulphur proteins appears to correlate with a loss of energy-conservation at site I (Poole & Haddock, 1975).

It is also possible to modify the respiratory chains of bacteria genotypically, and this is a potent tool in the study of energy conservation. A number of mutants of Escherichia coli with lesions in the respiratory chain have been isolated (see Haddock & Jones, 1977). These mutants are either defective in their ability to produce a particular redox component (e.g. ubiquinone, menaquinone, or various dehydrogenases), or in the iron-uptake systems; the latter have a similar phenotype to that produced by growth in iron-limitation.

1.9 Respiratory chain composition and respiration-linked proton translocation in bacteria

The fractionation of the mitochondrial respiratory chain into discrete respiratory complexes has greatly facilitated the investigation of the structure and function of mitochondrial redox components. In addition, the ability to make reasonably homogenous preparations of mitochondrial inner membrane vesicles of both normal and inverted orientations has allowed an intensive investigation of the redox centre topology of the mitochondrial respiratory chain, principally using membrane-impermeant reductants, oxidants, inhibitors and protein-labelling reagents (see DePierre & Ernster, 1977).

In contrast, methods for the fractionation of bacterial respiratory chains have not been developed to the same degree as those for mitochondria, and this has severely restricted investigations of the structure and function of bacterial redox components. Furthermore, extensive topological studies have been made only on a few specialised bacterial respiratory systems such as the hydrogenase (Jones, 1980) and ubiquinol:nitrate oxidoreductase (Jones *et al.*, 1978, 1980) systems of *E.coli*, the formate : fumarate oxidoreductase of *Vibrio succinogenes* (Kröger, 1978), and the simple ferrous-iron oxidising system of *Thiobacillus ferro-oxidans* (Ingledew *et al.*, 1977). Nevertheless, from the limited information available, it would appear that, although there are many similarities between bacterial and mitochondrial respiratory chains, the former tend to be composed of somewhat simpler proteins than their mitochondrial counterparts.

The types and spatial locations of the redox components of bacterial respiratory chains - along with the stoicheiometries of respiration-linked proton translocation - are considered below, with a view to obtaining information about the likely mechanisms of proton translocation at the four possible coupling sites (see section 1.6).

1.9.1 Site 0, nicotinamide nucleotide transhydrogenase

Bacterial transhydrogenases appear to be of two basic types (Hoek *et al.*, 1974; Rydström *et al.*, 1976):

(1) Soluble, or easily solubilised, energy-independent flavoproteins which are specific for the 4B-hydrogen atom of both NADH and NADPH.

(2) Membrane-bound, usually energy-linked, non-flavin - containing proteins which are specific for the 4A-hydrogen atom of NADH and the 4B-hydrogen atom of NADPH.

BB-specific soluble transhydrogenases have been purified from *Pseudomonas* sp. and *Azotobacter* sp., and found to consist of a single subunit of MW 40,000-58,000 which aggregates in a nucleotide-dependent manner into complexes with a molecular weight of several million (see Rydström et al., 1976).

The AB-specific membrane-bound transhydrogenase of bacteria has not yet been purified, though soluble factors which will restore activity to depleted membranes have been isolated from various bacteria (see Rydström, 1977). There appear to be strong similarities between the membrane-bound transhydrogenases of bacteria and mitochondria (e.g. Asano *et al.*, 1967) - both are devoid of obvious redox centres and hence probably act as Bohr-like proton pumps (see section 1.6). The stoicheiometry of proton translocation associated with transhydrogenase activity has been claimed to be $2H^+/2e^-$ in both bacteria (Scholes & Mitchell, 1970b), and mitochondria (Moyle & Mitchell, 1973).

1.9.2 Site I, NADH : quinone oxidoreductase

Membrane-bound NADH dehydrogenases (NADH : artificial acceptor oxidoreductases) have been purified to a reasonable degree of homogeneity (75-90%) from Acholeplasma laidlawii (Jinks & Matz, 1976), Photobacterium phosphoreum (Imagawa & Nakamura, 1978), and Bacillus caldotenax (Kawada et al., 1981). The enzymes from P.phosphoreum and B.caldotenax were found to contain FAD as prosthetic group in contrast to the FMN found in mitochondrial preparations. An FAD-linked NADH:DCPIP oxidoreductase has also been purified to 75% homogeneity from E.coli (Dancey et al., 1976; Dancey & Shapiro, 1976), but more recently Thomson & Shapiro (1981) and Jaworowski et al. (1981) have independently reported the purification of an NADH:quinone oxidoreductase, containing FAD and a variable amount of iron, which seems more likely to be the true respiratory chain dehydrogenase of this organism. Up to 95% of the protein in these preparations could be accounted for by a single band of MW 45,000-47,000 and, furthermore, Jaworowski et al. (1981) were able to reconstitute NADH oxidase activity by addition of their preparation to membrane vesicles from E.coli ndh mutants. Interestingly, these workers claim that NADH:ubiquinone-1 oxidoreductase activity is not dependent on the presence of iron, but the latter may well be required for interaction with the endogenous quinone, and indeed for energy conservation at site I.

The proton translocation mechanism of the mitochondrial complex I - a large (MW $\geqslant$ 600,000) multi-subunit complex containing FMN, ubiquinone and at least 16 iron-sulphur centres, which spans the coupling membrane (see Ragan, 1976) - has been studied in some detail. Mitchell (1972b) and Garland et al. (1972) have independently proposed that NADH:ubiquinone oxidoreductase forms a redox loop, in which FMN acts as a transmembrane

hydrogen carrier, and iron-sulphur centres (possibly N-1 and N-2) act as transmembrane electron carriers. However, although there is good evidence that the NADH and ubiquinone reduction sites are both situated towards the matrix-side of the coupling membrane, there is a great deal of controversy over the spatial organisation of the iron-sulphur centres (see Ohnishi, 1979), and other workers (e.g. Papa, 1976) favour a proton pump mechanism for this energy conserving site.

→ $H^+/2e^-$ quotients of greater than 2 at site I have not been convincingly demonstrated in bacteria, so NADH:ubiquinone oxidoreductase may indeed function as a redox loop. If the true → $H^+/2e^-$ quotient at site I is greater than 2, however, as has been claimed for mitochondria (see Lehninger et al., 1979), a true proton pump mechanism must be implicated.

1.9.3 Site II, quinol:cytochrome c oxidoreductase; quinol oxidase

A discrete quinol:cytochrome c oxidoreductase complex, analogous to complex III of mitochondria (which is a large [$\geq$250,000 D] multisubunit complex containing cytochromes b_{562}, b_{566}, c_1, ubiquinone and at least one iron-sulphur protein [see Trumpower & Katki, 1979]) has not yet been isolated from bacteria. Most bacteria do, however, contain quinone (either ubiquinone or menaquinone), and multiple species of cytochrome b. The function of non-haem iron in bacteria is poorly understood (Yoch & Carrithers, 1979), but it is probable that there is at least one iron-sulphur protein associated with the cytochrome b region of the respiratory chain (see Bragg, 1979).

The proton-translocation stoicheiometries of bacteria which contain a high-potential, membrane-bound cytochrome c are consistent with the operation of a Q-cycle at site II (Fig. 1.7a). The lower proton trans-

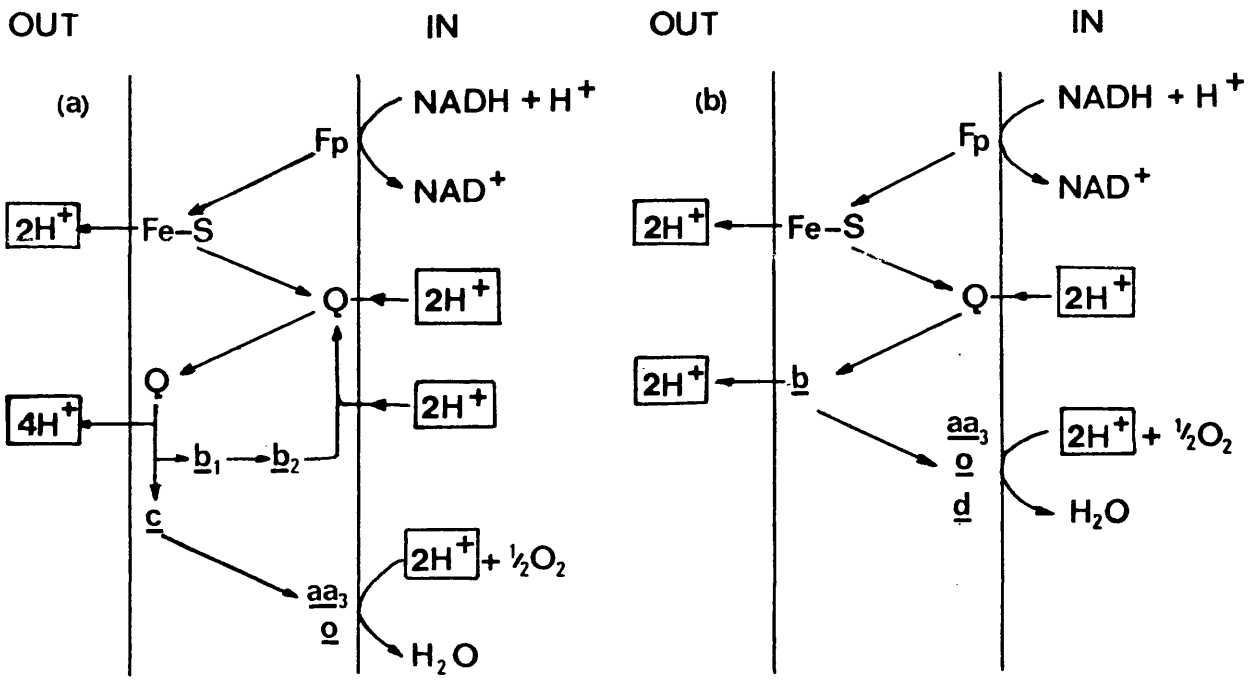


Fig. 1.7 Possible organisation of bacterial respiratory chains with (a), or without (b) cytochrome c. After Jones (1977).

location stoicheiometries observed in bacteria lacking cytochrome c, however, suggest that site II could only be associated with a Q-cycle mechanism in these organisms if the proton consumption site of the oxidase were external (see Jones, 1977). In bacteria lacking cytochrome c site II may well consist of a simple redox loop employing quinone as the transmembrane hydrogen-carrier and the b-type cytochromes plus cytochrome oxidase as the electron-transferring arm (Fig. 1.7b).

The validity of the Q-cycle mechanism has been critically examined for the mitochondrial complex III. The generally agreed $\rightarrow H^+/2e^-$ and $\rightarrow$ charge/$2e^-$ quotients at site II of 4 and 2 respectively (Mitchell, 1969; Lehninger et al., 1979; Pozzan et al., 1979), and the locations of the ubiquinone reduction site and cytochrome c at the inner and outer faces of the membrane, respectively (Trumpower & Katki, 1979), are consistent with the Q-cycle mechanism. Furthermore, the b-type cytochromes are known to be intrinsic membrane proteins, though there is as yet no evidence for a transmembrane arrangement (see Trumpower & Katki, 1979).

The major doubt concerning the validity of the Q-cycle mechanism is the ability of ubiquinone to function as required (see Trumpower, 1981). Although recent evidence suggests that ubiquinone is bound to protein, and hence relatively immobile within the mitochondrial membrane (see Yu et al., 1977), this does not preclude transmembrane hydrogen flow via a series of ordered bound quinones (Salerno et al., 1977). However, the existence of ubisemiquinone radicals which are both physically and functionally separated at the inner and outer faces of the membrane, and which are relatively stable to dismutation, as required by the Q-cycle mechanism, is a subject of controversy.

Recent evidence that ubisemiquinone may be stabilised by protein binding (Yu et al., 1978), by interaction with an iron-sulphur protein

(Ruzicka et al., 1975), or by interaction between a pair of bound ubisemiquinone radicals (Ruzicka et al., 1975; Salerno et al., 1977), has strengthened the position of the Q-cycle, but other mechanisms for proton-translocation at site II involving true redox-linked proton pumps have been proposed (Papa, 1976, von Jagow et al., 1978). Two types of mechanism may be envisaged - the first involving a proton pump with a stoicheiometry of $4H^+/2e^-$, and the second involving a proton pump with a stoicheiometry of $2H^+/2e^-$ plus a redox loop composed of ubiquinone and the b-type cytochromes as the hydrogen- and electron- transferring arms, respectively.

1.9.4 Site III, cytochrome c oxidase

As discussed in section 1.8.1, three cytochromes, aa_3, o and d, have been clearly shown to function as terminal oxidases in bacteria.

(1) Cytochrome aa_3

Bacterial a-type cytochrome oxidases have recently been purified from P.denitrificans (aa_3), Thiobacillus novellus (aa_3), Thermus thermophilus (c_1/aa_3) and the thermophile PS3 (c/a/o) (for review see Ludwig, 1980). These oxidases all appear to be considerably simpler proteins than the 7 subunit complex of mitochondria (see Capaldi, 1979) as they contain only 2 (P.denitrificans, T.novellus, PS3) or at the most 3 (T.thermophilus) subunits. The two subunits of the P.denitrificans oxidase appear to be similar to subunits I and II of the mitochondrial complex which probably carry the 2 haem a centres (a and a_3) and 2 atoms of copper (Azzi, 1980; Winter et al., 1980).

Little is known concerning the orientation of the bacterial cytochrome aa_3 within the coupling membrane, but haem a of the mitochondrial oxidase has been shown to be close to cytochrome c on the outer face of the membrane, whereas haem a_3 and the two copper atoms are close to the centre of the membrane (Ohnishi et al., 1979). It is often assumed, though without conclusive evidence, that the protons consumed in the oxidase reaction are derived from the internal phase, and cytochrome c oxidase thus forms an electron-transferring redox arm (see Hinkle & Mitchell, 1970). If the $\rightarrow H^+/O$ quotient at site III is equal to zero, then this may indeed be the sole electrogenic feature of cytochrome oxidase, though it is equally possible that the protons consumed in the oxidase reaction are derived from the external phase, having been delivered there by a proton pump mechanism.

There is some recent evidence, from whole cells of Bacillus stearothermophilus (Chicken et al., 1981) and P.denitrificans (van Verseveld et al., 1981), that cytochrome oxidase aa_3 may act to catalyse net proton translocation (i.e. $\rightarrow$ charge/O > 2, $\rightarrow H^+/O > 0$), as has been claimed for the mitochondrial oxidase (see Wikström & Krab, 1979). If this is the case then a true proton pump mechanism must be implicated regardless of whether the oxidase also forms a redox arm. The observation of net proton translocation at site III of P.denitrificans contrasts with the observation that the purified oxidase from this organism does not translocate protons after reconstitution into membrane vesicles (Ludwig, 1980). It is interesting in this context that the purified P.denitrificans oxidase does not have a subunit corresponding to subunit III of the mitochondrial oxidase which is claimed by Casey et al. (1980) to house the proton pump.

Furthermore, Wikström & Saraste (1980) have shown that a mitochondrial cytochrome oxidase preparation depleted of subunit III does not translocate protons.

The above observations have led to speculation that the purified oxidase from P.denitrificans is lacking a third subunit which is responsible for proton pumping in whole bacteria. This idea may have some credence in terms of the proposed evolution of the mitochondrion from an ancestral form of this bacterium (John & Whatley, 1975), as the genes for subunits I, II and III of the eukaryotic cytochrome oxidase are carried on the mitochondrial genome (Schatz & Mason, 1974). Interestingly, however, the purified two subunit oxidase from PS3 appears to translocate protons with a stoicheiometry of $2H^+/2e^-$ after reconstitution into liposomes (Sone, N. and Hinkle, P. personal communication cited in Ludwig, 1980).

(2) Cytochrome o

Cytochromes o have been purified from Vitreoscilla sp. (Tyree & Webster, 1978) and Azotobacter vinelandii (Yang & Jurtshuk, 1978; Yang et al., 1979), and each has been shown to contain two haems b. In A.vinelandii, the two haems are located on a single polypeptide of MW 28,000, whereas the Vitreoscilla cytochrome contains two identical subunits each of MW 13,000 and each containing one b-type haem. There is no evidence for the presence of copper in either of these preparations, so the four redox centres which are necessary for the reduction of molecular oxygen to water may be provided by two molecules of cytochrome o.

In bacteria containing a high redox potential, membrane-bound cytochrome c, cytochrome o may feature in an energy-conserving cytochrome c

oxidase (see Jones, 1977), or alternatively may accept electrons from cytochrome b in a branch of the respiratory chain associated with a lesser degree of energy conservation (e.g. in P.denitrificans; van Verseveld & Stouthamer, 1978). At least in the former case, it would appear that cytochrome o must be situated on the inner face of the coupling membrane in order to form an electron transferring arm at site III - the small size of cytochrome o makes a proton pump mechanism unlikely.

It should be noted that cytochromes o have a wide range of spectral properties (Lemberg & Barrett, 1973) and they may well vary appreciably in terms of their structure and energy-conserving properties.

(3) Cytochrome d

A large protein complex (approximate MW 350,000) has been purified from E.coli and found to contain two haems d plus two haems b (Reid & Ingledew, 1980). This complex is an intrinsic membrane protein, but nothing is known of the spatial orientation of the haem groups. There is no evidence that cytochrome d can accept electrons from cytochrome c (other than in the specialised cd_1 nitrite reductase; see Lemberg & Barrett, 1973), or function in an energy-conserving site III. Indeed, the induction of cytochrome d in bacteria containing cytochrome c plus cytochrome oxidases aa_3 and/or o generally leads to a reduced efficiency of respiratory chain energy conservation (see Jones, 1977).

1.10 ATP synthesis in bacteria

The structure of the bacterial ATP phosphohydrolase has been the subject of intensive investigations (see Haddock & Jones, 1977; Downie *et al.*, 1979). It is only necessary to note here that this complex consists of two multisubunit components - one an intrinsic membrane protein (BF_o), and the other easily solubilised (BF_1). BF_o probably acts as a proton channel conveying protons through the membrane to the catalytic component BF_1 which is situated on the inner face of the cytoplasmic membrane. Several reports have shown that ATP synthesis in bacteria may be driven by an artificially-imposed protonmotive force, or by either $\Delta\Psi$ or ΔpH alone (e.g. Wilson *et al.*, 1976).

Since the early experiments of the 1950's (Copenhaver & Lardy, 1952; Lehninger, 1954), it has been widely accepted that the passage of $2e^-$ through a coupling site results in the synthesis of one molecule of ATP. More recently, however, controversy over the magnitude of the $\rightarrow H^+/O$ quotient in mitochondria (see section 1.7), and the realisation that the exchange of ATP for ADP and phosphate is probably accompanied by the uptake of one proton per round of transport (Klingenberg & Rottenberg, 1977; but see Mitchell, 1979), has plunged this subject back into dispute. Table 1.3 summarises the positions held by some of the groups who are prominent in this controversy.

Bacteria do not generally transport adenine nucleotides, and they may, therefore, be expected to exhibit higher ATP/O quotients than mitochondria i.e. comparable with those which have been measured for ATP synthesis from intramitochondrial ADP and phosphate (Brand, 1979). The free energy of hydrolysis of ATP (ΔG_p, phosphorylation potential) supported by inverted membrane vesicles from bacteria, however, appears to be substantially

Laboratory	$\rightarrow H^+/O$ quotient	$\rightarrow H^+/ATP$ quotient via ATP'ase	$\rightarrow H^+/ATP$ quotient for transport	Overall ATP/O quotient	References
Mitchell	6	2	0	3	Mitchell (1979)
Hinkle	6	2	1	2	Hinkle & Yu (1979)
Brand	8	2	1	2.7	Brand *et al*. (1978) Brand (1979)
Lehninger	12	3	1	3	Alexandre *et al*. (1978)

Table 1.3 Stoicheiometry of ATP synthesis during NADH oxidation in mitochondria

greater than that supported by submitochondrial particles, thus suggesting that the $\rightarrow H^+$/ATP quotient of bacteria might be higher than that of mitochondria (Ferguson & Sorgato, 1977).

Three basic strategies have been employed for the measurement of ATP/O quotients in bacteria:

(1) Direct measurement of ATP synthesis and oxygen consumption.

(2) Comparison of the $\rightarrow H^+$/O and $\rightarrow H^+$/ATP quotients.

(3) Growth yield studies.

1.10.1 Direct measurement of ATP/O quotients

ATP/O quotients have been determined, in both whole bacteria and inverted membrane vesicles, by direct measurement of ATP synthesis and oxygen consumption. Experiments in whole bacteria have met with only limited success due to the difficulties of determining the intracellular concentrations of adenine nucleotides and phosphate, and due to the fact that the ATP-synthesising and utilising apparatus of bacteria are located in the same compartment. Indeed by this method, ATP/O quotients for NADH oxidation in various bacteria ranging from 0.3-3.0 mol ATP/g-atom O have been determined (e.g. Hempfling, 1970; Knowles & Smith, 1970; van der Beek & Stouthamer, 1973).

The use of inverted membrane vesicles overcomes many of the problems encountered with whole cells but usually yields only a minimum estimate for the ATP/O quotient as these preparations are rarely perfectly coupled (see Harold, 1972; Gel'man et al., 1975; Jones, 1977). Nevertheless, ATP/O quotients for NADH oxidation of approaching 3 mol ATP/g-atom O have been obtained using the particularly well-coupled vesicles derived from chemolithotrophs (e.g. Kiesow, 1964).

1.10.2 ATP/O quotients from a comparison of the $\rightarrow H^+/O$ and $\rightarrow H^+/ATP$ quotients

The determination of $\rightarrow H^+/O$ quotients has been discussed in some detail above (see section 1.7). $\rightarrow H^+/ATP$ quotients may be determined either directly (i.e. kinetically) by measuring proton translocation concomitant with ATP synthesis or hydrolysis (note that correction for scalar proton consumption/production must be made), or indirectly (i.e. thermodynamically) from a comparison of the protonmotive force and the phosphorylation potential.

Measurement of H^+ uptake concomitant with ATP synthesis in whole bacteria generally yields $\rightarrow H^+/ATP$ quotients of not less than 5 g-ion H^+/mol ATP (Maloney, 1977, 1978). These values are surely overestimated, however, due to the uncorrected consumption of ATP by various cellular reactions. On the other hand, ATP hydrolysis in inverted membrane vesicles is generally associated with low $\rightarrow H^+/ATP$ quotients (e.g. for E.coli, $\rightarrow H^+/ATP$ = 0.6g-ion H^+/mol ATP; West & Mitchell, 1974), presumably because of the poor coupling properties of these preparations. Nevertheless, a value for the $\rightarrow H^+/ATP$ quotient of approximately 2 g-ion H^+/mol ATP has been obtained using the ATP phosphohydrolase of the thermophile PS3 incorporated into liposomes with a very high phospholipid: protein ratio, and hence very low passive proton permeability (Sone et al., 1976).

The determination of the $\rightarrow H^+/ATP$ quotient from a comparison of the $\Delta\bar{\mu}H^+$ and ΔG_p is considered in detail in Chapter 6, but it is worth noting here that the values obtained are generally greater than 2g-ion H^+/mol ATP. A kinetic method related to this which involves measuring the

rate of ATP synthesis as a function of an artificially-imposed $\Delta\bar{\mu}H^+$, however, yields a $\rightarrow H^+$/ATP quotient of close to 2 for whole cells of Streptococcus lactis (Maloney & Schattschneider, 1980); and a similar value has been obtained from Rhodopseudomonas sphaeroides in short-flash kinetic experiments (Jackson et al., 1975; Petty & Jackson, 1979).

1.10.3 ATP/O quotients from growth studies

The growth efficiency of an aerobic energy-limited continuous culture can be described by the equation:

$$Q_{O_2} = \mu/Y_{O_2}^{max} + M_{O_2} \qquad (1.2)$$

(Pirt, 1965; Harrison & Loveless, 1971), where μ is the specific growth rate ($\equiv$ dilution rate [D], h^{-1}), Q_{O_2} is the in situ respiration rate (mol O_2/h per g dry wt. bacteria $\equiv \mu/Y_{O_2}$, where Y_{O_2} is the molar growth yield on oxygen, g dry wt. bacteria/mol O_2), $Y_{O_2}^{max}$ is the maximum molar growth yield (i.e. $\equiv Y_{O_2}$ at infinite μ), and M_{O_2} is the maintenance respiration rate ($\equiv Q_{O_2}$ at $\mu = 0$). Furthermore, in an energy-limited culture, $Y_{O_2}^{max}$ is proportional to the amount of ATP produced per mole of oxygen consumed (Bauchop & Elsden, 1960), and we can define Y_{ATP}^{max}, the maximum molar growth yield with respect to ATP (g dry wt. bacteria/mol ATP) such that:

$$Y_{O_2}^{max} = N.\ Y_{ATP}^{max} \qquad (1.3)$$

The proportionality constant N (mol ATP/mol O_2), once corrected for any substrate-level phosphorylation, is equivalent to twice the value of the ATP/O quotient.

$Y_{O_2}^{max}$ may be determined experimentally from plots of the Q_{O_2} against μ, but a value for Y_{ATP}^{max} is rather more difficult to obtain. In

facultative anaerobes Y_{ATP}^{max} may be determined from the $Y_{carbon\ substrate}^{max}$ (g dry wt. bacteria/mol carbon substrate) during anaerobic growth, as the fermentation pathways and ATP yields via substrate-level phosphorylation are well understood. The assumption that the Y_{ATP}^{max} will be similar during aerobic and anaerobic growth is a reasonable one - provided that growth is energy-limited under both conditions - but recent evidence that energy may be conserved from the efflux of fermentation products down their concentration gradients (Michels et al., 1979; Otto et al., 1980) suggests that it might not be correct in all cases. In obligate aerobes, Y_{ATP}^{max} can usually only be obtained from theoretical calculations of the energy requirement for biosynthesis of cell materials, or by comparison with values from facultative anaerobes.

Using these techniques, ATP/O quotients of approximately 3 and 2 mol ATP/g atom O respectively have been obtained for bacteria with and without a high-potential membrane-bound cytochrome c (Jones, 1977).

1.11 Single-cell protein (SCP)

'Single-cell protein' is the term which has been adopted to represent the cells of algae, bacteria, fungi and yeasts grown - principally for their protein contents - for human food or animal feed purposes. Interest in SCP grew through the 1960's in response to predictions of World shortages of food, and particularly of protein (see United Nations Economic and Social Council, 1967; U.S. President's Science Advisory Committee, 1967). The first international meeting, convened to discuss SCP in 1967 (see Mateles & Tannenbaum, 1968), considered mainly theoretical aspects of SCP production and utilisation, but by the second meeting in 1973 (see Tannenbaum & Wang, 1975) several industrial processes had been developed

to at least the pilot plant stage. The period between 1973 and the present day has seen would-be SCP producers faced with a number of problems, both of an economic and technical nature, but, though many industrial organisations have decreased their interest in SCP, others have initiated production on a commercial scale (see Litchfield, 1978).

There is no intention here to attempt to review the complex multidisciplinary field of SCP production and utilisation, but a few of the factors most closely related to the biochemical and physiological properties of the SCP organism will be discussed.

1.11.1 Choice of substrate for SCP production

Possible substrates for SCP production fall into three categories (Humphrey, 1975):

(1) Energy source materials - e.g. methane, methanol, n-alkanes, ethanol.

(2) Waste materials - e.g. bagasse, whey, molasses, sulphite waste liquor.

(3) Renewable source materials - e.g. starch, sugar, cellulose.

The applicability of the last two classes depends largely on local economic factors and will not be discussed further here. Of the energy source materials, methanol has recently been most often chosen as the substrate for large-scale production of bacterial SCP (e.g. Faust *et al.*, 1981; Smith, 1981; Urakami *et al.*, 1981). Methanol is somewhat more expensive than methane or n-alkanes, but it combines the advantages of being readily available in a highly purified form, non-explosive, very soluble in water, and readily washed from the product. Furthermore, due to the bioenergetic inefficiency of the monooxygenase reactions involved in methane and n-alkane dissimilation, the oxygen demand and heat production during growth on these substrates is greater than during growth on methanol (see Goldberg, 1977).

Both n-alkanes and ethanol have been extensively utilised for the production of yeast SCP. Despite its relatively high cost, ethanol has been much used as a substrate for food-yeast production due to its non-toxic nature and consumer acceptability.

1.11.2 Choice of organism for SCP production

Clearly the choice of organism for SCP production depends to a large extent on the choice of substrate, and the intended market for the product. Yeasts have been used for human consumption for over 50 years, but bacterial SCP has given very poor results in toxicological tests in humans (see Kharatyan, 1978). There has been relatively little research so far into the nutritional and toxicological properties of algal and fungal SCP (see Clément, 1975; Imrie & Vlitos, 1975).

For the production of animal feed, bacteria have the advantages over yeasts of a higher growth rate, higher growth yield, and higher protein content - though these factors are to some extent offset by the larger size of yeasts (which allows easier removal from suspension), and by the ability of yeasts to grow at low pH thus resisting bacterial contamination. Indeed, a number of industrial processes for the production of yeast SCP are operated non-aseptically at pH3-4 (see Litchfield, 1978).

There has been considerable controversy as to whether pure or mixed (but well-defined) cultures are preferable for the production of bacterial SCP (see Harrison, 1978). During growth on methane, there is evidence that non-methanotrophic components of mixed cultures are able to remove products (e.g. methanol) which would otherwise inhibit growth of the methanotroph (e.g. Wilkinson et al., 1974; Harrison, 1976b). Moreover, in a mixed culture, it might be expected that lysis products of the methylotroph(s)

would be utilised by the heterotroph(s) thus decreasing the amount of carbon lost in the supernatant; there is some dispute, however, as to whether mixed cultures grow with higher or lower growth yields than do pure cultures (Goldberg, 1977; Harrison, 1978). The decreased susceptibility of mixed cultures to outside contamination may be of some practical value in SCP production, but this is offset by the fact that it is easier to produce SCP from pure cultures to the constant specifications required by both consumers and regulatory agencies (Goldberg, 1977: Harrison, 1978).

Continuous culture has well-defined advantages over batch culture in terms of productivity per unit volume per unit time (Mateles, 1968), and the former has been used almost ubiquitously for SCP production. Due to the difficulties of achieving good mixing in a large fermenter, any SCP organism will be subjected to a varying regime of substrate, oxygen and carbon dioxide concentrations, as well as to varying temperature, pressure and pH (see Mateles, 1979). The ability to grow with near-optimal yields in this varying environment is a principal factor in the selection of an organism for SCP production.

1.11.3 I.C.I. SCP process

Since 1973, I.C.I. Agricultural Division has operated a pilot plant at Billingham, Cleveland with a productivity of up to 1,000 tonnes/year for the production of SCP from methanol. In 1980, the first commercial-scale plant, with a productivity of 50,000-75,000 tonnes/year was commissioned. The I.C.I. product, 'PRUTEEN', consists of the dried biomass of the methylotrophic bacterium M.methylotrophus. The nutritional and toxicological properties of PRUTEEN have been extensively investigated

(Stringer & Wilson, 1976), and it is now sold on the animal feed market as a milk replacer for calves, or to be included in compound feeds for piglets, laying hens, broilers and turkeys.

The methanol used in the PRUTEEN process is produced from gaseous mixtures of hydrogen and carbon oxides in the presence of a copper-based catalyst (The I.C.I. Low Pressure Methanol Process, undated). This process is extremely versatile as the reactants may be produced either by the steam reforming of natural gas (The I.C.I. Steam Naphtha Reforming Process, undated), or by the partial oxidation of heavy fuel oil or coal.

The heart of the PRUTEEN process is a pressure cycle fermenter with a volume in excess of $1000m^3$ (Fig. 1.8). The fermenter consists of a concentric riser and downcomer through which the culture is rapidly circulated, driven by compressed air introduced at the base of the riser. The advantages of this design over conventional stirred-tank technology for large-scale SCP production have been discussed elsewhere (Hatch, 1975; Gow et al., 1975). In order to obtain optimum yields, the bacteria are grown in methanol-limited continuous culture and the dissolved oxygen tension is maintained above a critical value. The fermenter effluent is treated to effect agglomeration prior to centrifugation, and the resulting slurry is granulated and dried in a flash drier; the liquid removed in the bacterial separation processes is recycled to the fermenter where fresh nutrients are added.

The major contributions to the operating costs for PRUTEEN production are methanol (59%), energy (23%; mainly for air-compression, cooling and product drying) and other constituents of the growth medium (17%) (Smith, 1981). The central importance of the growth yield of the organism to the economics of the process is reflected not only in the dominant contribution

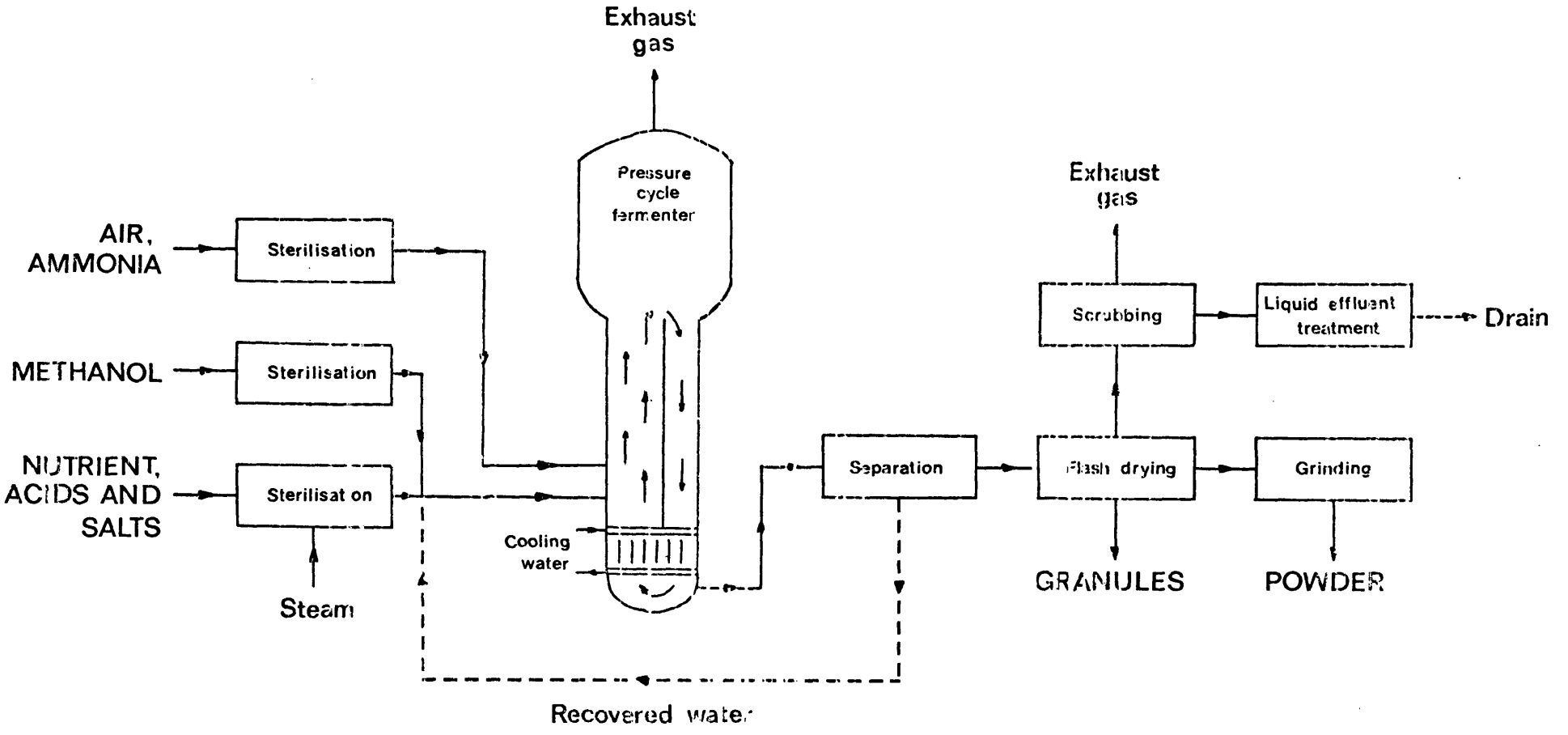


Fig. 1.8. Flow sheet for the PRUTEEN process. After Smith (1981).

of methanol to the operating costs, but also in the costs for air-compression and cooling, as the oxygen demand and heat production both depend on the efficiency of cell growth.

1.12 Objectives

The prime objective of this work was to obtain an understanding of respiratory chain energy conservation in M.methylotrophus. The growth yields of methylotrophs, compared to the heats of combustion of their substrates, are lower than those of most heterotrophs (Linton & Stephenson, 1977 and, on a broader level, a reason for this discrepancy was sought. It was hoped that this investigation would help to identify any bioenergetic inefficiencies in the growth of M.methylotrophus which might be open to remedy at a later date.

Three facets of respiratory chain energy conservation in M.methylotrophus have been investigated:

(1) The composition, sequential organisation, and activity of the respiratory chain (Chapter 3).

(2) Kinetic parameters of energy conservation such as the $\rightarrow H^+/O$ quotient (Chapter 4) and $\rightarrow H^+/ATP$ quotient (Chapter 6).

(3) Thermodynamic parameters of energy conservation such as the protonmotive force and the phosphorylation potential (Chapter 6).

In addition, the effect of the growth conditions on some of the above parameters has been studied (Chapter 5).

Accounts of this work have appeared in the literature (Dawson & Jones, 1980ab, 1981abc).

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and biochemicals

2.1.1 Sources of chemicals and biochemicals

2.1.2 Preparation and assay of stock solutions

2.2 Growth and maintenance of bacterial cultures

2.2.1 Maintenance of stock cultures

2.2.2 Preparation of inocula

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

2.2.4 Growth of M.methylotrophus in oxygen-limited continuous culture

2.2.5 Growth of M.methylotrophus in ammonium-limited continuous culture

2.3 Determination of the dry weights of bacterial suspensions

2.4 Preparation and fractionation of cell extracts

2.4.1 Preparation and fractionation of cell extracts used in the recording of cytochrome spectra

2.4.2 Preparation and fractionation of cell extracts used for transhydrogenase assays

2.5 Determination of cytochrome contents

2.5.1 Recording of reduced minus oxidised difference spectra

2.5.2 Recording of reduced plus CO minus reduced difference spectra

2.5.3 Quantitative estimation of cytochrome contents

2.6 Determination of respiration rates

2.7 Enzyme and metabolite assays

2.7.1 Nicotinamide nucleotide transhydrogenase assay by oxygen consumption

2.7.2 Nicotinamide nucleotide transhydrogenase assay by 3-acetylpyridine-NAD^+ reduction

2.7.3 Glucose-6-phosphate dehydrogenase assay

2.7.4 Formate assay

2.8 Determination of respiration-linked proton and charge translocation stoicheiometries

2.8.1 Determination of the stoicheiometry of respiration-linked proton translocation by the oxygen-pulse method

2.8.2 Determination of the stoicheiometries of respiration-linked proton and charge translocation by the initial-rate method

2.9 Determination of the electrical potential difference ($\Delta\Psi$) across the coupling membrane

2.10 Determination of the pH difference (Δ pH) across the coupling membrane

2.10.1 Determination of the intracellular buffering capacity

2.10.2 Determination of the Δ pH

2.11 Determination of the intracellular concentrations of adenine nucleotides and inorganic phosphate

2.11.1 Preparation of neutralised perchloric acid extracts

2.11.2 ATP assay using the luciferin/luciferase method

2.11.3 Conversion of AMP and ADP to ATP

2.11.4 Inorganic phosphate assay

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and biochemicals

2.1.1 Sources of chemicals and biochemicals

2,3,5,6-Tetramethyl-p-phenylene diamine ($DADH_2$) was obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset SP8 4JL.

L-ascorbic acid (vitamin C), sodium D-isoascorbate, L-malic acid, sodium pyruvate, Malachite Green, and Triton X-100 were obtained from BDH Ltd., Poole, Dorset BH12 4NN.

ATP (disodium salt), carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP), NADH (grade II, disodium salt), and NADPH (tetrasodium salt) were obtained from Boehringer Corporation (London) Ltd., Lewes, East Sussex BN7 1LG.

Sodium amytal was obtained from E. Lilly & Co. Ltd., Basingstoke, Hants.

Sodium D,L-lactate, sodium succinate, FisoFluor '2', Folin-Ciocalteu phenol reagent, and Fisons Trace Element solution were obtained from Fisons Ltd., Loughborough, Leics. LE11 ORG.

Duroquinol was obtained from ICN Pharmaceuticals, K & K Laboratories Division, Plainview, New York 11083, USA.

Polyoxyethylenesorbitan monolaurate (Tween 20) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Berks. SL3 OB2.

Oxoid Purified Agar was obtained from Oxoid Ltd., Basingstoke, Hants. RG24 OPW.

[1-^{14}C] Acetic acid (sodium salt, 40-60mCi/mmol), 5,5-dimethyl [2-^{14}C] oxazolidine-2,4-dione (DMO; 57mCi/mmol), and [^{14}C] methyltriphenylphosphonium iodide (TPMP$^+$, 9mCi/mmol in ethanol) were obtained from The Radiochemical Centre Ltd., Amersham, Bucks HP7 9LL.

AMP (type II, sodium salt), ADP (grade VI, sodium salt), NAD$^+$ (grade III), NADP$^+$ (sodium salt), 3-acetylpyridine-NAD$^+$ (grade I), D,L-isocitrate (trisodium salt), phosphoenolypyruvate (trisodium salt), glucose-6-phosphate (monosodium salt), N-ethylmaleimide (NEM), phenazine methosulphate (PMS), N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), N,N'-dicyclohexylcarbodiimide (DCCD), antimycin A, 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO), rotenone, dicumarol, valinomycin, glycylglycine (free base), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes, free acid), piperazine-N,N'-bis (2-ethanesulphonic acid) (Pipes, free acid), adenylate kinase (myokinase; grade III, 1,100-1,500 E.U./mg), alcohol dehydrogenase (yeast, 300-400 E.U./mg), catalase (bovine liver, 2,000-5,000 E.U./mg), carbonic anhydrase (bovine erythrocyte, 2,500 E.U./mg), cytochrome c (bovine heart, type IV), formate dehydrogenase (*Pseudomonas oxalaticus*, 0.3 E.U./mg), pyruvate kinase (type II, 350-500 E.U./mg), and dessicated firefly lanterns were obtained from Sigma (London) Chemical Co. Ltd., Poole, Dorset BH17 7NH.

Nigericin, [^{3}H] tetraphenylphosphonium bromide (TPP$^+$, 2mCi/μmol), and [1-^{14}C] phenylacetic acid (45mCi/mmol) were the generous gifts of Dr R.L. Hamil, Lilly Research Laboratories, E. Lilly and Co. Ltd., Indianapolis, Ind. 46206, U.S.A., Dr M. Drzyzga, Hoffman La Roche, Nutley, New Jersey 07110, U.S.A., and Dr R.A. Cooper, Dept. of Biochemistry, University of Leicester, Leicester LE1 7RH, respectively.

All other chemicals were of the ANALAR grade.

2.1.2 Preparation and assay of stock solutions

As ethanol acts as an electron donor to methanol dehydrogenase (Anthony & Zatman, 1965), all water-insoluble chemicals were dissolved in N,N'-dimethylformamide (DMF). Whenever DMF was used, control experiments were carried out to determine the effect, if any, of the solvent. The concentration of DMF in cell suspensions was never allowed to exceed 1% (v/v). The following chemicals were dissolved in DMF : antimycin A rotenone, duroquinol, FCCP, nigericin, and valinomycin. All other chemicals were dissolved in distilled water except amytal, HQNO and dicumarol, which were dissolved in dilute KOH, and $DADH_2$ which was dissolved in dilute HCl.

Adenine nucleotide standards, and HQNO, were assayed by A_{260} ($\varepsilon_{(AMP, ADP, ATP)_{260}}$ 15.3, 15.4, 15.4$mM^{-1}cm^{-1}$; Beaven et al., 1955; Bock et al., 1956), and A_{345} ($\varepsilon_{(HQNO)_{345}}$ 9.45 $mM^{-1}cm^{-1}$; Cornforth & James, 1956), respectively Formaldehyde was prepared by autoclaving 0.5g of paraformaldehyde plus 5ml of water in a sealed container for 3h, and assayed using NAD^+-linked alcohol dehydrogenase (Bernt & Bergmeyer, 1974) as follows. The 3ml reaction mixture contained 0.65M-potassium phosphate buffer (pH7.0), 75µM-NADH, 480 E.U. of alcohol dehydrogenase and 0-150nmoles of formaldehyde. After incubating for 30 min at room temperature, the A_{340} was measured against a reaction mixture with no added formaldehyde; and the difference was assumed to be due to the reduction of the total formaldehyde in the assay.

2.2 Growth and maintenance of bacterial cultures

2.2.1 Maintenance of stock cultures

M.methylotrophus (formerly Pseudomonas methylotropha; NCIB 10515; Byrom & 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 1l : K_2HPO_4, 1.9g; $NaH_2PO_4.2H_2O$, 1.56g; $(NH_4)_2SO_4$, 1.8g; $MgSO_4.7H_2O$, 20mg; $FeCl_3.6H_2O$, 9.7mg; Fisons Trace Element solution (Cu, 5p.p.m.; Mn, 24-25 p.p.m.; Zn, 22-23p.p.m.; Ca, 720p.p.m.), 1ml; Oxoid Purified Agar, 15g; methanol, 5ml; adjusted to pH7.2 with KOH.

2.2.2 Preparation of inocula

Bacteria used for the inoculation of continuous cultures were grown, at 37°C, as 100-150ml batch cultures in 500ml flasks which were aerated by shaking on a rotary shaker (L.H. Engineering Co. Ltd., Stoke Poges, Bucks. SL2 4EG) at approximately 300 r.p.m. The growth medium used was the methanol /salts solution described above, except that the methanol concentration was increased to 1% (v/v) (250mM). Bacteria were grown to a final density of approximately 1g dry wt. bacteria/l, and used within 12h of the end of the logarithmic growth phase.

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

The bacteria used for most of the experiments described in this thesis were grown at Leicester; however, those bacteria used for the determination of cytochrome contents (see sections 3.2, 5.2) were grown at I.C.I. Agricultural Division, Billingham. All bacteria were grown at pH7.0, 40°C, and at a dilution rate ($D \equiv \mu$, the specific growth rate) of $0.18h^{-1}$. The dissolved oxygen tension (D.O.T.) was monitored using a lead/silver galvanic electrode, and, for methanol-limited cultures, was maintained above 50% of air saturation. The purity of cultures was checked daily by plating on to nutrient agar and methanol/salts agar plates which were then incubated at 37°C for 72h.

At Leicester, cultures (approximate volume 970ml) were grown in a 1l laboratory fermenter (L.H. Engineering Co. Ltd.). The medium contained in 1l : $MgSO_4.7H_2O$, 0.27g; K_2SO_4, 35mg; K_2HPO_4, 0.113g; 1.1M-H_3PO_4, 3.75ml; Fisons Trace Element solution, 6ml; and methanol, 2ml (50mM). Iron was pumped into the fermenter vessel separately as $FeSO_4$ solution (0.16g of $FeSO_4.7H_2O$ plus 2ml of H_2SO_4/l) at a rate of 4ml/h to give an input concentration of 14.4μM. The medium described above is acidic (pH ≃ 3.0), and the pH was controlled at 7.0 ± 0.1 by automatic addition of 10% (w/v) NH_4OH which also acted as the nitrogen source for growth. Cell densities of approximately 0.65g dry wt. bacteria/l were routinely achieved.

At Billingham, cultures (approximate volume 2½l) were grown in a 5l Porton-style fermenter (Baker, 1968) to a substantially higher cell density (approximately 22g dry wt. bacteria/l) than achieved at Leicester. The medium contained in 1l: $MgSO_4.7H_2O$, 1.62g; K_2SO_4, 0.205g; K_2HPO_4, 0.685g; 1.1M-H_3PO_4, 21.5ml; Fisons Trace Element solution, 36ml. Methanol and iron were introduced as a methanolic $FeCl_3$ solution to give input

concentrations of 1.56M and 3.57mM respectively. The nitrogen-source was NH_3 gas which was also used for pH control.

2.2.4 Growth of M.methylotrophus in oxygen-limited continuous culture

Oxygen limitation was achieved at Leicester by increasing the concentration of methanol in the medium to 100mM, whereas at Billingham the methanol concentration was unchanged but the aeration rate was decreased. Otherwise, the conditions for growth in oxygen-limited continuous culture were the same as for growth in methanol-limited culture. Cell densities achieved in oxygen-limited continuous culture were approximately 1 and 19g dry wt. bacteria/l at Leicester and Billingham respectively.

During growth in oxygen-limited continuous culture the D.O.T. was close to zero, and no increase was observed after switching off the supply of methanol for 5 min.

2.2.5 Growth of M.methylotrophus in ammonium-limited continuous culture

Bacteria were grown in ammonium-limited continuous culture only at Billingham. The conditions were as for methanol-limited culture except that the input methanol concentration was decreased to 0.69M, 3.42g of $(NH_4)_2SO_4$/l was added to the medium, and pH control was achieved using 4M-NaOH/4M-KOH instead of NH_3 gas. A cell density of 7.5g dry wt. bacteria/l was routinely achieved.

During growth in ammonium-limited continuous culture the D.O.T. was maintained above 50% of air saturation but no increase was observed after switching off the supply of methanol for 5 min.

2.3 Determination of the dry weights of bacterial suspensions

In all the experiments reported in this thesis, dry weights of bacterial suspensions were determined from the A_{680}. The relationship between the dry weight and A_{680} was determined as follows. Washed cell suspensions (in distilled water) were prepared to a range of optical densities, then filtered under negative pressure through preweighed Sartorius membrane filters (pore dia. 0.45μM; Sartorius Membranfilter GmbH, Göttingen, West Germany). The filters were dried overnight in a drying oven (approximate temperature 80°C), allowed to cool, and then reweighed. Correction was made for a small loss in weight of unused filters dried in the same way. The A_{680} was found to be a linear function of the dry weight up to at least A_{680} 1 ≡ 0.63mg dry wt. bacteria/ml.

2.4 Preparation and fractionation of cell extracts

2.4.1 Preparation and fractionation of cell extracts used in the recording of cytochrome spectra

Bacteria were harvested (12,000g, 10 min), washed with 25mM-Hepes/KOH buffer (pH7.0), and resuspended in this same buffer. Cells were broken either by sonication (5, 1 min periods), or by passing twice through an Aminco French pressure cell (15,000psi; American Instrument Co., Maryland 20910, U.S.A.). Unbroken cells and cell debris were then spun-down (12,000g, 10 min) leaving the crude cell extract. Fractionation of the crude extract into membrane and soluble fractions was achieved by high-speed centrifugation (150,000g, 2h). After decanting the soluble fraction, the surface of the membrane pellet was washed with 20mM-KH_2PO_4/KOH buffer

(pH7.0), and the pellet was resuspended in this same buffer using a hand homogeniser. Throughout the preparation procedure cell extracts were kept at close to $0^{o}C$.

Protein contents were assayed by the method of Lowry et al. (1951) using the Folin-Ciocalteu phenol reagent.

2.4.2 Preparation and fractionation of cell extracts used for transhydrogenase assays

The procedure was essentially as above except that bacteria were washed and resuspended in 140mM-KCl, 10mM-glycylglycine/KOH buffer (pH7.0); the membrane pellet was also resuspended in this same buffer. Sonication was used exclusively for cell disruption; and protein contents were assayed using the Biuret reagent (Gornall et al., 1949), correction being made for a slight interference by glycylglycine.

2.5 Determination of cytochrome contents

2.5.1 Recording of reduced minus oxidised difference spectra

All spectra were recorded using a Hitachi Perkin-Elmer 557 split-beam/dual-wavelength spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, Bucks. HP9 1QA), with a slit width of 2nm. Spectra were recorded either at room-temperature, or at 77K using a liquid-nitrogen cooled attachment (cuvette path length 2mm; see Bonner, 1961).

For room-temperature spectra, the air-oxidised minus air-oxidised baseline was first recorded and stored in the spectrophotometer's micro-computer. The contents of the sample cuvette (path length 10mm) were then

reduced with a few grains of sodium dithionite, and the contents of the reference cuvette were oxidised with 5µl of 2 volume H_2O_2. After approximately 2 min, the reduced minus oxidised difference spectrum was recorded, automatic correction being made for the baseline spectrum. The procedure for low temperature spectra was similar except that no baseline correction was made.

2.5.2 Recording of reduced plus CO minus reduced difference spectra

For room-temperature spectra, the contents of both cuvettes were reduced with dithionite, and after approximately 2 min the reduced minus reduced baseline was recorded and stored. CO was bubbled for 1 min through the sample cuvette, and this was then incubated in the dark. After 10 min, the reduced plus CO minus reduced difference spectrum was recorded, automatic correction being made for the baseline spectrum. Again, the procedure for low temperature spectra was similar except that no baseline correction was made.

2.5.3 Quantitative estimation of cytochrome contents

Cytochrome contents were estimated both from the reduced minus oxidised difference spectrum and, where applicable, from the reduced plus CO minus reduced difference spectrum. For the former, the molar extinction coefficients used were those of the mammalian cytochromes aa_3 ($16mM^{-1}cm^{-1}$), b ($22mM^{-1}cm^{-1}$) and c ($19mM^{-1}cm^{-1}$) (see Chance, 1957) for the wavelength pairs 601-625nm, 558-575nm and 550-535nm respectively. The CO-binding cytochromes, aa_3, o and c_{co}, were estimated from the Δ absorbance between peaks at 428nm, 418nm and approximately 414nm respectively, and the absorption trough in the 430-450nm region of the reduced plus CO minus

reduced difference spectrum. The molar extinction coefficients of cytochromes $\underline{aa}_3$ and $\underline{o}$ were taken to be the same as those of mammalian cytochrome $\underline{aa}_3$ ($148mM^{-1}cm^{-1}$; Vanneste, 1965), and bacterial cytochrome $\underline{o}$ ($170mM^{-1}cm^{-1}$; Daniel, 1970), respectively. No accurate value for the molar extinction coefficient of cytochrome $\underline{c}_{co}$ is available, but a value of $55mM^{-1}cm^{-1}$, derived from the 3-haem cytochrome $\underline{c}_{553}$ of Chromatium (εmM $165mM^{-1}cm^{-1}$; Bartsch & Kamen, 1960), has been adopted for the cytochromes $\underline{c}_{co}$ of Beneckea natriegens (Weston & Knowles, 1974), and of some methylotrophs (e.g. Tonge et al., 1974). This value, however, surely represents a minimum estimate as it assumes that all three haems of the Chromatium cytochrome exhibit stoicheiometric (1:1) CO-binding; and instead a value of $159mM^{-1}cm^{-1}$, intermediate between the molar extinction coefficients of cytochromes $\underline{o}$ and $\underline{aa}_3$, has been used here.

2.6 Determination of respiration rates

Respiration rates were measured at 40°C using a polarographic oxygen electrode (Rank Bros., Bottisham, Cambridge CB5 9DA) fitted with a thin teflon membrane. The electrode had a 90% response time to the addition of dithionite of approximately 2.5s. Oxygen uptake traces were recorded on a Servoscribe 2s potentiometric recorder (Smith's Industries Ltd., London NW2 7UR). The reaction conditions are described for individual experiments.

2.7 Enzyme and metabolite assays

2.7.1 Nicotinamide nucleotide transhydrogenase assay by oxygen consumption

The assay mixture, which was maintained at 40°C, contained 25mM-phosphate (Na_2HPO_4, KH_2PO_4) buffer (pH7.4) plus cell fraction(s) (see section 2.4.2), as required, in a total volume of 2.4ml. The reaction was started by the addition of NADPH (1.5mM), and the oxygen uptake rate was measured as in section 2.6. When a steady rate was achieved, NAD^+ (1mM) was added, and any stimulation of oxygen uptake was noted.

2.7.2 Nicotinamide nucleotide transhydrogenase assay by 3-acetylpyridine-NAD^+ reduction

This assay is based on the reaction first described by Stein *et al.* (1959):

$$\text{NADPH} + \text{3-acetylpyridine-NAD}^+ \longrightarrow \text{3-acetylpyridine-NADH} + \text{NADP}^+ \qquad (2.1)$$

In the presence of transhydrogenase, NADPH will reduce 3-acetylpyridine-NAD^+ thus causing a shift in the absorption maximum. The 3-acetylpyridine-NADH formed is only slowly reoxidised by the respiratory chain. The 1ml reaction mixture, which was maintained at 40°C, contained 20mM-phosphate (Na_2HPO_4, KH_2PO_4) buffer (pH7.6), 2mM-3-acetylpyridine-NAD^+, and cell fraction (see section 2.4.2) as required. The reaction was started by the addition of NADPH (0.36mM), and 3-acetylpyridine-NAD^+ reduction was monitored by the increase in A_{375} ($\varepsilon_{(3\text{-acetylpyridine-NADH})_{375}}$ *minus* $\varepsilon_{(NADPH)_{375}} = 5.1 mM^{-1}cm^{-1}$).

2.7.3 Glucose-6-phosphate dehydrogenase assay

The total (NAD^+-plus $NADP^+$-linked) glucose-6-phosphate dehydrogenase activity was assayed, at 40°C, by $NAD(P)^+$ reduction:

$$\text{Glucose-6-phosphate} + NAD(P)^+ \longrightarrow \text{6-phosphogluconate} + NAD(P)H + H^+ \qquad (2.2)$$

The 3ml reaction mixture contained 0.1M-Tris/HCl buffer (pH8.0), 3.3mM-$MgCl_2$, 0.33mM-NAD^+, 0.33mM-$NADP^+$, and a sample containing glucose-6-phosphate dehydrogenase. The reaction was started by the addition of glucose-6-phosphate (0.33mM), and NAD(P)H formation was monitored by A_{340} ($\varepsilon_{(NAD(P)H)_{340}}$ $6.2mM^{-1}cm^{-1}$).

2.7.4 Formate assay

Formate was assayed, at room temperature, using NAD^+-linked formate dehydrogenase, by the method of Hopner & Knappe (1974):

$$HCOO^- + NAD^+ + H_2O \longrightarrow HCO_3^- + NADH + H^+ \qquad (2.3)$$

The 1ml reaction mixture contained 40mM-KH_2PO_4/KOH buffer (pH7.0), 1mM-NAD^+, and an aqueous sample containing 30-300nmoles of formate. The reaction was started by the addition of 0.4 E.U. of formate dehydrogenase, and NADH formation was monitored at 340nm against a blank containing no added formate. The net NADH formation was determined from the maximum of the extinction-time curve, this being reached within 3-5 min. Owing to the NADH and formate oxidase activities of the formate dehydrogenase, the formation of NADH is not stoicheiometric with formate removal but is strictly proportional to it. The proportionality factor was determined for each batch of enzyme (made up daily) by calibration with known amounts of sodium formate.

2.8 Determination of respiration-linked proton and charge translocation stoicheiometries

2.8.1 Determination of the stoicheiometry of respiration-linked proton translocation by the oxygen-pulse method

The oxygen-pulse technique used was essentially that of Mitchell & Moyle (1967a). The pH was monitored using a Beckman 4500 pH meter (Beckman Instruments Inc., California 92634, U.S.A.) fitted with a Russell combination micro-electrode (Russell pH Ltd., Auchtermuchty, Fife, Scotland K714 7DP), and recorded on a Servoscribe 2s potentiometric recorder. The micro-electrode was fitted through the perspex top of a Rank oxygen electrode chamber, only a very narrow aperture being open for the addition of reagents by microsyringes.

The conditions for the measurement of proton translocation stoicheiometries were optimised for the oxidation of endogenous substrates (see sections 4.2, 5.6). The 5ml reaction mixture contained 140mM-KCl, 1.5mM-glycylglycine/KOH buffer, 25-35mg dry wt. of washed bacterial suspension, 1μg of valinomycin/mg dry wt. bacteria, 250μg of carbonic anhydrase, and substrates and inhibitors as required. The K^+ ionophore, valinomycin, was present in order to allow collapse of the $\Delta\Psi$ which would otherwise build up and oppose further proton translocation; and carbonic anhydrase was present to prevent any pH overshoots which might be caused by slow reequilibration of the CO_2/HCO_3^- equilibrium after a pH change (see Scholes & Mitchell, 1970a).

Bacterial suspensions were maintained at 40°C and allowed to attain anaerobiosis by the oxidation of endogenous substrates. Valinomycin was added after anaerobiosis was achieved, and the suspensions were then

allowed to equilibrate for 30 min. Substrates and inhibitors, when required, were added, and the pH was adjusted to 6.2 by small additions of anaerobic KOH or HCl.

Oxygen was introduced in 5-50µl volumes of air-saturated 140mM-KCl, approximately 10 different volumes being used for each determination. The resulting acidifications of the external medium were calibrated by 1-10µl additions of anaerobic 10mM-KOH. Acidification was complete within 3-4s of adding oxygen, and the ΔpH was estimated by extrapolating back to the time at which half the pH rise had occurred (Mitchell & Moyle, 1965). (The solubility of oxygen in air-saturated 140mM-KCl was determined by measuring the fraction consumed by known amounts of NADH in the presence of PMS and catalase [Robinson & Cooper, 1970], and found to be 0.38µg-atom O/ml, at 40°C). The $\rightarrow H^+/O$ quotient was calculated from plots of the ΔpH, corrected by extrapolation, against the amount of oxygen added; and the half-time of decay of the ΔpH resulting from an oxygen pulse was determined from plots of log ΔpH against time.

2.8.2 Determination of the stoicheiometries of respiration-linked proton and charge translocation by the initial-rate method

Respiration-linked proton translocation stoicheiometries were also determined by a method, previously applied to mitochondria (Reynafarje *et al.*, 1976), in which initial rates of respiration and acidification were measured following the addition of substrate to aerobic suspensions of bacteria; the pH of the substrate being accurately matched to that of the bacterial suspension.

Cell suspensions were incubated at 40°C in screw-topped bottles, under anaerobic conditions, in the same reaction mixture as used in the oxygen-

pulse method. After 30 min, the cell suspensions were aerated by shaking briefly, and transferred to the chamber of a Rank oxygen electrode which was fitted with a pH electrode as above (section 2.8.1). Once the electrode outputs had stabilised (approximately 5 min), the substrate was added, and the resulting acidification and oxygen consumption were recorded and calibrated as before (sections 2.6, 2.8.1). The $\rightarrow H^+/O$ quotient was calculated from the ratio of the initial rates of acidification and oxygen consumption, corrected for the endogenous rates.

The stoicheiometry of respiration-linked charge translocation was determined in a similar procedure by monitoring the concentration of the counter-ion K^+ (in the presence of valinomycin; Reynafarje & Lehninger, 1978), using a K^+ selective electrode and sulphate reference electrode (Chemetric Ltd., Sherborne, Dorset DT9 5ER). In order to accommodate the additional electrodes, the perspex top of the Rank oxygen electrode chamber was replaced by parafilm. The reaction mixture was the same as that used for the measurement of $\rightarrow H^+/O$ quotients except that the KCl concentration was lowered to 10mM (140mM-LiCl was present to maintain the ionic strength), and the buffer concentration was increased to 10mM-glycylglycine/LiOH. Both the pH and p$\underline{K}$ of added substrates were accurately matched to those of the bacterial suspension. K^+ movements were calibrated by 5-25µl additions of 100mM-KCl, and simultaneous H^+ movements, when measured, were calibrated by 1-10µl additions of 100mM-LiOH. (The solubility of oxygen in air-saturated 10mM-KCl, 140mM-LiCl was found to be 0.41µg-atom O/ml at 40°C; Robinson & Cooper, 1970).

2.9 Determination of the electrical potential difference ($\Delta\Psi$) across the coupling membrane

The $\Delta\Psi$ component of the protonmotive force was estimated from the uptake of either of the permeant cations [^{14}C] TPMP$^+$ or [^{3}H] TPP$^+$ (e.g. Ramos *et al.*, 1976; Rottenberg, 1979). The concentration of the free cation in the reaction mixture was monitored by flow dialysis using apparatus similar to that described by Colowick & Womack (1969). The flow dialysis cell consisted of a vertical arrangement of two reaction chambers which were both maintained at 40°C by immersing in a water bath. The upper chamber, which was open to the air, was separated from the lower chamber (volume 1.3ml) by Visking dialysis tubing (pore dia. 2.4nm; Scientific Supplies Co. Ltd., London EC1R 5EB) which had previously been boiled for 10 min in 10mM-EDTA.

The upper chamber of the flow dialysis apparatus contained initially 0.8ml of buffer (see individual experiments) plus either 20nmoles (when [^{14}C] TPMP$^+$ was used), or 50nmoles (when [^{3}H] TPP$^+$ was used) of methanol. The experiment was started by the addition of either 4µl of [^{14}C] TPMP$^+$ (11.25mM in ethanol), or 10µl of [^{3}H] TPP$^+$ (16µM). After approximately 5 min, 200µl of a cell suspension (15 mg dry wt./ml in buffer) was added to the reaction chamber, and this was followed 7 min later by 25nmoles of FCCP. The experiment was terminated approximately 6 min after addition of the FCCP.

Throughout this procedure, the same buffer which was used in the reaction mixture was flowed through the lower chamber at a constant rate of 2ml/min using an LKB 12000 Varioperpex peristaltic pump (LKB-Produkter AB, S-16125 Bromma 1, Sweden), and 1ml buffer samples were collected manually every 30s, 4ml of FisoFluor '2' was added to each sample, and the radioactivity was then counted (5000 c.p.m. total) using the pre-set channels

of a Hewlett-Packard 3385 Tri-carb liquid scintillation counter (Packard Instrument Ltd., Caversham, Bucks. RG4 7AA). Correction was made for the background radiation which rarely exceeded 2% of the total counts.

The internal concentration of the radiolabelled permeant cation was calculated from the amount of radioactivity released from the bacteria after the addition of 25μM-FCCP, using a value for the internal volume of M.methylotrophus of 1.06 ± 0.04(4)μl/mg dry wt. bacteria which was obtained by the method of Murastugu et al. (1979) using [^{14}C] mannitol and [^{3}H] H_2O (McKay, A.M., unpublished results). The accumulation ratio was thus determined, and the $\Delta\Psi$ was calculated from this using the Nernst equation:

$$\Delta\Psi_{(in-out)} = 2.303\frac{RT}{F}\log_{10}\left(\frac{[cation]_{(out)}}{[cation]_{(in)}}\right) \qquad (2.4)$$

2.10 Determination of the pH difference (ΔpH) across the coupling membrane

2.10.1 Determination of the intracellular buffering capacity

The intracellular buffering capacity (dH^+/dpH; ng-ion H^+/pH unit/5ml of bacterial suspension) was determined as the difference between the extracellular buffering capacity, measured in an intact cell suspension, and the total (extracellular plus intracellular) buffering capacity, measured after rendering the bacteria fully permeable to protons (Mitchell & Moyle, 1969; Collins & Hamilton, 1976). Buffering capacities were determined as a function of the extracellular pH by varying the latter from approximately pH6.0 to 8.0 in steps of 0.1 to 0.2pH units using suitable amounts (10-20μl) of anaerobic 20mM-NaOH. The buffering capacity could thus be determined by dividing the amount of NaOH added by the resulting pH change.

The determination of buffering capacities was carried out in the chamber of a Rank oxygen electrode which was fitted with a pH electrode, sealed, and maintained at 40°C. The pH was measured and recorded as described in section 2.8.1. The 5ml reaction mixture contained 140mM-KCl, 15mg dry wt. of washed bacterial suspension and 250µg of carbonic anhydrase.

Prior to the measurement of the external buffering capacity (B^E; the superscripts E, I and T refer to the extracellular, intracellular and total [intracellular plus extracellular] phases respectively), cell suspensions were rendered anaerobic by respiration following the addition of 1mM-methanol, then allowed to equilibrate for 15 min. After the measurement of B^E, as above, the pH was returned to approximately 6.0, and the bacteria were fully permeabilised by the addition of 10µM-FCCP, 1µg of valinomycin/mg dry wt. bacteria and 0.4% (v/v) Triton X-100. Following a 15 min re-equilibration period, the total buffering capacity (B^T) was determined. It should be noted, however, that B^T, determined as above, includes the buffering capacities of the reagents added to permeabilise the cells, and must be corrected accordingly. The buffering capacity of the FCCP plus valinomycin plus Triton X-100 (B^R) was thus determined as a function of pH in a medium containing 140mM-KCl plus 250µg of carbonic anhydrase only. B^R was calculated as the difference in the buffering capacities determined before and after the addition of these reagents.

The intracellular buffering capacity of <u>M.methylotrophus</u> is thus given by the equation:

$$B^I = (B^T - B^R) - B^E \qquad (2.5)$$

This expression actually represents an approximation to the internal buffering capacity; the true value being given by:

$$(B^T - B^R) - B^E = B^I \left[1 - (d\Delta pH/_{dpH^E}) \right] \qquad (2.6)$$

where ΔpH is the transmembrane pH gradient under the conditions of determination of the extracellular buffering capacity (see Mitchell & Moyle, 1969).

2.10.2 Determination of the ΔpH

The ΔpH was determined from the change in extracellular pH occurring after the transition of a respiring bacterial suspension from aerobic to anaerobic conditions (Mitchell & Moyle, 1969; Collins & Hamilton, 1976). The 5ml reaction mixture, which was maintained at 40°C in the chamber of a Rank oxygen electrode, contained buffer (see individual experiments), approximately 15mg dry wt. of washed bacterial suspension, 50mM-methanol, 250µg of carbonic anhydrase and 100µg of catalase.

The pH was adjusted as required and, after a 15 min equilibration period, the cell suspension was rendered aerobic by the addition of 50µl of 2 volume H_2O_2. The pH and oxygen concentrations were recorded as described previously (see sections 2.6, 2.8.1), and the latter was maintained above 50% of air-saturation by further additions of H_2O_2 as necessary (50µl of 2 volume H_2O_2 was sufficient to maintain aerobiosis for approximately 2.5 min). After approximately 7.5 min the cell suspension was allowed to become anaerobic, and the pH was monitored for a further 20 min. The pH changes occurring between the onset of anaerobiosis (pH_0^E ; the subscripts A, N and O, refer to aerobic conditions, the final anaerobic state, and the transition between aerobiosis and anaerobiosis,

respectively) and the final anaerobic state (pH_N^E) were calibrated by 10-20µl additions of anaerobic 20mM-NaOH. From these calibrations, the total proton movements occurring after anaerobiosis (δH^{+E}) could be determined. The internal pH of respiring cells (pH_A^I) could thus be calculated by manual integration of the internal buffering capacity which was measured in the same batch of cells (see section 2.10.1):

$$\delta H^{+E} = \int_{pH_N^I}^{pH_A^I} B^I dpH^I \qquad (2.7)$$

(Provided that there is no ΔpH in the final anaerobic state, $pH_N^I = pH_N^E$). The ΔpH of aerobic energised cells is thus given by:

$$\Delta pH = pH_A^I - pH_o^E \qquad (2.8)$$

2.11 Determination of the intracellular concentrations of adenine nucleotides and inorganic phosphate

Determinations were carried out either directly on methanol-limited continuous cultures of M.methylotrophus, or on washed bacterial suspensions. Unless otherwise stated, the latter (5ml volume) were incubated at 40°C, with rapid stirring, either aerobically in open 25ml beakers, or anaerobically in small bottles (20ml nominal volume) fitted with Suba-Seal stoppers through which oxygen-free nitrogen gas was flowed via two syringe needles. The conditions and periods of incubation are described for individual experiments.

2.11.1 Preparation of neutralised perchloric acid extracts

Growing cells were sampled and quenched directly from the chemostat using a spring-loaded syringe which drew 1.5ml of culture into 0.5ml of cold 25% (v/v) perchloric acid. Bacterial suspensions were likewise quenched by the addition of cold 25% (v/v) perchloric acid to a final concentration of 6% (v/v), but the acid was added to the cell suspension via a conventional syringe. The acid-quenched mixtures were kept on ice for at least 10 min in order to ensure complete inactivation of cellular enzymes, and then centrifuged (12,000g, 10 min) to remove precipitated material. The supernatants were approximately neutralised (pH6.8-7.2), on ice with constant stirring, using a mixture of saturated KOH/2M-Tris base/distilled water in the volumetric proportions 30/60/10. After 5 min on ice to ensure complete precipitation, the $KClO_4$ was removed by centrifugation (12,000g, 10 min), and the neutralised extracts were stored at 0-4°C.

The levels of AMP, ADP, ATP and inorganic phosphate, were found to be unchanged after several hours storage on ice of either acid-quenched cells, or neutralised extracts; and furthermore the latter could be frozen overnight with no change in the concentrations of these metabolites. AMP, ADP, ATP or phosphate, added immediately after quenching, could be completely recovered from neutralised extracts (recoveries were 98%, 109%, 100% and 95% respectively), thus indicating that there was no significant loss of these metabolites by adsorption to the $KClO_4$ precipitate, under these conditions (see Wiener et al., 1974).

The efficiency of quenching was checked by comparing the concentrations of AMP, ADP, ATP and inorganic phosphate obtained after quenching anaerobic cell suspensions with either aerobic, or nitrogen-sparged, perchloric acid

(see Hempfling, 1970) - no difference between the results obtained by these two procedures was observed. Likewise, similar results were obtained when aerobic, energised bacterial suspensions were quenched using a spring-loaded syringe (approximate sampling time 100ms; Niven et al., 1977), or by adding acid more slowly from a conventional syringe (approximate quenching time 1s). A second perchloric acid extraction of the original perchloric acid-precipitated material released only a further 2% of the total amount of adenine nucleotides released in the first extraction, and very little phosphate.

The concentrations of adenine nucleotides and phosphate in cell extracts were converted into intracellular concentrations, after taking into account the dilution involved in the quenching procedure, using a value of 1.06 µl/mg dry wt. bacteria for the intracellular volume of M.methylotrophus (see section 2.9).

2.11.2 ATP assay using the luciferin/luciferase method

In the presence of excess 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, 1947; McElroy & Strehler, 1949; Strehler & Trotter, 1952; Kimmich et al., 1975). The basic reaction is shown in equations (2.9, 2.10); only a transient flash of light is produced due to the complex end-product inhibition observed, particularly with crude enzyme preparations (see McElroy & DeLuca, 1973; DeLuca, 1976).

$$\text{ATP} + \text{luciferin} \underset{Mg^{2+},\ \text{luciferase}}{\rightleftharpoons} \text{adenyl-luciferin} + \text{PPi} \qquad (2.9)$$

$$\text{adenyl-luciferin} + O_2 \longrightarrow \text{oxyluciferin} + \text{AMP} + CO_2 + h\nu \qquad (2.10)$$

The assay conditions used were essentially those of Kimmich et al. (1975) except that an Aminco Chem-Glow photometer (American Instruments Co., Maryland 20910, U.S.A.) was used to measure the initial flash intensity, the response being recorded on a Servoscribe potentiometric recorder.

The luciferin/luciferase solution was prepared and 'activated' as per Kimmich et al. (1975). 1g of firefly lanterns was ground with 100ml of ice-cold 20mM-glycylglycine, 20mM-$MgSO_4$, 50mM-sodium arsenate/H_2SO_4 buffer (pH7.4). The residue was removed by centrifugation (12,000g, 10 min), and the extract was mixed with 1.6g of $Ca_3(PO_4)_2$, and then swirled for 10 min at room temperature. After this period, the $Ca_3(PO_4)_2$ was spun-down (12,000g, 2 min) and the supernatant was retreated with fresh $Ca_3(PO_4)_2$, as above. After removing the $Ca_3(PO_4)_2$ again (12,000g, 10 min), the clear straw-coloured supernatant was frozen in aliquots. (This preparation was found to be adequately stable for about 2 months when frozen).

The assay mixture contained, in a 6x50mm tube, 250µl of 5mM-sodium arsenate, 4mM-$MgSO_4$, 20mM-glycylglycine/NaOH buffer (pH8.0), and a 50µl sample containing 0-50 pmoles of ATP. The tube was entered into the reaction chamber of the photometer, and 100µl of the luciferin/luciferase solution (equilibrated to room temperature) was injected into the assay mixture using a Hamilton repeating dispenser (Hamilton-Bonaduz, Bonaduz, Switzerland). The peak-height of the flash produced was found to be directly proportional to the amount of ATP in the sample (Fig. 2.1).

The luciferin/luciferase reaction is readily inhibited by a number of common anions, including Cl^- and ClO_4^- (Strehler & Trotter, 1952; Denburg & McElroy, 1970; Lundin & Thore, 1975), and so ATP contents were calibrated by internal standardisation. This procedure involved duplicate assays -

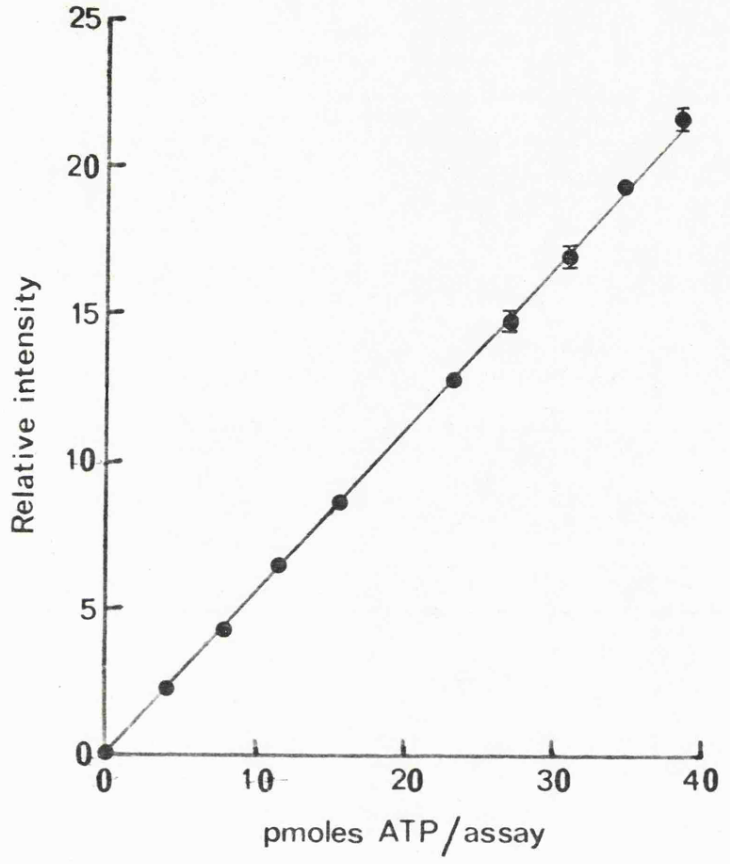


Fig. 2.1 Relationship between the ATP content and light emission in the luciferin/luciferase assay. The assay was carried out as described in section 2.11.2. Each point on the graph is the mean of three determinations, and the standard error is shown by error bars.

20 pmoles of standard ATP was added to one of the assay mixtures, and the ATP content was then calibrated using the difference between the readings for the duplicate assays. The use of internal calibration also corrected for the slow loss of luciferase activity during the time course of the determinations (approximately 10%/hr). A further small correction was made for the flash intensity obtained on injecting luciferin/luciferase into an ATP-free assay mixture.

Control experiments confirmed that zero readings were obtained when either AMP or ADP replaced ATP in the assay mixture. Furthermore, the presence of high levels of AMP or ADP did not affect the reading obtained for ATP, under these conditions (see McElroy & DeLuca, 1973).

2.11.3 Conversion of AMP and ADP to ATP

AMP and ADP levels were determined after conversion of either ADP alone (pyruvate kinase), or AMP plus ADP (pyruvate kinase and adenylate kinase), to ATP as follows:

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow[\text{pyruvate kinase}]{} \text{ATP} + \text{pyruvate} \qquad (2.11)$$

$$\text{AMP} + \text{ATP} \underset{\text{adenylate kinase}}{\rightleftharpoons} 2\text{ADP} \qquad (2.12)$$

The 500µl reaction mixture contained 400µl of an adenine nucleotide-containing sample, 1mM-phosphoenolpyruvate, 50 E.U. of pyruvate kinase, 50 E.U. of adenylate kinase (only present for the conversion of AMP plus ADP to ATP), 3mM-$MgSO_4$, and 21mM-Tris/acetate buffer (pH7.3). Mixtures were incubated at 30°C for 1h, then stored on ice and assayed within 2h. Almost quantitative conversions of AMP and ADP standards to ATP were achieved (97 ± 3(6)% and 95 ± 4(6)% respectively). It was found that some

published conversion procedures (e.g. Pradet, 1967; Niven et al., 1977) worked adequately with AMP and ADP standards, but failed to achieve complete conversion of adenine nucleotides in perchloric acid-extracts. Using the increased enzyme levels shown here, however, AMP and ADP added to cell extracts could be completely recovered (see section 2.11.1). Control experiments confirmed that, in the absence of adenylate kinase, there was no conversion of AMP to ATP.

2.11.4 Inorganic phosphate assay

The assay used for the determination of inorganic phosphate was based on that described by Itaya & Ui (1966), in which a phosphomolybdate complex is used to shift the absorption maximum of the dye, Malachite Green. To make the colour-developing reagent, 1 volume of ammonium molybdate/5M-HCl was added to 3 volumes of 0.05% (w/v) Malachite Green solution. The mixture was then allowed to stand at room temperature for 30 min before filtering to remove a green precipitate from the straw-coloured reagent. The assay mixture consisted of a 1ml sample containing 0-100nmoles of inorganic phosphate, and 5ml of the colour-developing reagent. This mixture was incubated on ice for 30 min before the addition of 0.2ml of 1.5% (w/v) Tween 20. The assay mixture was then transferred to room temperature for 15 min before measuring the A_{650} of the green colour formed against a water blank. A basically linear relationship between the A_{650} and inorganic phosphate content was observed (Fig. 2.2).

Control experiments confirmed that the acid conditions of the assay did not cause the release of inorganic phosphate from AMP, ADP or ATP standards.

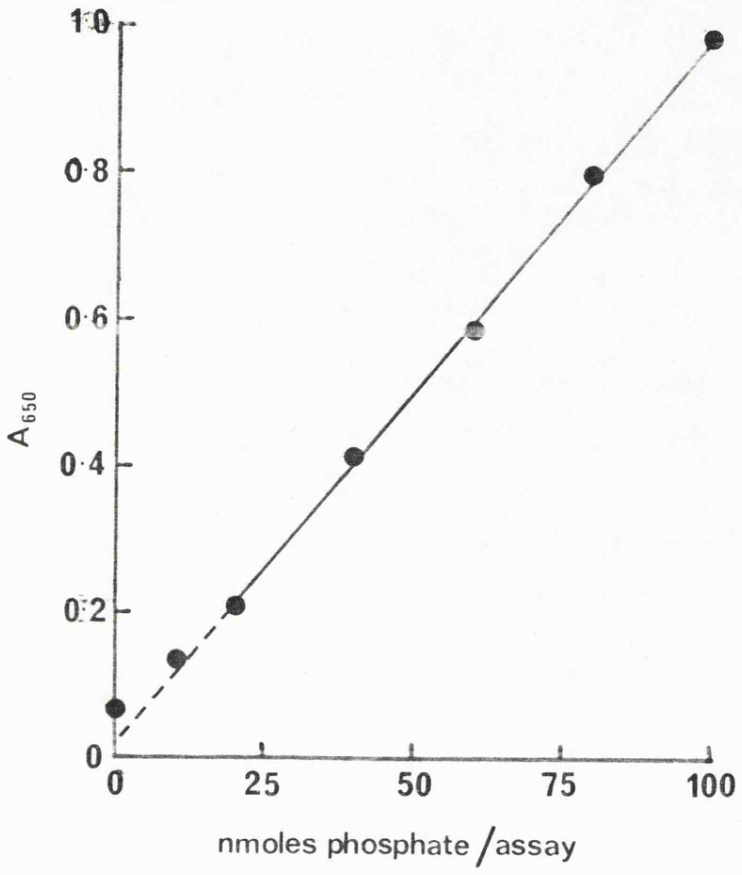


Fig. 2.2 Calibration curve for the inorganic phosphate assay. The assay was carried out as described in section 2.11.4. The inorganic phosphate standards were made from KH_2PO_4 which had been dried overnight in a drying oven.

All the numerical results in this thesis are given as the mean $\pm$ the standard error of the mean, with the number of determinations in parenthesis.

CHAPTER 3

RESPIRATORY CHAIN OF *METHYLOPHILUS METHYLOTROPHUS* GROWN IN METHANOL-LIMITED CONTINUOUS CULTURE

3.1 Introduction

3.2 Cytochrome content of *M.methylotrophus*

3.3 Nicotinamide nucleotide transhydrogenase activity of *M.methylotrophus*

3.4 Respiratory activity of whole cells of *M.methylotrophus*

3.5 Effect of inhibitors on the respiratory activity of whole cells of *M.methylotrophus*

3.5.1 Inhibition of the respiratory chain prior to the terminal oxidase region

3.5.2 Inhibition of the terminal region of the respiratory chain by cyanide and azide

3.6 Discussion and conclusions

CHAPTER 3

RESPIRATORY CHAIN OF *METHYLOPHILUS METHYLOTROPHUS* GROWN IN METHANOL-LIMITED CONTINUOUS CULTURE

3.1 Introduction

The cytochrome content of a large number of methylotrophic bacteria has now been investigated (Anthony, 1970, 1975a; Ribbons *et al.*, 1970; Hersh *et al.*, 1971; Davey & Mitton, 1973; Tonge *et al.*, 1974; Monosov & Netrusov, 1975; Weaver & Dugan, 1975; Babel & Steudel, 1977; Drabikowska, 1977; Bamforth & Quayle, 1978; Cross & Anthony, 1978; van Verseveld & Stouthamer, 1978). Although some early investigations failed to detect any *b*-type cytochromes from difference spectra of whole organisms (Ribbons *et al.*, 1970; Davey & Mitton, 1973; Weaver & Dugan, 1975), it soon became clear that this was due to masking by large relative amounts of cytochrome *c*. Indeed, all methylotrophs studied so far are now known to contain cytochromes of the *a*-, *b*- and *c*-types. Studies involving cell fractionation (Anthony, 1970, 1975a; Monosov & Netrusov, 1975; Drabikowska, 1977; Bamforth & Quayle, 1978; Cross & Anthony, 1978) have shown that cytochromes of the *a*- and *b*-types are exclusively membrane-bound, whereas cytochrome *c* is found both in the membrane and soluble fractions. It is not yet clear, however, whether the cytochrome *c* found in the soluble fraction is genuinely soluble *in vivo*, or bound loosely to the membrane and released during cell breakage.

All methylotrophic bacteria studied so far have been found to contain multiple CO-binding cytochromes. A CO-binding cytochrome of the *c*-type (cytochrome c_{CO}) is present during methylotrophic growth of all bacteria,

and in addition CO-binding cytochromes of the a-type (cytochrome aa_3) and b-type (cytochrome o) are often found, though the latter is rather difficult to identify positively in the presence of cytochromes aa_3 and c_{co}. Although CO-binding by cytochrome c is a relatively unusual feature, cytochromes c_{co} have now been found in a wide range of bacteria including aerobes, anaerobes, facultative anaerobes, symbiotic bacteria and photoautotrophs, as well as in algae and diatoms (see Weston & Knowles, 1973). Cytochrome c_{co} is present in Paracoccus denitrificans only during methylotrophic growth, but this cytochrome is present in the facultative methylotrophs Pseudomonas AM1 and Pseudomonas extorquens also during heterotrophic growth, though at reduced levels (Tonge et al., 1974; Widdowson & Anthony, 1975; Higgins et al., 1976; Keevil & Anthony, 1979b). Indeed, evidence has recently been found for the coordinate regulation of cytochrome c_{co} and enzymes of the serine pathway in Methylobacterium organophilum, though the gene for cytochrome c_{co} appears to be located in a separate linkage group (O'Connor & Hanson, 1978).

The status of cytochrome c_{co} as an oxidase in methylotrophic bacteria is a subject of some controversy. No photoaction spectra of methylotrophs have been reported, and the kinetic competence of cytochrome c_{co} to act as an oxidase has not been investigated. A peak in the photoaction spectrum of the marine bacterium Beneckea natriegens at 412nm, however, suggests that the CO-binding cytochrome c of this organism may indeed have some oxidase activity (Weston & Knowles, 1974). In addition, the purified cytochrome c_{co} from Methylosinus trichosporium has considerable ascorbate oxidase activity, though the K_M for oxygen (250μM; Tonge et al., 1977b) is extremely high for a physiological oxidase. In contrast to that of M.trichosporium, the cytochrome c_{co} of Pseudomonas AM1 is only slowly

autoxidised (O'Keefe & Anthony, 1980b), and Widdowson & Anthony (1975) suggest that their slow interaction with CO precludes an oxidase function for the cytochromes c_{co} of Pseudomonas AM1, P.extorquens and Hyphomicrobium X. Cytochromes c have now been purified from M.trichosporium, P.extorquens (Higgins et al., 1976; Tonge et al., 1977b), Methylomonas methanica (Patel et al., 1977) and Pseudomonas AM1 (Widdowson & Anthony, 1975). This last cytochrome was later shown to be a mixture of two cytochromes c differing in respect of molecular weight and isoelectric point (O'Keefe & Anthony, 1979, 1980b). The purified cytochromes c from different sources appear to vary somewhat in CO-binding characteristics and autoxidation rates. In view of these differences it is possible that cytochrome c_{co} may have an oxidase function in some bacteria but not in others. It is notable, however, that no methylotroph has yet been found without other potential cytochrome oxidases (aa_3 and/or o) in addition to cytochrome c_{co}, and also that cytochrome c_{co} is present during anaerobic growth on methanol and nitrate (Widdowson & Anthony, 1975). Indeed, partly in view of their induction during methylotrophic growth (see above), it has been suggested that the ability of the methylotroph cytochromes c to bind CO may reflect an interaction with methanol dehydrogenase rather than with oxygen (O'Keefe, 1980; O'Keefe & Anthony, 1980a).

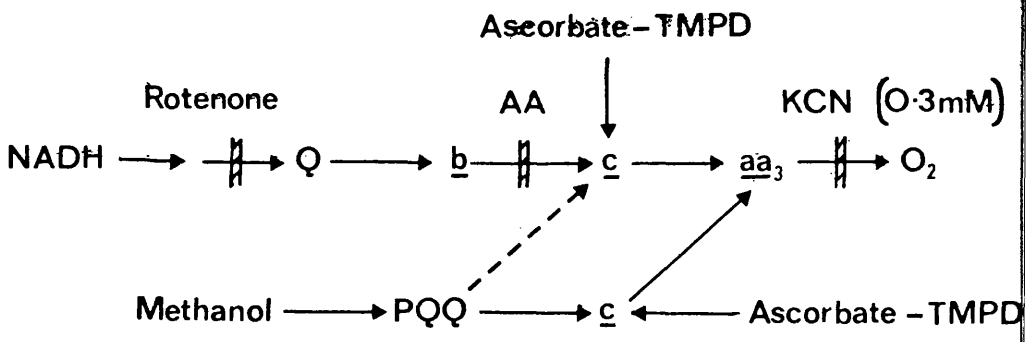
The a-type cytochrome has never been purified from a methylotroph, so the degree of resemblance to the mitochondrial cytochrome oxidase aa_3 is uncertain, though for convenience this label is used here. Pseudomonas AM1 has been shown to contain at least two species of b-type cytochrome (Keevil & Anthony, 1979a); one species is probably the CO-binding cytochrome o, and up to two species may be involved in electron transfer between the quinone pool and cytochrome c or cytochrome oxidase.

Cytochrome d has not yet been detected during methylotrophic growth, but this cytochrome is found during heterotrophic growth of the facultative methylotroph P.extorquens (Tonge et al., 1974).

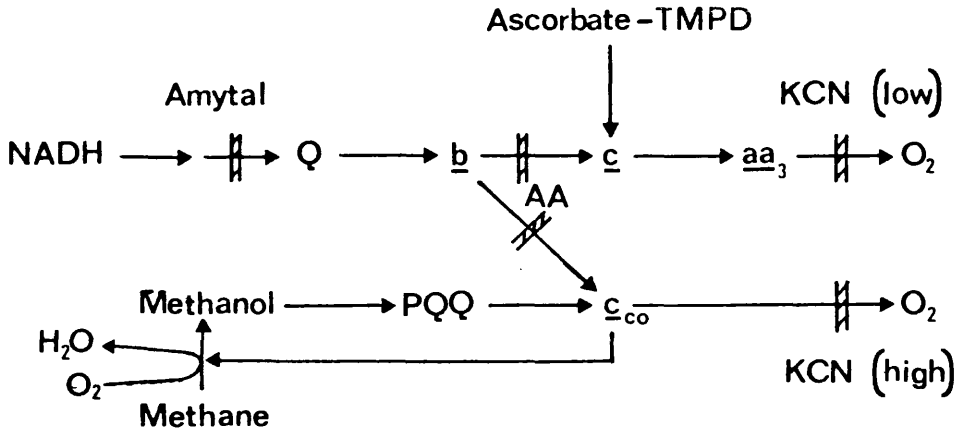
The non-cytochrome components of the respiratory chains of methylotrophs have been little studied except that the quinones of M.trichosporium and Methylomonas PII have been identified as ubiquinone-10 and ubiquinone-8 respectively (Higgins et al., 1976; Drabikowska, 1977).

Schemes for the respiratory chains of three methylotrophs, Pseudomonas AM1 (Fig. 3.1a), M.trichosporium (Fig. 3.1b) and P.denitrificans (Fig. 3.1c), have now been proposed, based largely on cytochrome composition and the sites of action of inhibitors. The sensitivity of the methylotrophs both to respiratory chain inhibitors such as rotenone, amytal and HQNO, and to terminal oxidase inhibitors such as cyanide and azide is very varied. As might be expected, methylotrophs appear to be resistant to inhibition by formate (Zakharova et al., 1980) which is known to be an inhibitor of the cytochrome oxidases of mitochondria (Nicholls, 1976) and of some bacteria (Zakharova, 1979). Fig. 3.1 abc reveals a good deal of diversity amongst the proposed schemes for the respiratory chains of methylotrophs, particularly in the terminal region. Cytochrome c_{co} is not considered to have an oxidase function in Pseudomonas AM1 or P.denitrificans but may do so in M.trichosporium, where it also seems to act as an electron donor to methane monooxygenase, and in P.extorquens (Higgins et al., 1976, 1977; Tonge et al., 1977b). The CO-binding cytochrome b of Pseudomonas AM1 is not thought to act as an oxidase, but that of P.denitrificans apparently does (though see Scholes & Smith, 1968; Lawford et al., 1976), the branch-point being at the level of cytochrome b. Despite this diversity, however, there are important common features, notably the site of interaction of

(a)


Ascorbate-TMPD
Rotenone
AA
KCN (0·3mM)
NADH
Q
b
c
aa3
O2
Methanol
PQQ
c
Ascorbate-TMPD

(b)


Ascorbate-TMPD
Amytal
KCN (low)
NADH
Q
b
c
aa3
O2
AA
Methanol
PQQ
c co
O2
H2O
O2
Methane
KCN (high)

(c)

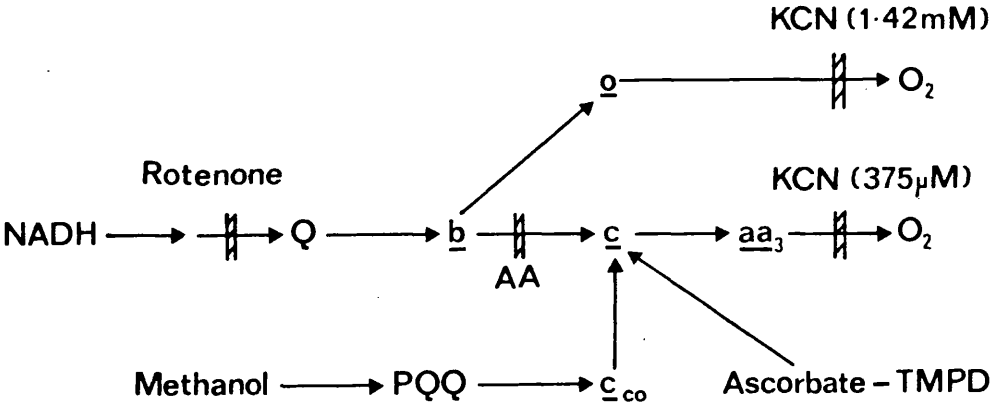

KCN (1·42mM)
o
O2
Rotenone
KCN (375μM)
NADH
Q
b
c
aa3
O2
AA
Methanol
PQQ
c co
Ascorbate-TMPD

Fig. 3.1 The respiratory systems of methylotrophic bacteria

(a), carbon-limited Pseudomonas AM1 (after Keevil & Anthony, 1979b); (b), M.trichosporium (after Higgins et al., 1977); and (c), P.denitrificans (after van Verseveld & Stouthamer, 1978).

Symbol : ⋚ , denotes inhibition (the figures in parentheses are the inhibitor concentrations required for 90% inhibition). Abbreviations : AA, antimycin A; Q, ubiquinone; PQQ, pyrrolo-quinoline quinone (prosthetic group of methanol dehydrogenase).

methanol dehydrogenase with the respiratory chain. Drabikowska (1977) has suggested that cytochrome b is involved in methanol oxidation in Methylomonas PII, but a large body of evidence has now been assembled favouring the interaction of methanol dehydrogenase with the respiratory chain at the level of cytochrome c. Indeed, methanol oxidation is not usually sensitive to the mid-chain inhibitors antimycin A and HQNO (Higgins et al., 1976, 1977; Tonge et al., 1977c; O'Keefe & Anthony, 1978; van Verseveld & Stouthamer, 1978); mutants of Pseudomonas AM1 (Anthony, 1975a) and P.denitrificans (Willison & John, 1979) lacking cytochrome c are unable to grow on methanol whereas growth on other substrates is unaffected; and Duine et al. (1979a) have recently been able to observe methanol-dependent cytochrome c reduction in purified preparations from Hyphomicrobium X. There appears to be a high degree of similarity between the methanol dehydrogenases of methylotrophs (see Yamanaka, 1981), and thus it seems likely that in Methylomonas PII the interaction of methanol dehydrogenase with the respiratory chain will also be at the level of cytochrome c; the methanol-dependent reduction of cytochrome b observed by Drabikowska (1977) was thus probably due to the reoxidation of NADH formed via the further oxidation of formaldehyde.

In a preliminary report, Cross & Anthony (1978) showed that M.methylotrophus contains cytochromes b, c, and sometimes aa_3; the presence of this last cytochrome being variable. In addition, these authors identified three CO-binding cytochromes, aa_3, o and c_{co}. This chapter describes an investigation of the composition of the respiratory chain of M.methylotrophus, grown in methanol-limited continuous culture. The sequential organisation of the respiratory chain, particularly the terminal oxidase region, was also studied using classical respiratory inhibitors.

Some of the diversity amongst the sensitivities of the terminal oxidase systems of methylotrophs to inhibitors may stem from the use of only one or two inhibitor concentrations at a single rate of flux through the oxidase system. This can be particularly misleading when uncompetitive inhibition is encountered, and results from substrates oxidised at quite different rates are compared. The type of inhibition kinetics, as well as the K_i for inhibition, can be useful diagnostically (Meyer, 1972; Jones, 1973); and the detailed kinetics of inhibition of the terminal region of the respiratory chain of M.methylotrophus, by cyanide, are considered here.

Since this work was largely completed a full report of the respiratory chain of M.methylotrophus has been published (Cross & Anthony, 1980b), and this work is discussed in the conclusion to this chapter (section 3.6).

3.2 Cytochrome content of M.methylotrophus

Fig. 3.2a shows the reduced minus oxidised difference spectrum of whole cells of M.methylotrophus. The presence of cytochrome c is clearly indicated by the strong peaks at 550nm and 520nm, and in addition the small peak at 602nm and the slight shoulder in the Soret region at 440nm are indicative of cytochrome aa_3. The low temperature spectrum of the membrane fraction (Fig. 3.3a) shows that a peak due to cytochrome b (553nm) was masked in whole cell spectra by the large excess of cytochrome c. (As is usual with low temperature spectra, an intensification and shift to shorter wavelength of 2-3nm were observed [Keilin & Hartree, 1949]). Cytochromes aa_3 (peaks 599, 438nm) and c (peaks 547, 518nm) were also found to be membrane-bound, but the latter was also observed as the only cytochrome in the soluble fraction (Fig. 3.3b).

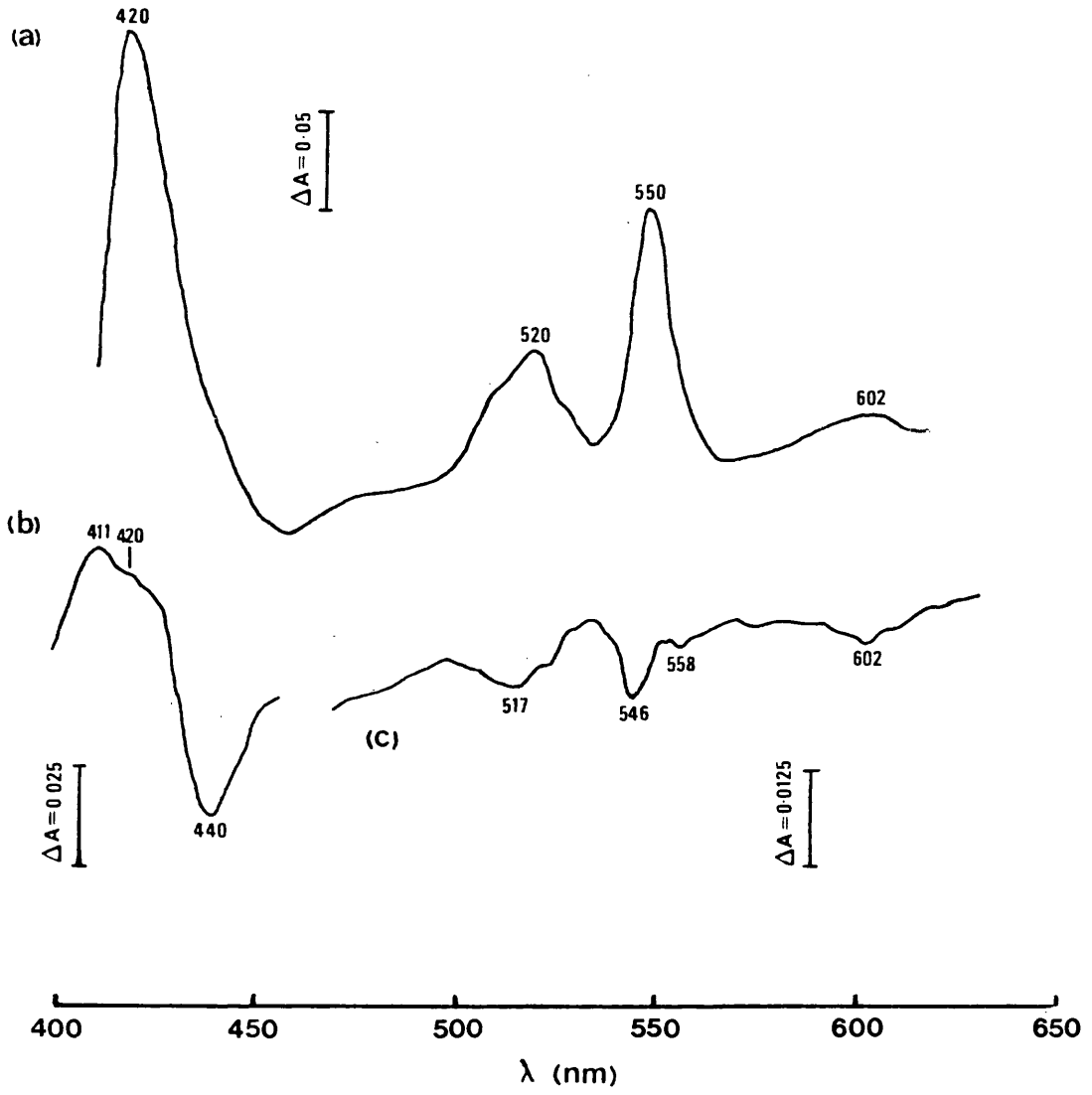


Fig. 3.2 Room temperature spectra of whole cells of M.methylotrophus

(a), Reduced minus oxidised (H_2O_2) difference spectrum (11.6 mg dry wt. bacteria/ml); (b), (c) reduced plus CO minus reduced difference spectrum ((b), 4.3 mg dry wt. bacteria/ml; (c), 31.1 mg dry wt. bacteria/ml).

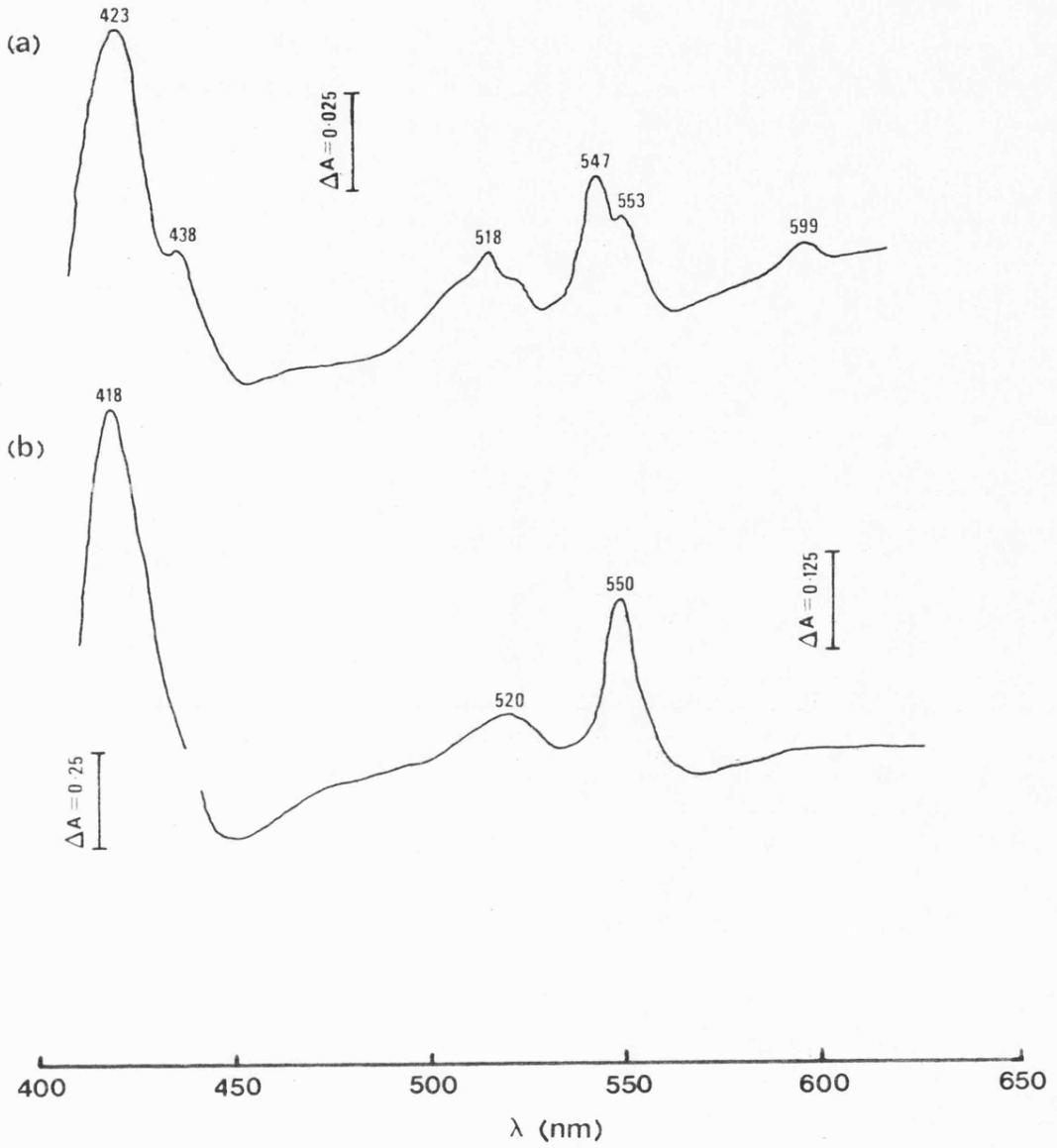


Fig. 3.3 Reduced minus oxidised (H_2O_2) difference spectra of the membrane and soluble fractions. (a), Low temperature spectrum of the membrane fraction (12.9 mg protein/ml); (b) room temperature spectrum of the soluble fraction (15.5 mg protein/ml).

Reduced plus CO minus reduced difference spectra of whole cells (Fig. 3.2bc) and membranes (Fig. 3.4ab) indicated the presence of three membrane-bound CO-binding cytochromes; viz, cytochromes aa_3 (troughs 600-602nm, 440-442nm), o (trough 557-558nm, shoulder 420nm) and c_{co} (trough 546-548nm, peak 411-413nm). The soluble cytochrome c also bound a small amount of CO (Fig. 3.4cd).

Interestingly, the fermenter supernatant contained large amounts of cytochrome c, and also of a material which absorbed strongly in the oxidised form at about 448nm, and was possibly flavin (see Beinert, 1960). The amounts of both substances found were far too large to be accounted for by cell lysis.

Quantitative estimates of the cytochrome contents of the membrane and soluble fractions of M.methylotrophus are shown in Table 3.1. The values derived from reduced plus CO minus reduced difference spectra are necessarily very approximate due to the overlapping of the bands from different cytochromes in the Soret region, and the uncertainty over the values of the molar extinction coefficients used (see section 2.5.3). (It should be noted that the experiments described in this section were carried out on bacteria grown at Billingham, but preliminary experiments have shown that the cytochrome content of bacteria grown at Leicester is at least qualitatively similar.)

3.3 Nicotinamide nucleotide transhydrogenase activity of M.methylotrophus

Crude extracts of M.methylotrophus were found to have an NADPH oxidase rate equal to approximately half that of NADH oxidase (see Table 3.2). No stimulation by NAD^+ was observed, but it is likely that this substance would already be present in the crude extract to the catalytic level required for transhydrogenase function. Very little NADPH oxidase

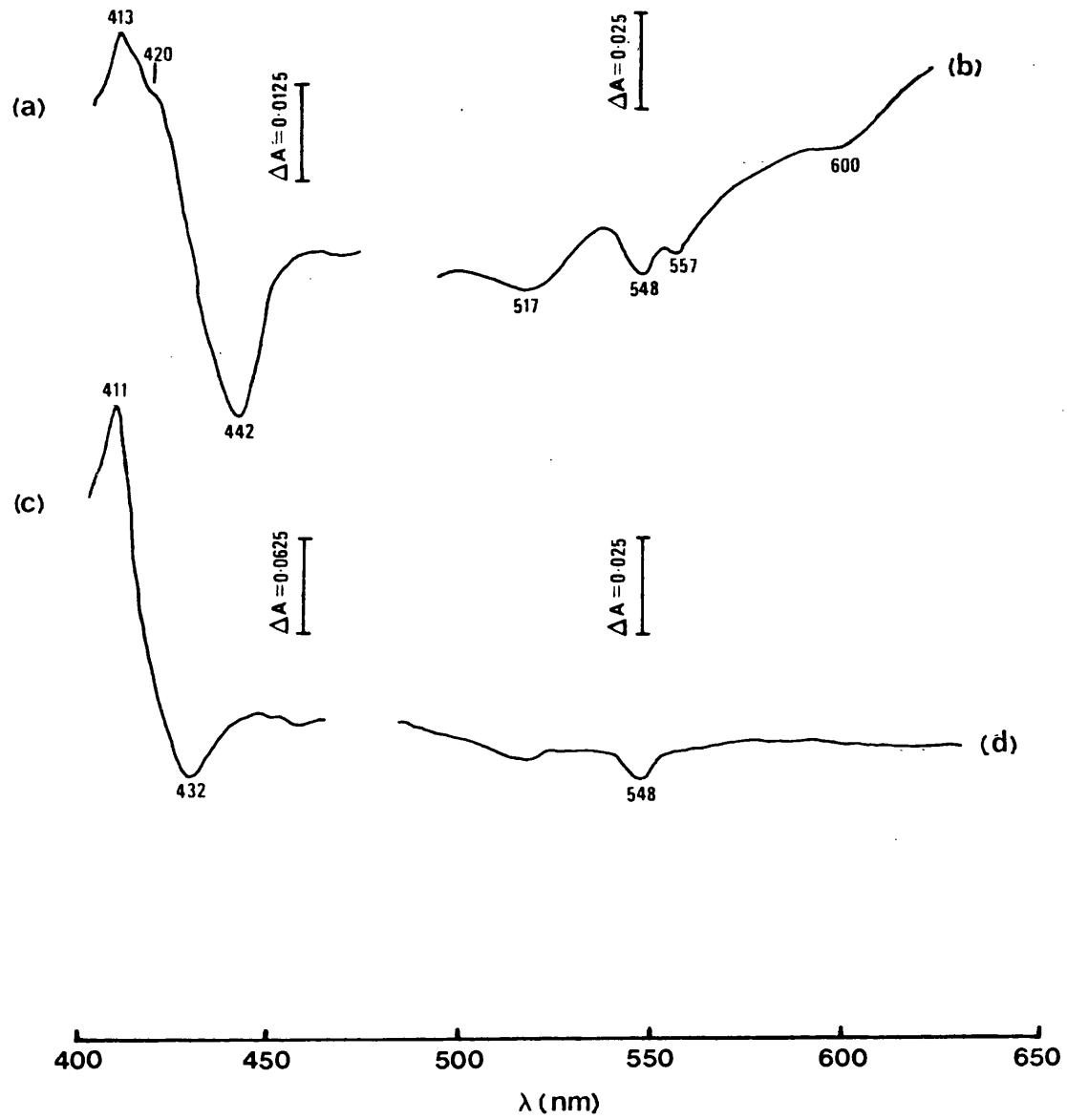


Fig. 3.4 Room temperature reduced plus CO minus reduced difference spectra of the membrane and soluble fractions. (a), (b), Membrane fraction ((a), 17.6 mg protein/ml; (b) 29.2 mg protein/ml); (c), (d), soluble fraction ((c), 13.0 mg protein/ml; (d), 21.3 mg protein/ml).

Membrane fraction						Soluble Fraction	
aa_3*	b*	c*	aa_3†	o†	c_{co}†	c*	c_{co}†
				(nmoles/g protein)			
59 ± 9 (7)	243 ± 23 (7)	444 ± 37 (7)	18 ± 3 (3)	33 ± 6 (3)	38 ± 8 (3)	1023 ± 85 (7)	18 ± 4 (3)

Table 3.1 Cytochrome content of methanol-limited M.methylotrophus. The fractions were prepared as described in section 2.4.1. Cytochromes marked * were estimated from reduced minus oxidised difference spectra, whereas those marked † were estimated from reduced plus CO minus reduced difference spectra. The wavelength pairs and molar extinction coefficients used are listed in section 2.5.3.

Cell Fraction	Donor	Acceptor	NAD(P)H oxidation rate (nmoles NADH oxidised/min per mg protein)
Crude extract	NADH	O_2	15.2 ± 1.7 (3)
	NADPH	O_2	7.9 ± 2.6 (3)
	NADPH + NAD^+	O_2	7.5 ± 3.1 (2)
	NADPH	3-acetylpyridine-NAD^+	5.6
Membrane	NADH	O_2	55.5 ± 11 (4)
	NADPH	O_2	2.3 ± 0.2 (3)
	NADPH + NAD^+	O_2	1.5 ± 0.5 (2)
	NADPH	3-acetylpyridine-NAD^+	0
Soluble	NADPH	3-acetylpyridine-NAD^+	6.7
Soluble + Membrane	NADPH	O_2	19.6 ± 3.9 (2)*
	NADPH + NAD^+	O_2	19.6 ± 3.9 (2)*

Table 3.2 Nicotinamide nucleotide transhydrogenase activity of *M.methylotrophus*. Cell fractions were prepared and assayed as described in sections 2.4.2 and 2.7.1, 2.7.2, respectively.

* nmoles NADH oxidised/min per mg soluble protein.

activity was found in the membrane fraction (with or without NAD^+), but this activity could be reconstituted by the addition of soluble fraction. The presence of a soluble transhydrogenase, only, was confirmed by 3-acetylpyridine-NAD^+ reduction using NADPH as substrate.

3.4 Respiratory activity of whole cells of M.methylotrophus

Whole cells of M.methylotrophus readily oxidised methanol, ethanol, butanol, formaldehyde, formate, NADH, ascorbate-TMPD, $DADH_2$ and duroquinol; methylamine, trimethylamine, glucose, fructose, glycerol, ferrocyanide and ascorbate-mammalian cytochrome c were oxidised less rapidly; and isocitrate, lactate, malate, pyruvate, acetate and succinate were not oxidised. The respiration rates from selected substrates are shown in Table 3.3.

3.5 Effect of inhibitors on the respiratory activity of whole cells of M.methylotrophus

3.5.1 Inhibition of the respiratory chain prior to the terminal oxidase region

Whole cells of M.methylotrophus were found to be considerably less susceptible to inhibition by conventional respiratory inhibitors than mitochondria, or the mitochondrion-like bacterium P.denitrificans (see John & Whatley, 1977). Rotenone (1mM) failed to significantly inhibit respiration from the NAD^+-linked substrate formate. Amytal, on the other hand, caused some inhibition of formate oxidation, but only at high inhibitor concentrations (5mM) when it also affected

Substrate	Respiration rate (ng-atom O/min per mg dry wt.) bacteria
Endogenous	5.8 ± 0.8 (5)
Methanol	292 ± 37(9)
Formaldehyde	315 ± 28(9)
Formate	37 ± 6 (5)
NADH	182 ± 16(5)
Ascorbate-TMPD	1400
$DADH_2$	480
Duroquinol	181 ± 3 (5)

Table 3.3 Respiratory activity of whole cells of M.methylotrophus

Respiration rates were determined as described in section 2.6 in a reaction mixture containing 140mM-KCl, 10mM-glycylglycine (pH7.0) plus sufficient washed cell suspension to give a convenient rate of oxygen consumption. All substrates were used at saturating concentrations (methanol, 20mM; formaldehyde, 2mM; formate, 5mM; NADH, 2mM; ascorbate, 3mM-TMPD, 1mM; $DADH_2$, 1mM; duroquinol, 2mM). The values of the respiration rates from ascorbate-TMPD and $DADH_2$ were taken from plots of the respiration rate (corrected for autoxidation) versus the substrate concentration. Respiration rates from added substrates were corrected for endogenous respiration.

respiration from substrates feeding reducing equivalents into the respiratory chain below the level of NADH dehydrogenase. The mid-chain inhibitors antimycin A and HQNO had similar relative effects on respiration, i.e. they caused substantial inhibition of respiration from formate and duroquinol (duroquinol interacts with the respiratory chain at the level of the quinone pool or cytochrome b [Boveris et al., 1971; Kroger et al., 1973]), but little inhibition of respiration from methanol or ascorbate-TMPD. Indeed, 300μM-antimycin A inhibited formate oxidation by 70% but methanol oxidation by only 3%; the concentration dependence of inhibition by HQNO is shown in Fig. 3.5. The relative effects of inhibitors on respiration from different substrates were similar to those seen with other methylotrophs (section 3.1), and consistent with the hypothesis that methanol donates reducing equivalents to the respiratory chain at the level of cytochrome c. Intermediate inhibition of formaldehyde oxidation was observed as this substrate, at least in vitro, may be oxidised either via the reduction and reoxidation of NAD^+ or directly via methanol oxidase (see section 1.3.3).

Various substances were tested as potential specific inhibitors of methanol oxidase. However, only dicumarol had some specific effect, and the uncompetitive kinetics and time-dependence of the dicumarol inhibition rule this out as a useful inhibitor.

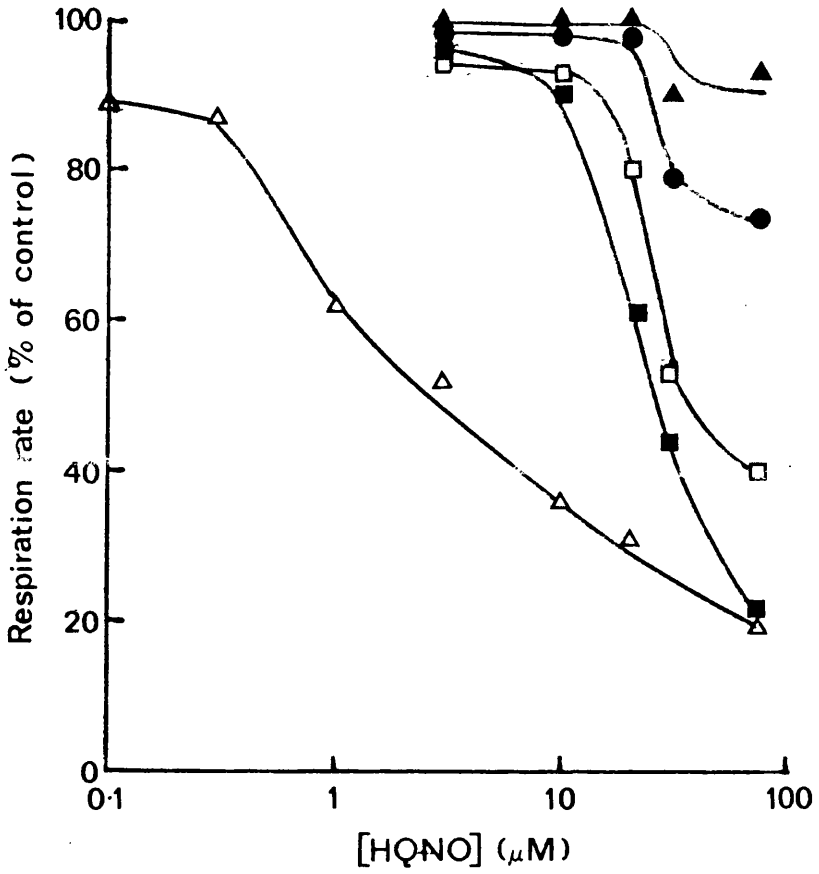
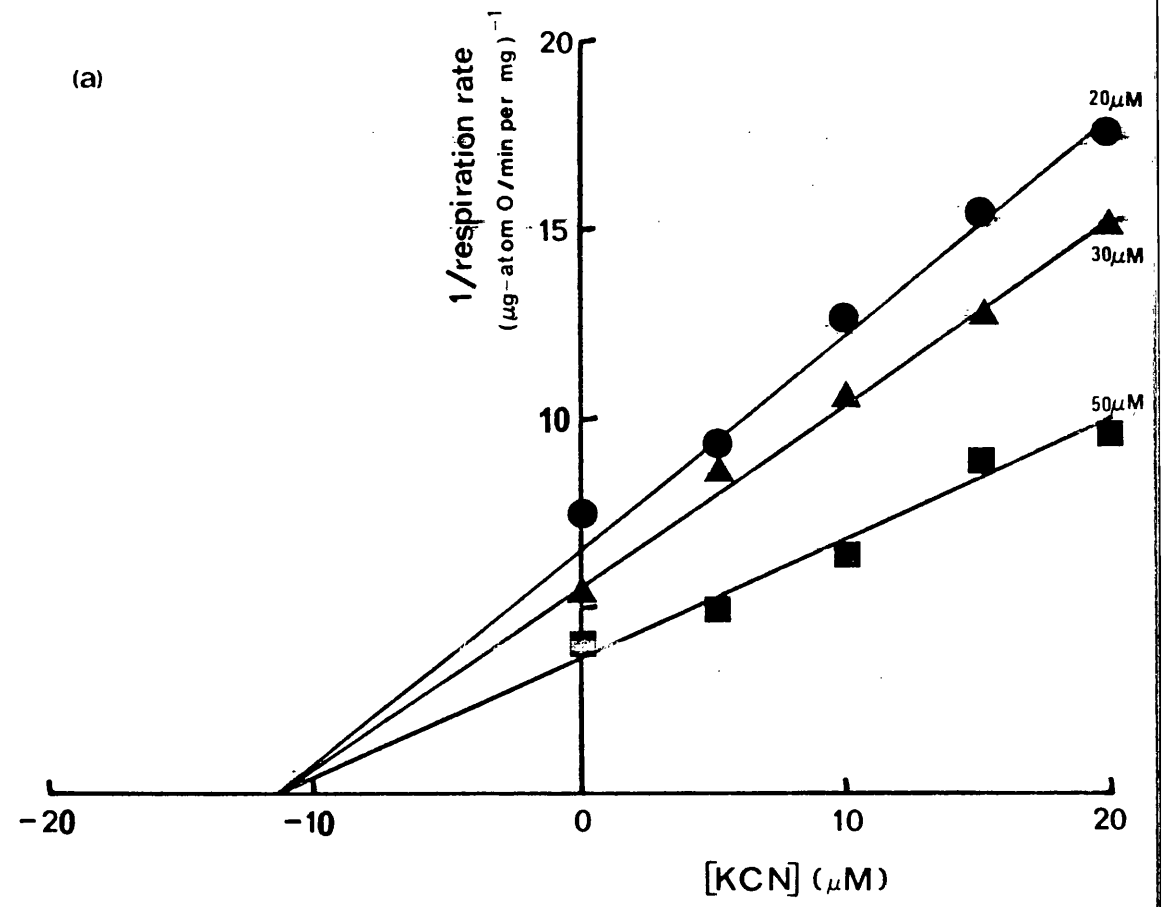


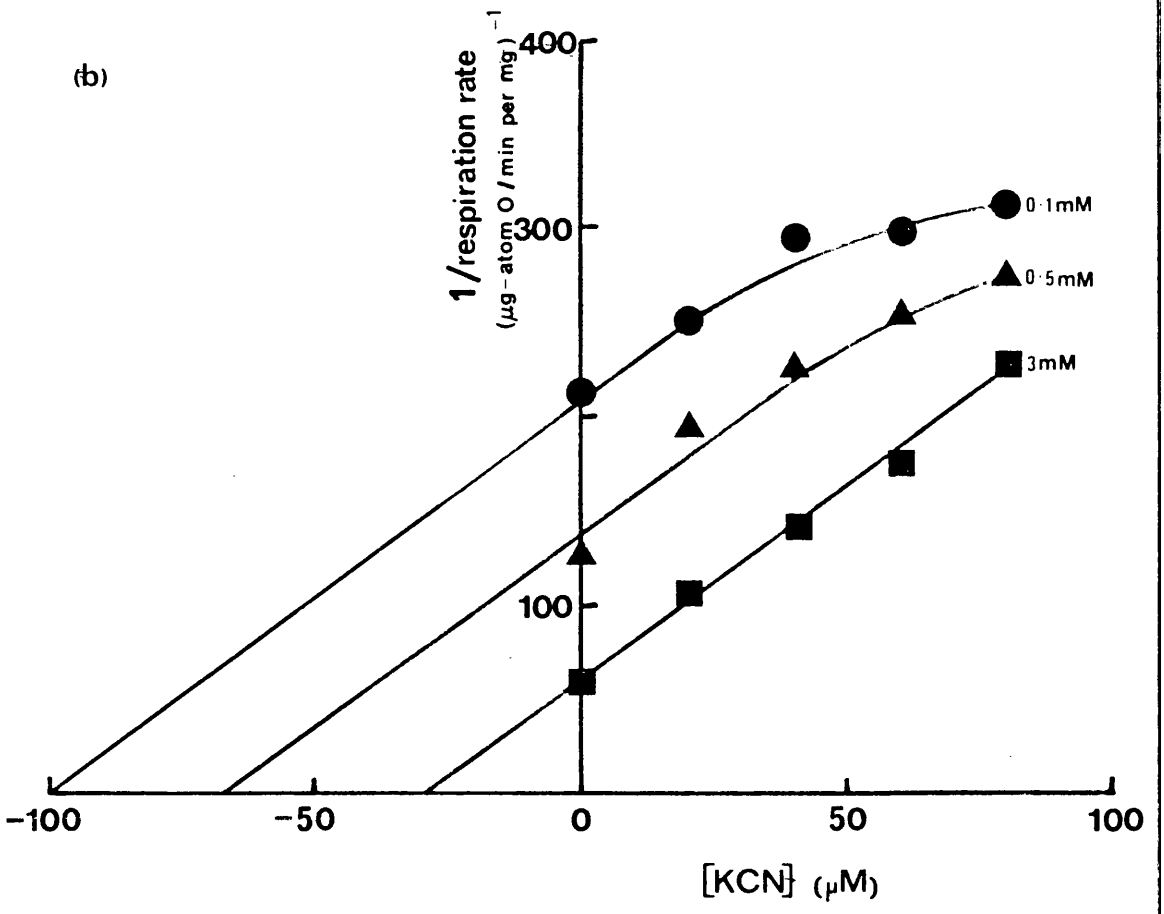
Fig. 3.5 Effect of HQNO on the oxidation of formate, formaldehyde, methanol, duroquinol and ascorbate-TMPD. Respiration rates were measured as described in section 2.6 in a reaction mixture containing 140mM-KCl, 10mM-glycylglycine (pH7.0) plus sufficient washed cells to give a convenient rate of oxygen consumption. Substrates (all 1mM) : ■ , formate; □ , formaldehyde; ● , methanol; △ , duroquinol; ▲ , ascorbate (3mM)-TMPD (1mM).

3.5.2 Inhibition of the terminal region of the respiratory chain by cyanide and azide

The oxidation of methanol by whole cells of M.methylotrophus was inhibited by cyanide in a non-competitive manner ($K_i \simeq 10\mu M$; Fig. 3.6a), whereas inhibition of respiration from the NAD^+-linked substrate formate was uncompetitive ($K_i \leq 27\mu M$; Fig. 3.6b). Detailed analyses of cyanide inhibition kinetics during bacterial respiration have previously shown that non-competitive and uncompetitive inhibition are diagnostic of cytochrome oxidases o and aa_3 respectively (Meyer, 1972; Jones, 1973). These current studies therefore indicate that cytochrome o is the least sensitive oxidase during methanol oxidation (V_{max} = 290ng-atom O/min per mg dry wt. bacteria), whereas cytochrome aa_3 is the least sensitive oxidase during the much slower formate oxidation (V_{max} = 17ng-atom/min per mg dry wt. bacteria). Plots of 1/fractional inhibition versus 1/[KCN] for the oxidation of methanol, and high concentrations of formate, extrapolate to one on the abscissa indicating complete inhibition, but the curving of the Dixon plots at lower formate concentrations (Fig. 3.6b) may indicate the presence of an additional relatively cyanide-insensitive oxidase, but with an activity too low to be physiologically significant. The inhibition of cytochrome c_{co} has not previously been examined in detail, but this cytochrome appears to be relatively insensitive to cyanide (Higgins et al., 1976; Tonge et al., 1977b,c), and thus it is likely that this cytochrome has at the most only very slight oxidase activity in M.methylotrophus.

The inhibition of ascorbate-TMPD oxidation by cyanide (Fig. 3.6c) showed either non-competitive (K_i = 2.5μM) or uncompetitive ($K_i \leq 2.5\mu M$)


(a)
1/respiration rate
(μg-atom O/min per mg)$^{-1}$
20
15
10
20μM
30μM
50μM
-20
-10
0
10
20
[KCN] (μM)


(b)
1/respiration rate
(μg-atom O/min per mg)$^{-1}$
400
300
100
0·1mM
0·5mM
3mM
-100
-50
0
50
100
[KCN] (μM)

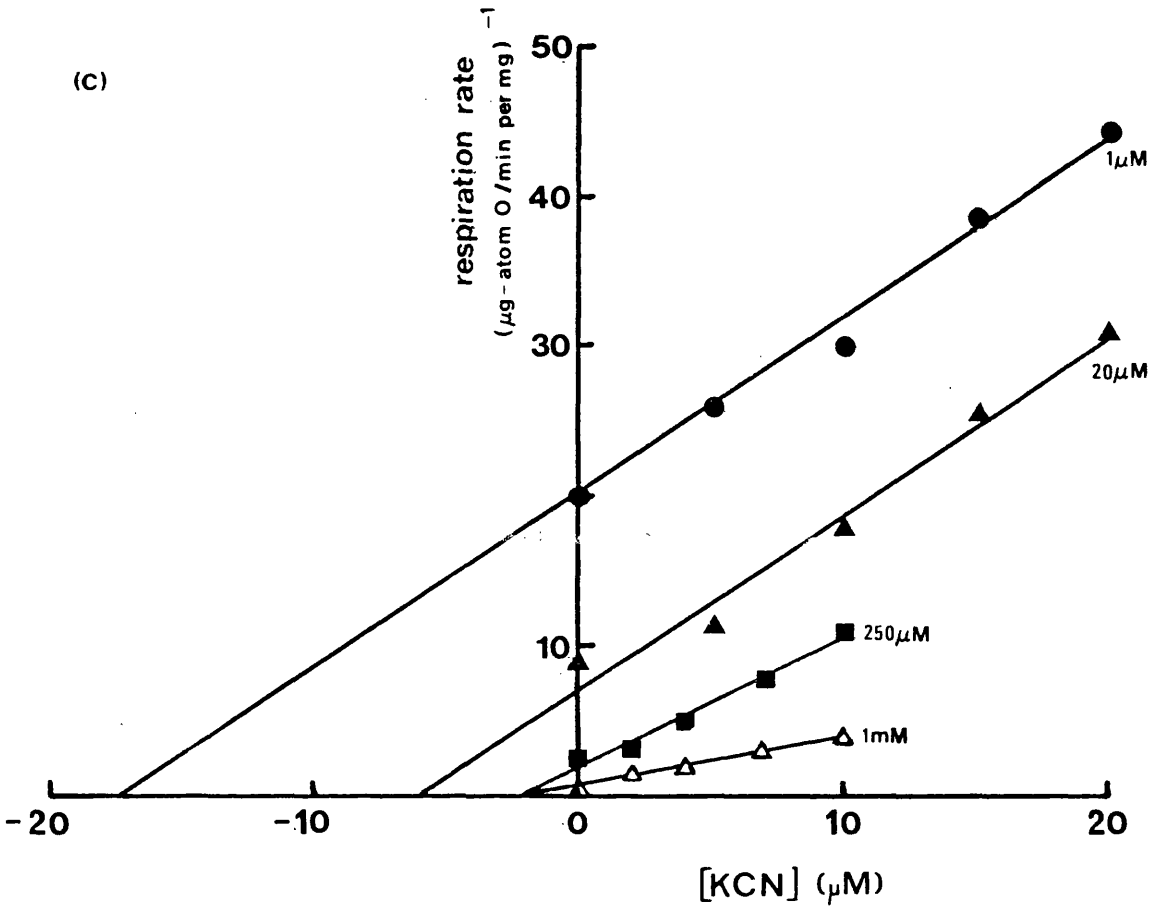


Fig. 3.6 The effect of cyanide on respiration from methanol, formate and ascorbate-TMPD (Dixon plots). Respiration rates were measured as described in section 2.6, in a reaction mixture containing 140mM-KCl, 20mM-glycylglycine (pH7.4) plus sufficient washed cell suspension to give a convenient rate of oxygen consumption. The cyanide was added 2 min prior to the addition of substrate. The substrates used, at the concentrations shown on the plots, were (a), methanol; (b), formate; and (c), ascorbate-TMPD (the ascorbate concentration was 3mM throughout and the TMPD concentration was varied as shown; correction was made for the autoxidation of this substrate).

kinetics depending on the concentration of TMPD, and hence on the respiration rate. Cytochrome o was again the least sensitive oxidase at high respiration rates (V_{max} = 1.10μg-atom O/min per mg dry wt. bacteria), since the I_{50} for cytochrome aa_3 is very low under these conditions. However, as the respiration rate is decreased, the I_{50} of cytochrome aa_3 increases until it exceeds the K_i of cytochrome o, and hence cytochrome aa_3 became the least cyanide-sensitive oxidase at low respiration rates ($V \leqslant$ 330ng-atom O/min per mg dry wt. bacteria).

As with cyanide, azide inhibition kinetics also suggested the involvement c two terminal oxidases in ascorbate-TMPD oxidation (Fig. 3.7). An azide-sensitive oxidase (I_{50} = 75μM) was apparent at high respiration rates, whereas at lower respiration rates all the flux to oxygen could be carried by an azide-insensitive oxidase (K_i = 1.2mM; non-competitive). Detailed information on the effect of azide on bacterial terminal oxidases is both sparse and inconsistent, so no unambiguous identification of the cytochromes responsible for the two phases of inhibition is possible. Mitochondrial cytochrome aa_3 is known to be inhibited in a non-competitive manner by azide (Minnaert, 1961; Yonetani & Ray, 1965), but the inhibition kinetics of the bacterial cytochromes o and c_{co} have not been examined.

3.6 Discussion and conclusions

Methanol-limited M.methylotrophus was found to contain cytochromes of the a-, b- and c-types, from reduced minus oxidised difference spectra, and cytochromes aa_3, o (b-type) and c_{co}, from reduced plus CO minus reduced difference spectra, in agreement with the preliminary report of Cross & Anthony (1978). Cytochromes of the a- and b-types were found to be exclusively membrane-bound, whereas a substantial proportion of the cytochrome c

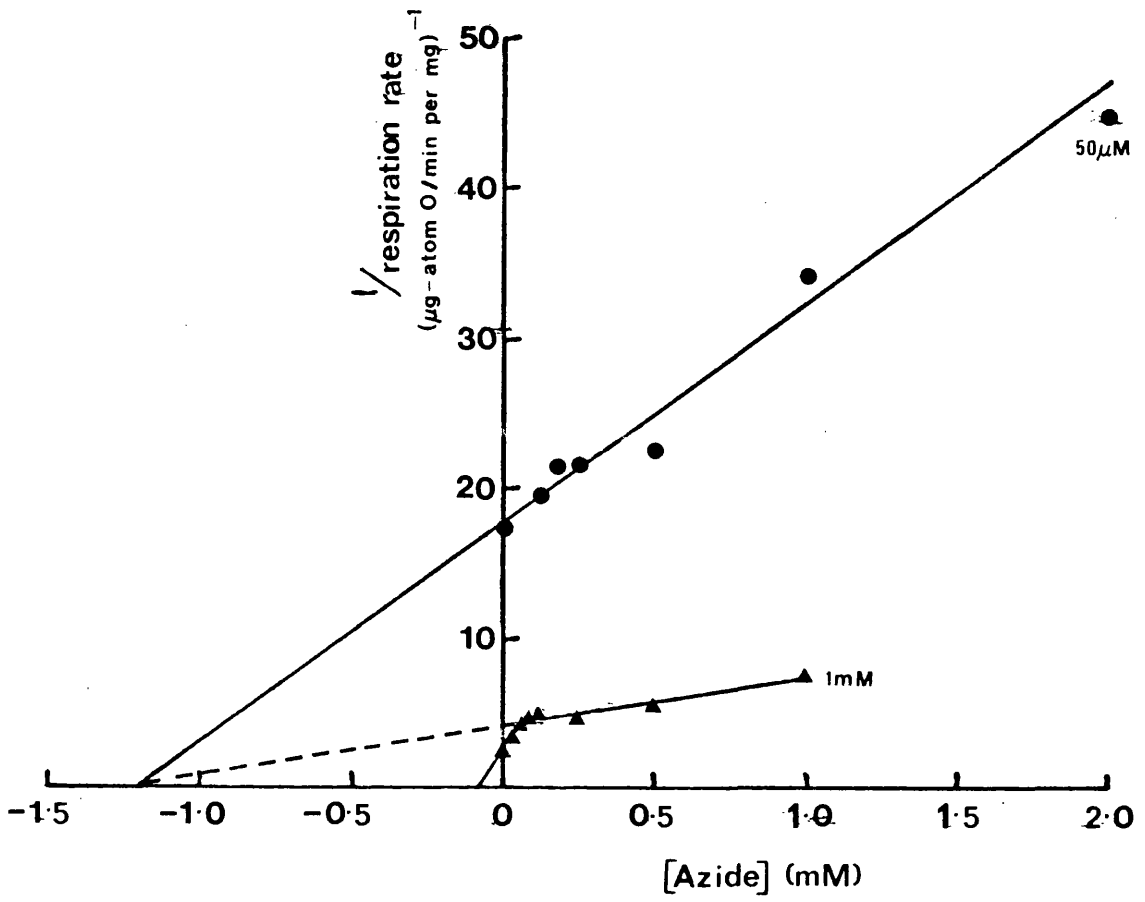


Fig. 3.7 The effect of azide on respiration from ascorbate-TMPD

The conditions were as described in the legend to Fig. 3.6. Again the inhibitor was added 2 min prior to the addition of substrate. The ascorbate concentration was 3mM throughout and the concentration of TMPD was as shown on the plots (correction was made for autoxidation).

was found in the soluble fraction after cell breakage. The concentrations of the membrane-bound cytochromes relative to unity for cytochrome c were aa_3, 0.13 : b, 0.55 : c, 1.00; and the CO-binding cytochromes were present in the approximate ratio aa_3, 0.47 : o, 0.87 : c_{co} : 1.00. These figures suggest that there are at least two species of both cytochrome b and cytochrome c. Indeed, a detailed report by Cross & Anthony (1980b), published after much of this work had been completed, showed that this organism contains up to three species of both cytochrome b (b_{60}, b_{110} and b_{260} [o?]) and cytochrome c (c_{310}, c_{356} and c_{373}; the subscripts to both b- and c-type cytochromes refer to the mid-point redox potentials of the membrane-bound cytochromes). Cross & Anthony (1980a) have also purified three species of soluble cytochrome c which may be the same as the membrane-bound cytochromes. These cytochromes were found to differ in terms of the molecular weight, isoelectric point, redox potential and CO-binding properties; none of the cytochromes c was rapidly autoxidisable, though some methanol oxidase activity was detected in the soluble fraction, in which cytochrome c is the only cytochrome present. The presence of cytochrome c in fermenter supernatants was also noted by Cross & Anthony (1980b) who showed that this cytochrome was reversibly photo-oxidised. A material reported here to absorb strongly in the oxidised form at 448nm is possibly the flavin responsible for catalysis of this photo-oxidation.

Little is known of the non-cytochrome components of the respiratory chain of M.methylotrophus but, being Gram negative, this organism is likely to contain ubiquinone (Bishop et al., 1962) in common with the other methylotrophs studied so far (see section 3.1). M.methylotrophus was found not to contain a membrane-bound transhydrogenase, but a soluble transhy-

drogenase was detected. This organism probably produces sufficient NADPH for biosynthesis from the oxidative ribulose monophosphate cycle (see section 1.3.3), and the purpose of the soluble transhydrogenase is thus to allow oxidation of excess reducing equivalents via the respiratory chain.

The lack of oxidation of carboxylic acids such as succinate, malate and pyruvate by M.methylotrophus is very probably due to a permeability barrier, as dehydrogenases for at least some of these substrates must be present. In common with other methylotrophs, M.methylotrophus oxidised multicarbon primary alcohols, presumably via a loose-specificity methanol dehydrogenase (Anthony & Zatman, 1965). Cross & Anthony (1980b) reported NADH oxidation by whole cells of M.methylotrophus, and this observation was repeated here. Oxidation of NADH by whole bacteria is unusual, but has been previously observed in Haemophilus parainfluenzae (White & Sinclair, 1970) and Acetobacter suboxydans (Daniel, 1970). NADH oxidation by whole cells of M.methylotrophus was insensitive to inhibition by HQNO, as well as by rotenone (Cooke, S.E. unpublished results), so it is possible that externally-supplied NADH is oxidised via a different route from internally-generated NADH. The role of the external NADH-oxidising system in this organism is far from understood, and clearly worthy of further investigation.

The insensitivity of whole cells of M.methylotrophus to respiratory inhibitors appears also to be due, at least in part, to a permeability barrier; membrane preparations are somewhat more sensitive than whole bacteria to antimycin A and HQNO, and are also sensitive to inhibition by high levels of rotenone (50% inhibition of NADH oxidase activity occurring at 0.5mM; Cross & Anthony, 1980b). The results obtained with whole cells were, however, consistent with the hypothesis that methanol donates reducing equivalents to the respiratory chain of M.methylotrophus at the

level of cytochrome c, as in other methylotrophs (see section 3.1).

The simplest interpretation of the cyanide inhibition data is that the respiratory chain of M.methylotrophus is branched at the level of cytochrome c, one branch leading to cytochrome oxidase aa_3 and the other to cytochrome oxidase o; both oxidases are accessible to all substrates, the relative oxidase activity in the presence of cyanide depending on the rate of electron flux. An alternative possibility is that this organism contains two respiratory chains with functionally distinct pools of cytochrome c, such that NADH is oxidised only via cytochrome aa_3 and methanol is oxidised only via cytochrome o, whereas both oxidases are involved in the oxidation of ascorbate-TMPD. However, this latter suggestion is rendered less likely by the finding that cytochrome aa_3 as well as cytochrome o is readily reduced by methanol in aerobic whole-cell suspensions (Cross & Anthony, 1980b). There was little or no evidence for any cyanide-insensitive respiration which might have occurred via cytochrome c_{co} but, on the other hand, substantial azide-resistant respiration was observed (Fig. 3.7). Cross & Anthony (1980b) implicated cytochrome c_{co} in this azide-resistant respiration but, in view of the findings from the cyanide inhibition work, it seems more likely that the two phases of inhibition by azide are due to cytochromes o and aa_3.

Fig. 3.8 represents the simplest scheme for the respiratory chain of methanol-limited M.methylotrophus which is consistent with all the results presented here. The scheme proposed by Cross & Anthony (1980b) is in very good agreement with that depicted here, the major difference being that these authors suggest a direct interaction between ascorbate-TMPD and cytochrome o. Cyanide inhibition kinetics, however, indicate that respiration from ascorbate-TMPD may be routed to oxygen via either cyto-

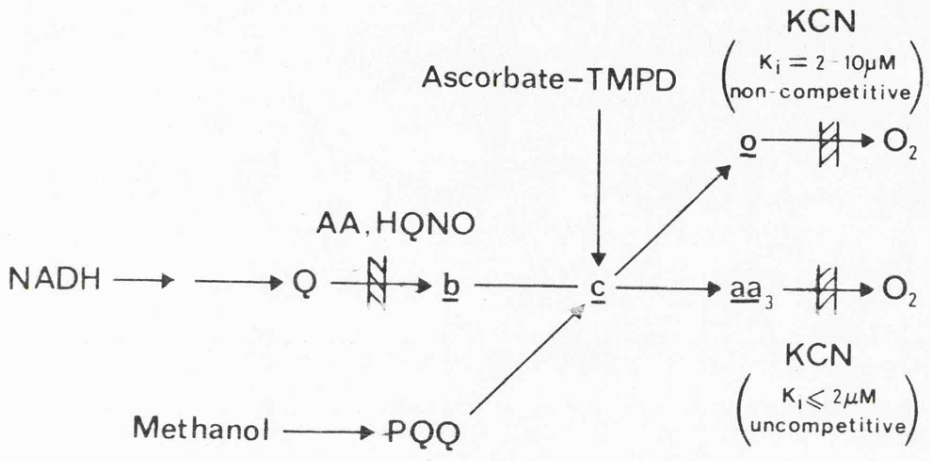


Fig. 3.8 The respiratory system of methanol-limited M.methylotrophus

The symbols and abbreviations are as for Fig. 3.1.

chrome o or aa_3 (Fig. 3.6c); and as the oxidation of ascorbate-TMPD was not inhibited by antimycin A or HQNO, it is most probable that this substrate is interacting with the respiratory chain at the level of cytochrome c. Indeed, there is an overwhelming body of evidence that ascorbate-TMPD interacts preferentially with cytochrome c in both bacteria and mitochondria (Packer & Mustafa, 1966; Tyler et al., 1966), and widespread use of the Kovacs oxidase test (Kovacs, 1956) has shown that only those bacteria containing a high redox potential cytochrome c readily oxidise this substrate (see Jurtshuk et al., 1975; West et al., 1978; Willison & John, 1979).

The respiratory system of methanol-limited M.methylotrophus was thus found to be closely similar to that of methanol-limited Pseudomonas AM1 (Fig. 3.1a) except that there is an extra oxidase, viz. cytochrome o, in the former. There are also some similarities with P.denitrificans (Fig. 3.1c), the branch-point in the terminal region of the respiratory chain of this latter organism, however, occurring at the level of cytochrome b rather than cytochrome c.

CHAPTER 4

RESPIRATION-LINKED PROTON TRANSLOCATION IN METHANOL-LIMITED *METHYLOPHILUS METHYLOTROPHUS*

4.1 Introduction

4.2 Proton translocation linked to endogenous respiration in *M.methylotrophus*

4.3 Stoicheiometry of proton translocation linked to the oxidation of added substrates in *M.methylotrophus*, measured by the oxygen-pulse method

4.4 Determination of proton and charge translocation stoicheiometries in *M.methylotrophus*, using the initial-rate method

4.4.1 Stoicheiometry of respiration-linked proton translocation, measured by the initial-rate method

4.4.2 Stoicheiometry of respiration-linked charge translocation, measured by the initial-rate method

4.5 Proton and charge translocation stoicheiometries in the cytochrome *c* oxidase region of the respiratory chain of *M.methylotrophus*

4.5.1 Stoicheiometry of proton translocation linked to the oxidation of ascorbate-TMPD and $DADH_2$, measured by the oxygen-pulse method

4.5.2 Stoicheiometry of proton and charge translocation linked to the oxidation of ascorbate-TMPD, measured by the initial-rate method

4.5.3 Effect of cyanide on the stoicheiometry of proton and charge translocation linked to the oxidation of ascorbate-TMPD

4.6 Discussion and conclusions

CHAPTER 4

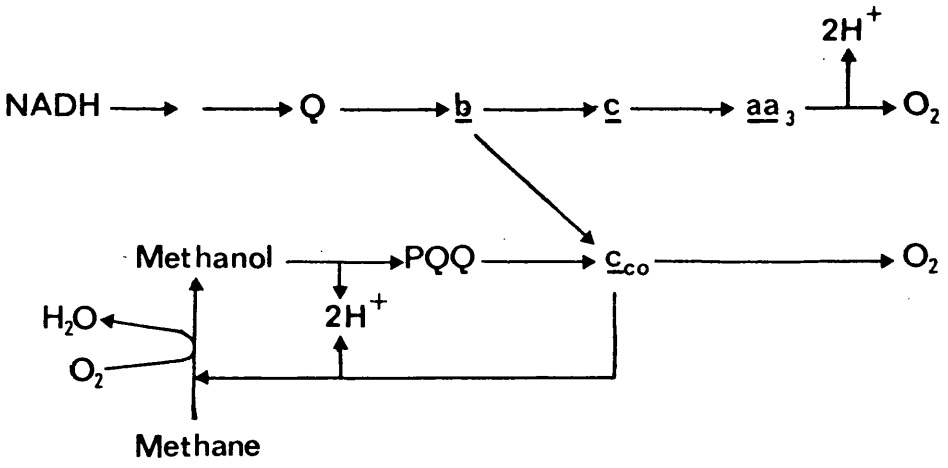
RESPIRATION-LINKED PROTON TRANSLOCATION IN METHANOL-LIMITED *METHYLOPHILUS METHYLOTROPHUS*

4.1 Introduction

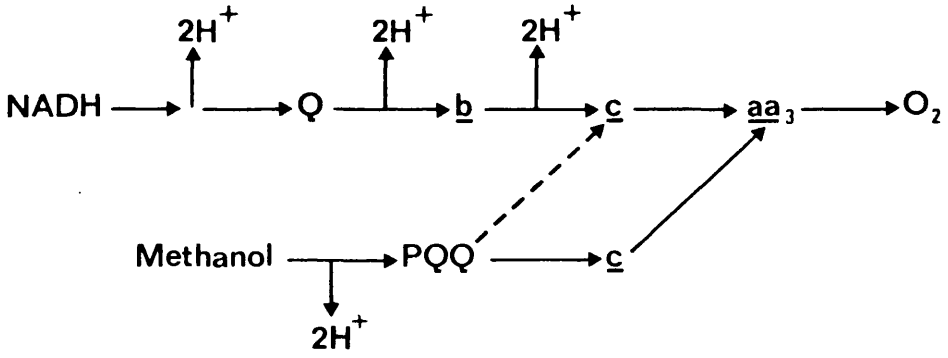
The ability of various methylotrophic bacteria to catalyse respiration-linked proton translocation has recently been investigated in several laboratories. Whole cells of the Type I methanotroph, *Methylococcus capsulatus*, failed to give a typical pH response on addition of an oxygen-pulse to an anaerobic suspension of bacteria (Tonge *et al.*, 1976). This was, perhaps, due to the location of the respiratory chain of this organism within the disc-like internal membrane system (Monosov & Netrusov, 1975; Wolfe & Higgins, 1979). Respiration-linked proton translocation has, however, now been demonstrated in whole cells of the Type II methanotroph, *Methylosinus trichosporium* (Tonge *et al.*, 1977a; Type II methanotrophs have a peripheral membrane system [see section 1.1.1]), and in several methanol-utilisers (Barnes *et al.*, 1976; Hammond & Higgins, 1978; O'Keefe & Anthony, 1978; van Verseveld & Stouthamer, 1978; Keevil & Anthony, 1979b) which lack an extensive internal membrane system (Rokem *et al.*, 1978; see section 1.1.1).

From a comparison of these studies, there would appear to be substantial diversity amongst the methylotrophs, as regards proton translocation stoicheiometries. Schemes incorporating respiration-linked proton translocation have now been proposed for the respiratory chains of three methylotrophs: *M.trichosporium* (Fig. 4.1a), *Pseudomonas AM1* (Fig. 4.1b)

(a)


2H+
NADH
Q
b
c
aa3
O2
Methanol
PQQ
cco
O2
H2O
2H+
O2
Methane

(b)


2H+
2H+
2H+
NADH
Q
b
c
aa3
O2
Methanol
PQQ
c
2H+

(c)

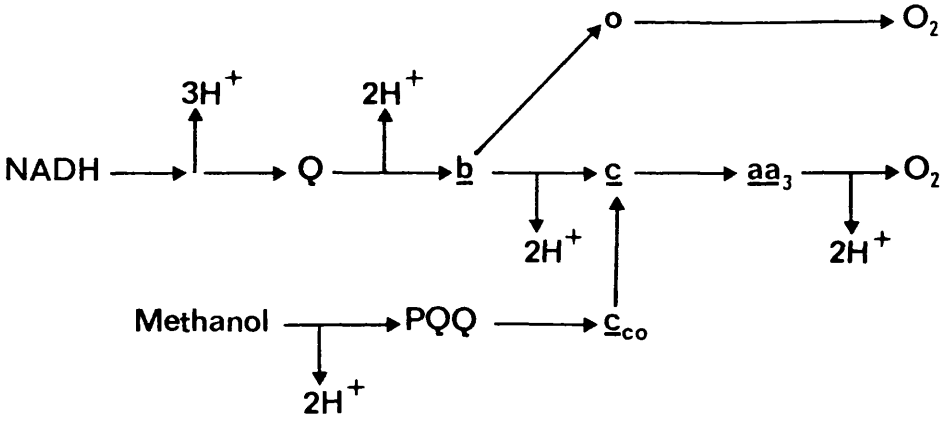

o
O2
3H+
2H+
NADH
Q
b
c
aa3
O2
2H+
2H+
Methanol
PQQ
cco
2H+

Fig. 4.1 Respiration-linked proton translocation in methylotrophic bacteria. (a) M.trichosporium (after Higgins et al., 1977), (b) carbon-limited Pseudomonas AM1 (after Keevil & Anthony, 1979b), (c) P.denitrificans (after van Verseveld et al., 1981). Abbreviations: PQQ, pyrrolo-quinoline quinone (prosthetic group of methanol dehydrogenase); Q, ubiquinone.

and Paracoccus denitrificans (Fig. 4.1c). M.trichosporium exhibits $\rightarrow H^+/O$ quotients of approximately 2 for the oxidation of ascorbate-TMPD, methane, methanol, formaldehyde and formate (Tonge et al., 1977a). In order to explain these low proton-translocation stoicheiometries, it has been proposed (Higgins et al., 1976, 1977) that the NADH : ubiquinone oxidoreductase region (site I) of the respiratory chain of this organism is not energy conserving; reducing equivalents from NADH pass to oxygen via either cytochrome c_{co} or cytochrome aa_3, proton translocation occurring between cytochromes b and c_{co}, or at the level of cytochrome aa_3, but not between cytochromes b and c in the branch of the respiratory chain terminated by cytochrome aa_3. The bulk of the reducing equivalents from methanol is considered to be conducted to oxygen via cytochrome c_{co}, energy conservation occurring between methanol dehydrogenase and this cytochrome. An added complication in this methanotroph is that cytochrome c_{co} may also act as an electron donor to methane monooxygenase (Tonge et al., 1975, 1977b; Higgins et al., 1976; but see Stirling & Dalton, 1979; Higgins et al., 1981), two protons being translocated per electron pair donated (Fig. 4.1a). Like M.trichosporium, Pseudomonas EN also exhibits equal proton translocation stoicheiometries for the oxidation of methanol, formaldehyde and formate (Barnes et al., 1976), but in this latter organism the $\rightarrow H^+/O$ quotient is approximately 3. These values are, however, difficult to interpret in the absence of further information on the respiratory chain composition of this organism.

Pseudomonas AM1, grown in methanol-limited continuous culture, exhibits $\rightarrow H^+/O$ quotients for the oxidation of endogenous substrates (NADH), and ascorbate-TMPD of approaching 6, and 2, respectively (Keevil & Anthony, 1979b). The $\rightarrow H^+/O$ quotient for methanol oxidation was not measured since

the $\Delta\Psi$-collapsing agent (see section 1.7) used in this work, SCN^-, substantially inhibited methanol oxidase activity, and other agents (e.g. K^+/valinomycin, NO_3^-) did not successfully collapse the $\Delta\Psi$ (O'Keefe & Anthony, 1978). However, the ATP/O quotient for respiration from methanol, determined in inverted membrane vesicles from both Pseudomonas sp.2 (Netrusov et al., 1977) and Pseudomonas AM1 (Netrusov & Anthony, 1979), was found to be consistent with the operation of only a single site of energy conservation during the oxidation of this substrate. In contrast to that of M.trichosporium, therefore, the respiratory chain of Pseuodomonas AM1 appears to contain three energy conserving sites : site I (NADH : ubiquinone oxidoreductase) acts to translocate $2H^+/2e^-$, and sites II plus III (ubiquinol oxidase) together act to translocate $4H^+/O$. In concordance with the thesis that methanol dehydrogenase interacts with the respiratory chain at the level of cytochrome c (see section 3.1), only site III is involved in methanol oxidation; the oxidation of this substrate is, thus, probably associated with a $\rightarrow H^+/O$ quotient of 2 (Fig. 4.1b). The facultative methylotroph, Pseudomonas extorquens, exhibits $\rightarrow H^+/O$ quotients which are closely comparable with those from Pseudomonas AM1 (Hammond & Higgins, 1978), and hence the number and arrangement of proton translocating sites in these two organisms is probably similar.

Methanol-grown P.denitrificans exhibits proton translocation stoicheiometries which are substantially higher than those of the other methylotrophs studied so far (van Verseveld & Stouthamer, 1978; van Verseveld et al., 1981); indeed the values determined in this organism, which has a mitochondrion-like respiratory chain (John & Whatley, 1977), are amongst the highest so far reported in any bacterium (see Jones, 1977). P.denitrificans exhibits $\rightarrow H^+/O$ quotients for the oxidation of endogenous

substrates (NAD(P)H), methanol and ascorbate-TMPD of approximately 7-10.5, 3.5-4 and 3, respectively. In order to explain these high proton translocation stoicheiometries, and particularly those for the oxidation of methanol and ascorbate-TMPD, it has been proposed that the cytochrome oxidase $\underline{aa}_3$ of this organism acts as a proton pump (see section 1.9.4; the phrase 'proton pump' in the context of cytochrome oxidase is generally used to denote net proton translocation i.e. $\rightarrow H^+/O > 0$, $\rightarrow K^+/O > 2$. Cytochrome oxidases which do not catalyse net proton translocation i.e. $\rightarrow H^+/O=0$, $\rightarrow K^+/O=2$ would not be regarded as 'proton pumps' in this terminology, even if their mechanism involved genuine transmembrane proton translocation). The complete respiratory chain of P.denitrificans is thus proposed to contain three energy-conserving sites : site I (NADH: ubiquinone oxidoreductase) acts to translocate $3H^+/2e^-$, site II (ubiquinol : cytochrome c oxidoreductase) translocates $4H^+/2e^-$, and site III (cytochrome c oxidase) translocates $2H^+/O$ (Fig. 4.1c).

Two major potential sources of error in the determination of bacterial proton translocation stoicheiometries are insufficient attention to growth conditions, and the inadequacy of the oxygen-pulse method (Mitchell & Moyle, 1967a) which has been ubiquitously used for the measurement of $\rightarrow H^+/O$ quotients in bacteria. As regards the growth of bacteria for these studies, the use of continuous culture to provide cells grown under reproducible, well-defined conditions is essential, as proton translocation stoicheiometries are known to vary both with the growth-phase and the nature of the limiting nutrient (Lawford et al., 1976; Haddock & Jones, 1977; see sections 1.8.2, 5.1).

The inadequacies of the oxygen-pulse method as regards measurement of proton and charge translocation stoicheiometries have been discussed in

section 1.7.1. However, it is worth stressing here that it is very difficult to identify the substrate which is oxidised during the short burst of respiration elicited by an oxygen pulse; and in order to obtain unambiguous $\rightarrow H^+/O$ quotients for the oxidation of added substrates, bacterial suspensions must be completely starved of endogenous substrates. This is frequently difficult to achieve and, even when it is possible, prolonged incubation in the absence of carbon substrate may damage the cytoplasmic membrane and/or the respiratory system. The oxygen-pulse method for the evaluation of $\rightarrow H^+/O$ quotients in mitochondria has also been criticised on the grounds that extrapolation procedures are inadequate to correct for a rapid phase of decay of the ΔpH, due to proton symport on the phosphate carrier (see section 1.7.1). Bacteria, however, do not require such rapid phosphate transport systems as mitochondria which must transport one molecule of phosphate for each molecule of ATP synthesised; and interestingly Cox & Haddock (1978) have shown that a mutant of Escherichia coli, which is unable to transport phosphate, exhibits a similar $\rightarrow H^+/O$ quotient to the wild-type organism. Nevertheless, it is possible that rapid movements of protons, in symport with other endogenous anions, may lead to underestimation of $\rightarrow H^+/O$ quotients in bacteria.

All the experiments reported in this chapter were performed using bacteria from well-defined methanol-limited continuous cultures ($D = 0.18h^{-1}$, see section 2.2.3). In order to determine the proton translocation stoicheiometries for various substrates, in M.methylotrophus, $\rightarrow H^+/O$ quotients have been measured by the oxygen-pulse method, and both $\rightarrow H^+/O$ and $\rightarrow$ charge/O (measured as $\rightarrow K^+/O$) quotients have been determined by an initial-rate procedure previously applied to mitochondria (see section 1.7.2). One advantage of the initial-rate method, over the oxygen-pulse method, is that respiration is initiated by the addition of

substrate, and hence there can be no ambiguity over the identification of the respiratory substrate. In addition, the $\rightarrow$ charge/O quotient should be unaffected by movements of protons in symport with anions, and hence may be used to determine whether the $\rightarrow H^+/O$ quotients are caused to be underestimated by secondary proton movements.

M.methylotrophus is known to contain three CO-binding cytochromes, aa_3, o and c_{co}, two of which (cytochromes aa_3 and o) probably function as terminal oxidases (see section 3.6). In view of this potential complexity, particular attention has been paid to proton translocation in the terminal region of the respiratory chain. $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients for the oxidation of ascorbate-TMPD have been determined by the initial-rate method (see Sigel & Carafoli, 1978, 1979, 1980), in the presence and absence of cyanide, this inhibitor being used to control the relative activities of the two oxidases.

4.2 Proton translocation linked to endogenous respiration in M.methylotrophus

The addition of air-saturated KCl to an anaerobic suspension of M.methylotrophus resulted in rapid acidification of the external medium (Fig. 4.2). The extent of the ΔpH was maximal at pH6.2 (Fig. 4.3a), and in the presence of 1µg of valinomycin/mg dry wt. bacteria (Fig. 4.3b); this K^+ ionophore was present to allow collapse of the $\Delta\Psi$ which would otherwise build up and oppose further proton translocation. The decay of the ΔpH showed first-order kinetics for at least 1 min, but after this period the rate of decay slowed, and the pH often failed to return to the original baseline (Fig. 4.2). The half-time ($t_{1/2}$) of the first-order region of decay was 104 $\pm$ 10(6)s in the absence of valinomycin, decreasing to 54 $\pm$ 7(7)s in the presence of 1µg of valinomycin/mg dry wt. bacteria.

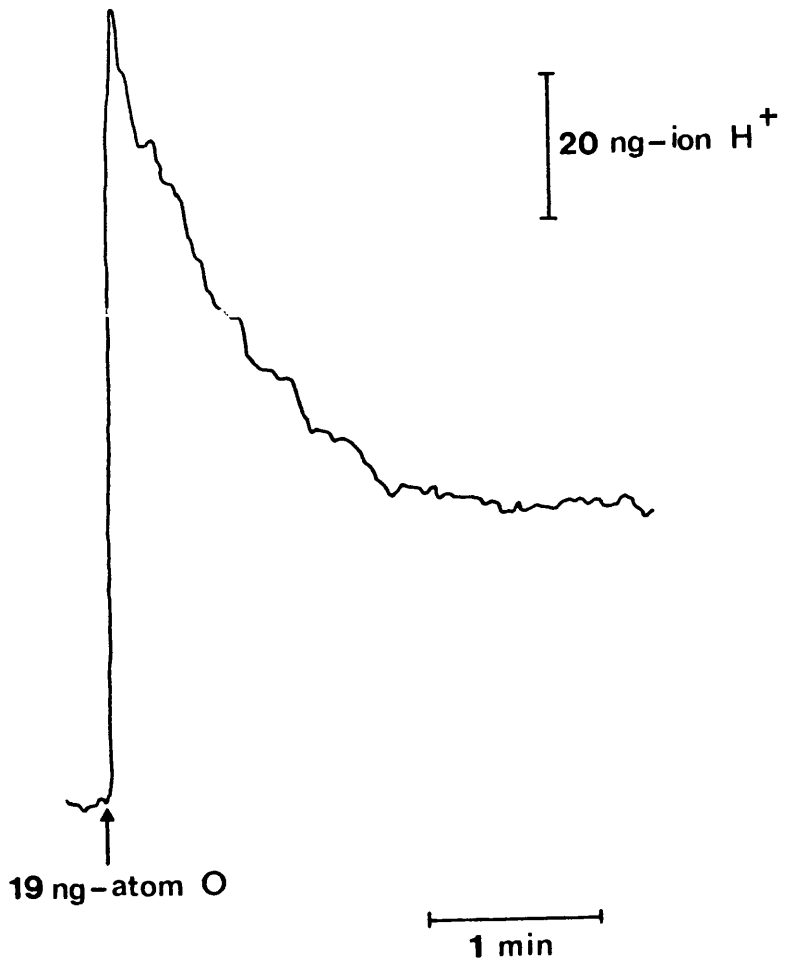
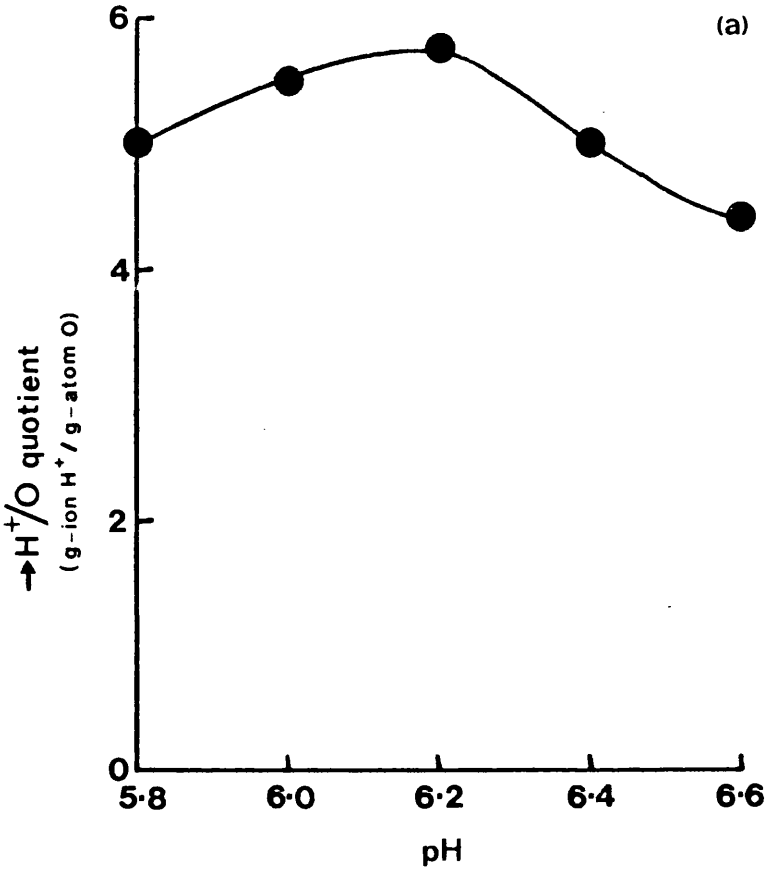


Fig. 4.2 Typical pH response to an oxygen-pulse. The conditions were as described in section 2.8.1 in the presence of endogenous substrates alone.


(a)
6
4
2
0
→H+/O quotient
(g-ion H+/g-atom O)
5·8
6·0
6·2
6·4
6·6
pH

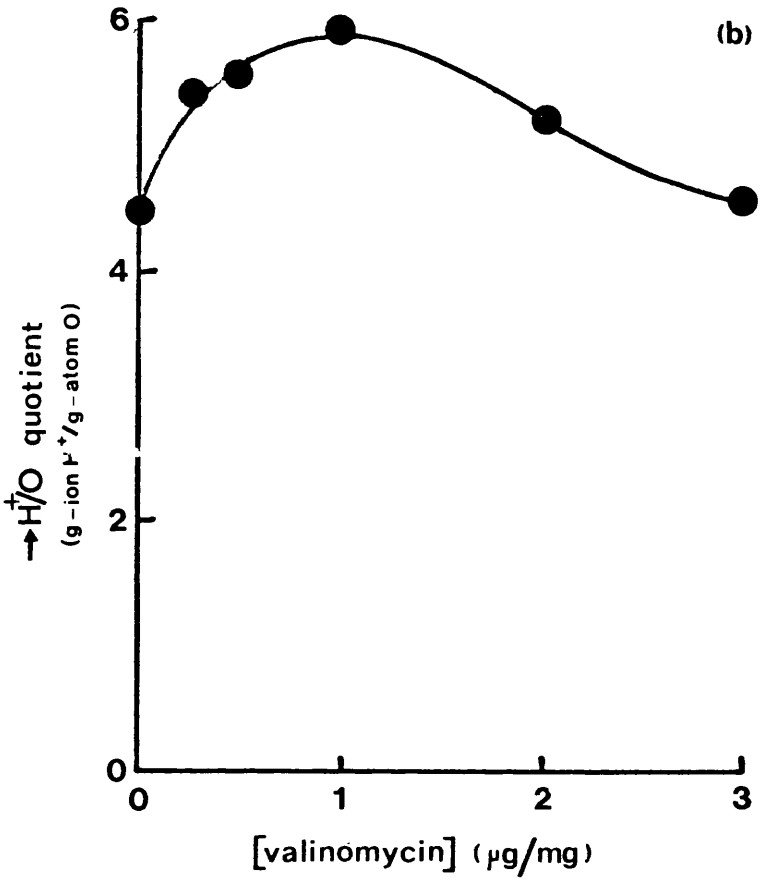


Fig. 4.3 Effect of pH and valinomycin concentration on the $\rightarrow H^+/O$ quotient measured by the oxygen-pulse method. $\rightarrow H^+/O$ quotients for the oxidation of endogenous substrates were determined as described in section 2.8.1, except that in (a) the pH was varied, and in (b) the concentration of valinomycin was varied.

The magnitude of the $\rightarrow H^+/O$ quotient was less when KSCN was used in place of valinomycin to collapse the $\Delta\Psi$ component of the proton motive force; this was probably due to the rapid rate of decay of the ΔpH observed in the presence of thiocyanate (in the presence of 120mM-KSCN, $t_{1/2} = 10.9 \pm 2.2(8)$s). The rate of decay of the ΔpH was increased by low concentrations of the uncoupling agent (H^+ ionophore) FCCP (10nM-FCCP caused a 50% decrease in the $t_{1/2}$ of decay, and 1μM-FCCP totally abolished the response to subsequent additions of oxygen). Bacterial suspensions which had been incubated with 1μg of nigericin / mg dry wt. bacteria for 15 min, in the presence or absence of valinomycin, showed no pH response to the addition of oxygen. (Nigericin facilitates H^+/K^+ antiport, and hence allows the collapse of the ΔpH).

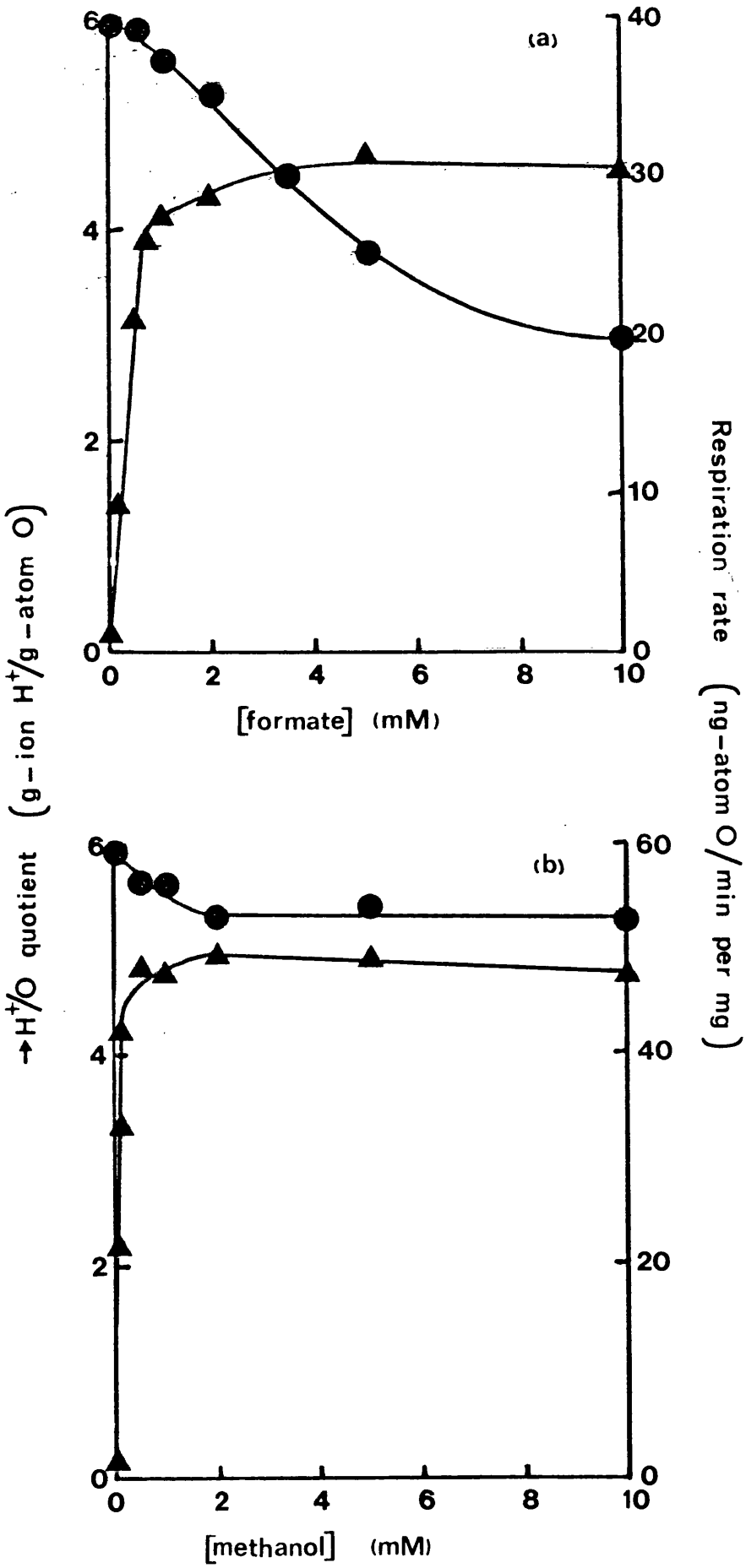
Under the optimum conditions, the $\rightarrow H^+/O$ quotient for the oxidation of endogenous substrates was found to be $5.92 \pm 0.17(9)$ g-ion H^+/g-atom O. This value was not significantly affected when bacterial suspensions were washed under anaerobic conditions in an attempt to deplete the cells of permeant anions which might otherwise cause underestimation of the $\rightarrow H^+/O$ quotient (see section 1.7.1; after two washes, $\rightarrow H^+/O = 6.04 \pm 0.18(5)$ g-ion H^+/g-atom O). N-ethylmaleimide, a thiol blocking reagent which increases the value of the $\rightarrow H^+/O$ quotient in mitochondria, possibly by inhibiting H^+/phosphate symport (see section 1.7.1), caused the $\rightarrow H^+/O$ quotient for the oxidation of endogenous substrates in M.methylotrophus to decrease steadily at concentrations above 10nmol/mg dry wt. bacteria (in the presence of 100 nmol of N-ethylmaleimide/mg dry wt. bacteria, $\rightarrow H^+/O = 1.6$ g-ion H^+/g-atom O).

4.3 Stoicheiometry of proton translocation linked to the oxidation of added substrates in M.methylotrophus, measured by the oxygen-pulse method

Although preferable when performing these experiments, it did not prove possible to starve bacteria of all their endogenous substrates by shaking bacterial suspensions in 140mM-KCl, 1.5mM-glycylglycine (pH7.0) for several hours. When bacteria were sufficiently starved to give no pH response to an oxygen pulse, it was not possible to restore the response by the addition of substrate. Bacteria harvested from methanol-limited continuous cultures, however, exhibited very low rates of respiration from endogenous substrates ($1.4 \pm 0.1(10)$ng-atom O/min per mg dry wt. bacteria after 30 min incubation with valinomycin) which could be markedly stimulated by the addition of substrate.

Fig. 4.4a-d shows the effects of formate, methanol, formaldehyde and duroquinol on the whole-cell respiration rate and $\rightarrow H^+/O$ quotient. The rates of respiration from formate and duroquinol, measured after the 30 min incubation with valinomycin, were comparable to those reported in Table 3.3, but the rates of oxidation of methanol and formaldehyde were significantly lower here. This decrease appears to be due to the lability of methanol oxidase during incubation at 40°C, rather than the composition of the reaction mixture. The assumption must be made, in the interpretation of the $\rightarrow H^+/O$ quotients for respiration from methanol and formaldehyde, that the activity remaining after incubation is representative of the total, in terms of its coupling to proton translocation.

The rate of decay of the ΔpH which resulted from an oxygen-pulse, in the presence of these substrates, was in all cases similar to that


(a)
(b)
[formate] (mM)
[methanol] (mM)
→H+/O quotient (g-ion H+/g-atom O)
Respiration rate (ng-atom O/min per mg)
0
2
4
6
8
10
20
30
40
60

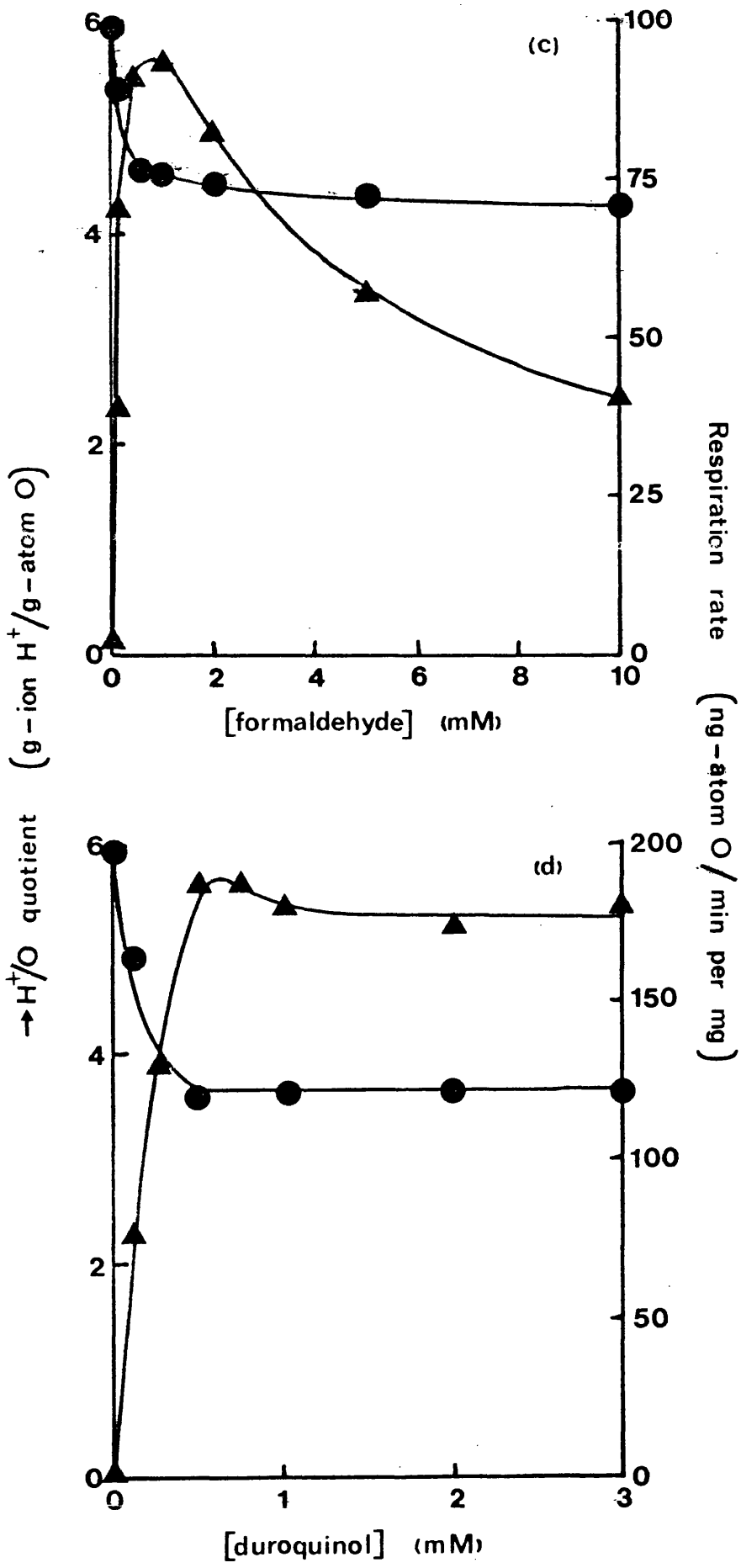

(c)
(d)
[formaldehyde] (mM)
[duroquinol] (mM)
→ H+/O quotient (g-ion H+/g-atom O)
Respiration rate (ng-atom O/min per mg)
0
2
4
6
8
10
1
3
25
50
75
100
150
200

Fig. 4.4 $\rightarrow H^+/O$ quotients, measured by the oxygen-pulse method, and respiration rates for the oxidation of added substrates. $\rightarrow H^+/O$ quotients and respiration rates were measured as described in sections 2.8.1 and 2.6 respectively. Respiration rates were determined in the same reaction mixture, and after similar incubation periods, as used for the oxygen-pulse method. The substrates used were (a), formate; (b), methanol; (c), formaldehyde; and (d), duroquinol (the rate of respiration from this substrate was corrected for autoxidation). Symbols : ▲, respiration rate; ●, $\rightarrow H^+/O$ quotient.

observed in the presence of endogenous substrates alone; and, whatever the substrate, the presence of FCCP (1μM) abolished the pH response to an oxygen-pulse.

The $\rightarrow H^+/O$ quotient for respiration from the NAD^+-linked substrate, formate (Fig. 4.4a), decreased as a function of increasing substrate concentration, even beyond the region where there was a large increase in respiration rate with increasing concentration of formate. There are several lines of evidence which indicate that the uptake of formate by M.methylotrophus is linked to the entry of a proton i.e. as the neutral species, formic acid. Firstly, small alkalinisations of the external medium were observed when formate (as an anaerobic solution) was added to an anaerobic suspension of bacteria. Secondly, formate added during the early stage of decay of the ΔpH induced by an oxygen-pulse caused the rapid collapse of the ΔpH. Thirdly, formate oxidation was inhibited by nigericin, which dissipates the ΔpH across the membrane, and by FCCP which dissipates both the ΔpH and $\Delta\Psi$, but not by valinomycin which dissipates only the $\Delta\Psi$ component of the protonmotive force. Garland et al. (1975) observed biphasic decays of the ΔpH induced by an oxygen-pulse in E.coli, in the presence of formate, and attributed the initial rapid phase of decay to movement of formic acid into the bacteria, in response to the ΔpH established by proton translocation. If this initial phase of decay were more rapid in M.methylotrophus than in E.coli, it might not be resolved in these experiments. Furthermore, should the extent of the rapid phase of decay vary in accordance with the formate concentration, then the outcome would be a decrease in $\rightarrow H^+/O$ quotient with increasing formate concentration, as seen here (Fig. 4.4a). Attempts to resolve an initial rapid phase of decay by decreasing the temperature of the assay were unsuccessful.

The presence of methanol had little effect on the stoicheiometry of proton translocation, causing only a slight decrease in the $\rightarrow H^+/O$ quotient below the endogenous value (Fig. 4.4b). A somewhat lower stoicheiometry of proton translocation had been expected for respiration from methanol, as this substrate is thought to donate reducing equivalents to the respiratory chain at the level of cytochrome c (see sections 3.1, 3.6). However, since the bacteria could not be fully starved of endogenous substrates, it is not possible completely to rule out interference by endogenous substrate oxidation during the measurement of the $\rightarrow H^+/O$ quotient for respiration from methanol, despite the 30-fold stimulation of the respiration rate observed on the addition of this substrate.

In the presence of 75μM-HQNO to inhibit flux through the respiratory chain prior to cytochrome c (see section 3.5.1), proton translocation linked to the oxidation of endogenous substrates was slow, and the extent of proton movement was very small. However, after the addition of methanol (1-10mM), proton translocation became rapid and increased in extent to give an $\rightarrow H^+/O$ quotient of $1.59 \pm 0.11(5)$ g-ion H^+/g-atom O. There was some increase in the rate of decay of the ΔpH, in the presence of HQNO, making extrapolation of the pH response difficult (see section 2.8.1), but this could not totally account for the low $\rightarrow H^+/O$ quotient that was observed for methanol oxidation. (Antimycin A could not be used in place of HQNO as this inhibitor also caused the rapid collapse of the ΔpH following an oxygen pulse).

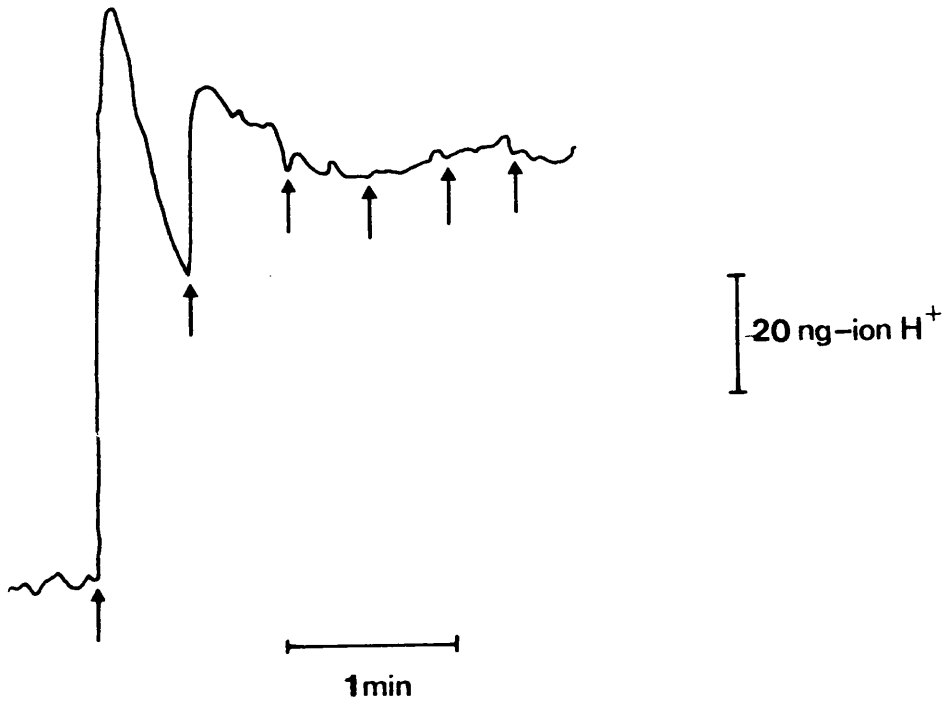
It is evident, from the work discussed above, that endogenous substrate oxidation may interfere with the measurement of proton translocation stoicheiometries for the oxidation of added substrates, even when addition of substrate causes a very large stimulation of the respiration rate. In

the anaerobic incubation period between the administration of oxygen pulses, reduced redox carriers (principally NAD(P)H) will slowly accumulate owing to reduction by endogenous substrates. This pool of reductant will then be rapidly oxidised when an oxygen pulse is introduced. Respiration from added substrates must compete with the rapid oxidation of the reductant pool, rather than with the slow endogenous respiration measured continuously under aerobic conditions. Indeed, this latter rate is insufficient to account for the rapid proton translocation elicited by an oxygen pulse in the presence of endogenous substrates alone (Fig. 4.2). Experimentally, it was possible to deplete the reductant pool by frequently introducing large oxygen pulses, such that endogenous respiration was insufficiently rapid to refill the pool between pulses. In the absence of added substrates, there was little pH response to added oxygen after the first two pulses (Fig. 4.5a); however, in the presence of methanol, further oxygen pulses yielded $\rightarrow H^+/O$ quotients in the range 2-3 g-ion H^+/g-atom O, after the reductant pool had been depleted by the first two pulses (Fig. 4.5b). An estimate of 2nmol NADH/mg dry wt. bacteria can be made for the size of the reductant pool.

No change in pH was observed when methanol (as an anaerobic solution) was added to an anaerobic suspension of bacteria, thus suggesting that methanol may cross the coupling membrane, if necessary, without H^+ cotransport. In contrast to formate oxidation, respiration from methanol was slightly stimulated by FCCP, possibly owing to the alleviation of respiratory control.

The oxidation of formaldehyde in M.methylotrophus may occur, at least in vitro, either via methanol oxidase, or via the reduction and re-oxidation of NAD^+ (see sections 1.3.3, 3.5.1). Measurement of the $\rightarrow H^+/O$

(a)



(b)

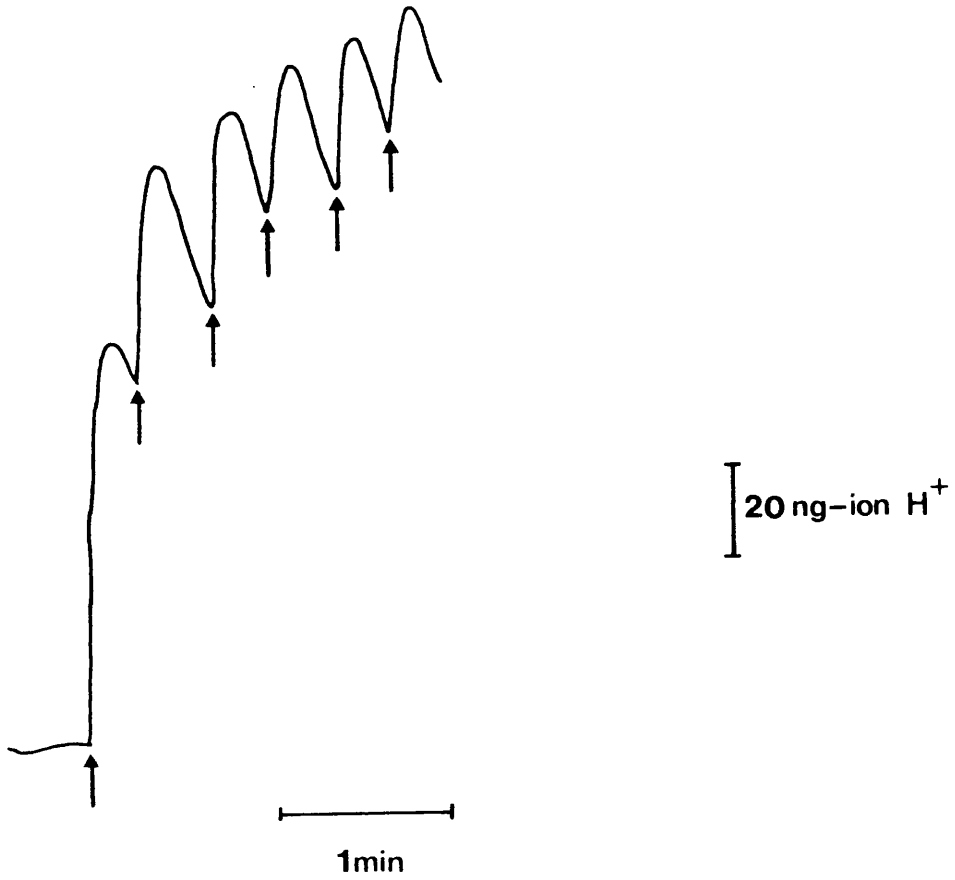


Fig. 4.5 pH response of an anaerobic suspension of M.methylotrophus to frequent, large oxygen pulses, in the presence or absence of methanol. Bacterial suspensions (2-3 mg dry wt. bacteria/ml) were incubated as for the measurement of $\rightarrow H^+/O$ quotients by the oxygen-pulse method (see section 2.8.1); (a), with no added substrate; and (b), in the presence of 1mM-methanol. The arrows indicate the addition of 50µl of air-saturated 140mM-KCl (19ng-atom O).

quotient for formaldehyde oxidation might also be expected to be subject to interference by endogenous substrate oxidation, as discussed above. The $\rightarrow H^+/O$ quotient of approximately 4.5g-ion H^+/g-atom O for the oxidation of 'saturating' concentrations of formaldehyde (Fig. 4.4c; concentrations of formaldehyde above 1mM were found to be inhibitory) should thus be regarded as a maximum estimate. A value for the $\rightarrow H^+/O$ quotient for the oxidation of formaldehyde via methanol oxidase may be obtained in the presence of HQNO – a value of 2.08 ± 0.13(5) g-ion H^+/g-atom O was observed under these conditions, although it should be noted that the inhibitor again caused an increase in the rate of decay of the ΔpH. Small acidifications were observed on the addition of formaldehyde (as an anaerobic solution) to an anaerobic suspension of bacteria. The source of these acidifications is unclear, but they were also observed in Pseudomonas AM1 by O'Keefe & Anthony (1978). FCCP caused a slight stimulation in the rate of formaldehyde oxidation, again possibly owing to the alleviation of respiratory control.

Duroquinol donates reducing equivalents to the respiratory chain at the level of the endogenous quinone, or cytochrome b (see section 3.5.1). The high rate of respiration from duroquinol (Fig. 4.4d; K_M = 0.15mM, V_{max} = 190ng-atom O/min per mg dry wt. bacteria), compared to that from the physiological substrates, suggests that the oxidation of this substrate should compete effectively with oxidation of the endogenous reductant pool during an oxygen pulse. The $\rightarrow H^+/O$ quotient for the oxidation of saturating concentrations of duroquinol was 3.65 ± 0.10(11) g-ion H^+/g-atom O.

4.4 Determination of proton and charge translocation stoicheiometries in *M.methylotrophus*, using the initial-rate method

4.4.1 Stoicheiometry of respiration-linked proton translocation, measured by the initial-rate method

Fig. 4.6 shows typical traces of the changes in pH and oxygen concentration which were obtained when methanol was added to an aerobic suspension of bacteria, in the presence of K^+/valinomycin. The pH trace was linear for approximately 10s and, after an initial lag of 2-3s, a constant rate of oxygen uptake was observed (this rate being used in the determination of the $\rightarrow H^+/O$ quotient). Values for the $\rightarrow H^+/O$ quotient were constant over a wide range of oxygen-uptake rates, thus suggesting that the response times of the electrodes were not limiting under these conditions.

Table 4.1 shows the values of the $\rightarrow H^+/O$ quotient for respiration from formate, methanol, formaldehyde and duroquinol, measured by the initial-rate method. There is no possibility of interference by endogenous substrate oxidation when using this method since, in contrast to the situation in the oxygen-pulse method, the reductant pools are oxidised before the addition of substrate. Furthermore, as only initial rates are considered, there are no problems due to respiration from the products of the first substrate oxidation. In the presence of 1μM-FCCP, no rapid pH changes were observed in response to the addition of any of the above substrates.

The value of the $\rightarrow H^+/O$ quotient for formate oxidation of approximately 3.5g-ion H^+/g-atom O (Table 4.1) is probably underestimated owing to the movement of formic acid across the membrane, as discussed in

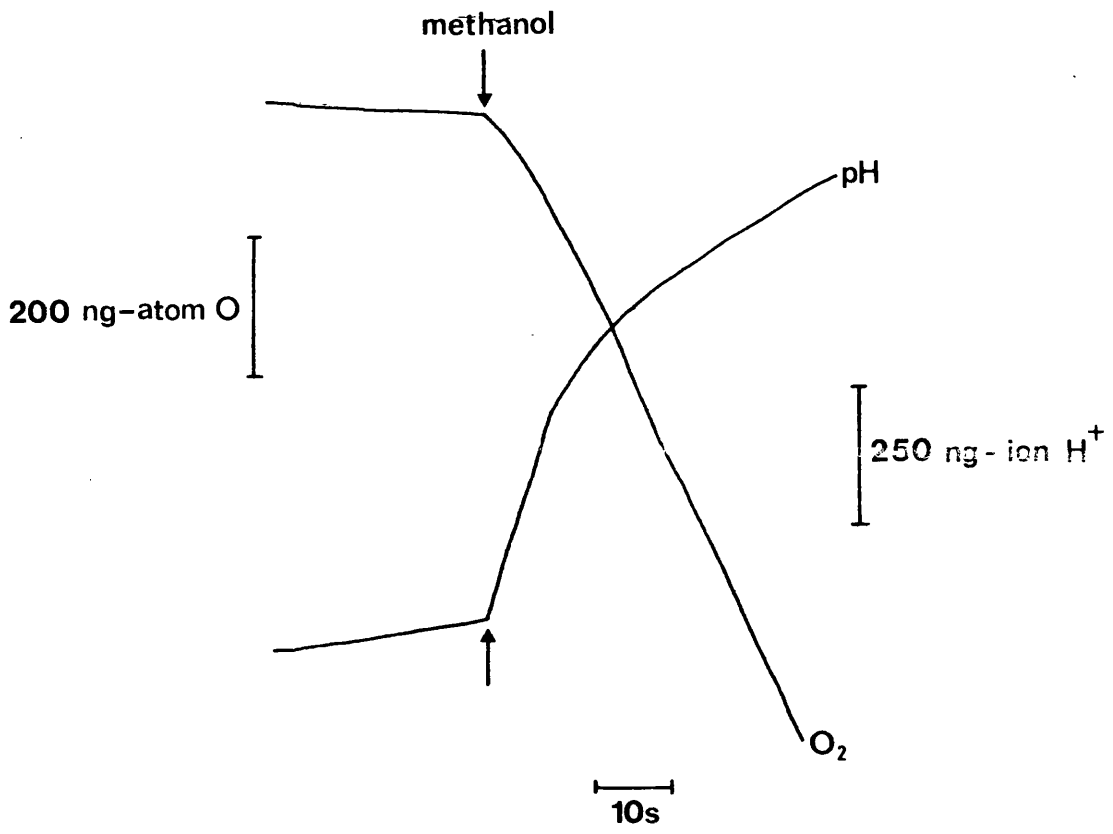


Fig. 4.6 Initial-rate method for the measurement of → H^+/O quotients.

The changes in pH and oxygen concentration were recorded as described in section 2.8.2. The arrows indicate the addition of methanol to a final concentration of 1mM.

Respiratory substrate	$\rightarrow H^+/O$ quotient (g-ion H^+/g-atom O)	$\rightarrow K^+/O$ quotient (g-ion K^+/g-atom O)
Formate	3.46 ± 0.28 (10)	6.27 ± 0.14 (39)
Duroquinol	3.48 ± 0.13 (8)	n.d.
Formaldehyde	3.42 ± 0.34 (8)	3.12 ± 0.16 (11)
Methanol	1.99 ± 0.19 (9)	1.78 ± 0.09 (8)

Table 4.1 $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients for the oxidation of formate, duroquinol, formaldehyde and methanol, measured by the initial-rate method. The $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients were determined in separate experiments, by the initial-rate method, in the presence of 140mM-KCl or 10mM-KCl plus 140mM-LiCl, respectively (see section 2.8.2). The final concentration of substrate was in all cases 1mM, except for measurement of the $\rightarrow K^+/O$ quotient for formate oxidation, when the formate concentration was varied between 1 and 10mM.

Abbreviation: n.d., not determined.

the previous section (section 4.3). However, the $\rightarrow H^+/O$ quotient of approximately 3.4g-ion H^+/g-atom O for the oxidation of formaldehyde is consistent with the view that this substrate may be oxidised *in vitro* either via the reduction and reoxidation of NAD^+, or directly via methanol oxidase (see sections 1.3.3, 3.5.1). Furthermore, the $\rightarrow H^+/O$ quotient of approximately 3.5g-ion H^+/g-atom O for respiration from duroquinol is similar to that obtained using the oxygen-pulse method (Fig. 4.4d), and indicates that up to $4H^+$ may be translocated during the passage of $2e^-$ from the quinone region of the respiratory chain to oxygen. The $\rightarrow H^+/O$ quotient of close to 2 for methanol oxidation was not significantly affected by N-ethylmaleimide up to a concentration of 100nmol/mg dry wt. bacteria.

4.4.2 Stoicheiometry of respiration-linked charge translocation, measured by the initial-rate method

Unfortunately, it was not possible to measure $\rightarrow$ charge/O quotients (as $\rightarrow K^+/O$ quotients) by the oxygen-pulse method, as consistent movements of neither K^+ nor H^+ could be obtained at the very low K^+ concentrations (less than 1mM) which were necessary to allow adequate sensitivity of the K^+-selective electrode in this procedure. The magnitude of the ion movements measured in the initial-rate method, however, is considerably greater than in the oxygen-pulse method, thus allowing a higher K^+ concentration to be used. The $\rightarrow K^+/O$ quotient for the oxidation of methanol was found to be independent of the K^+ concentration between 10 and 20mM, though at concentrations of K^+ below 10mM, the $\rightarrow K^+/O$ quotient was significantly decreased (20mM was the highest concentration of K^+ that was compatible with use of the K^+-selective electrode in this procedure).

Table 4.1 shows the $\rightarrow K^+/O$ quotients that were obtained for the oxidation of formate, methanol and formaldehyde in the presence of 10mM-KCl (140mM-LiCl being present to maintain the ionic strength). No K^+ movements were observed in response to the addition of any of these substrates in the presence of 1μM-FCCP. The $\rightarrow K^+/O$ quotient for the oxidation of the NAD^+-linked substrate, formate, was close to 6g-ion K^+/g-atom O, and was independent of the formate concentration between 1 and 10mM. This result confirms that $6H^+$ are translocated during the oxidation of one molecule of NADH, and that the $\rightarrow H^+/O$ quotient for formate oxidation, measured both by the initial-rate method and, at higher formate concentrations by the oxygen-pulse method, was underestimated owing to the uptake of formic acid.

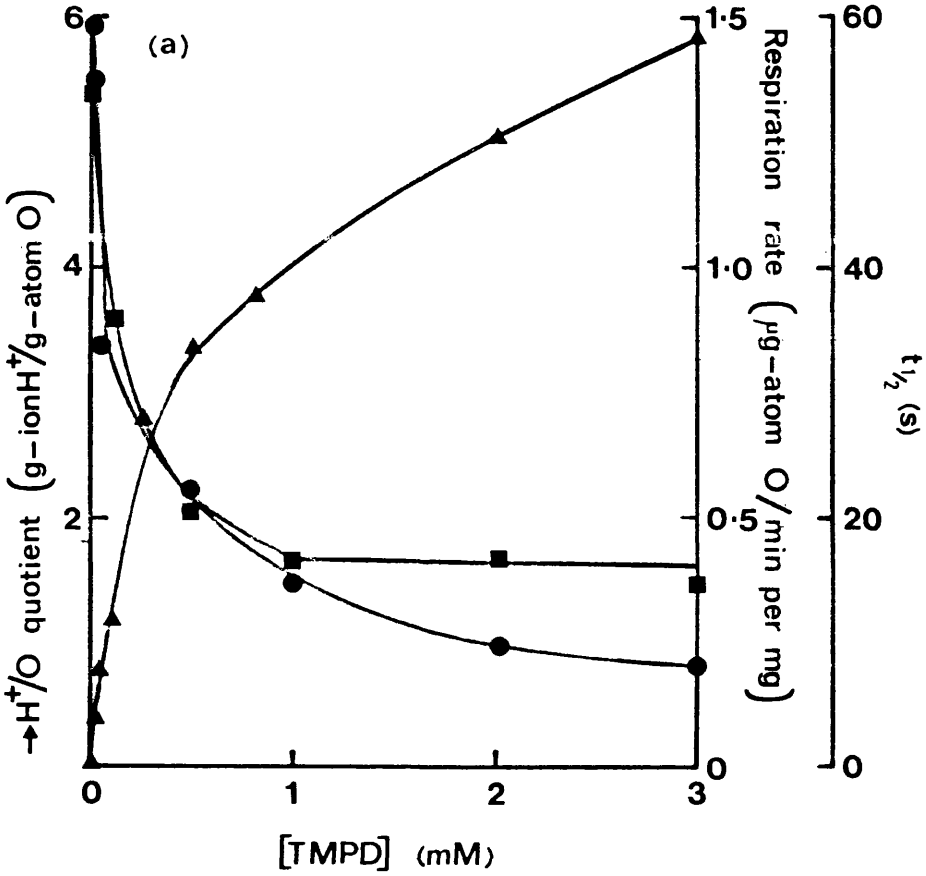
The $\rightarrow K^+/O$ quotients for the oxidation of methanol and formaldehyde were similar in magnitude to the $\rightarrow H^+/O$ quotients reported in the previous section (section 4.4.1), i.e. approximately 1.8 and 3.1g-ion K^+/g-atom O respectively. The $\rightarrow K^+/O$ quotient for methanol oxidation was not significantly affected by the presence of up to 100nmol of N-ethylmaleimide/mg dry wt. bacteria. Unfortunately, it was not possible to measure the $\rightarrow K^+/O$ quotient for duroquinol oxidation since the organic solvent (DMF) in which this substrate was dissolved appeared to interfere with the response of the K^+-selective electrode.

4.5 Proton and charge translocation stoicheiometries in the cytochrome c oxidase region of the respiratory chain of M.methylotrophus

4.5.1 Stoicheiometry of proton translocation linked to the oxidation of ascorbate-TMPD and $DADH_2$, measured by the oxygen-pulse method

Ascorbate-TMPD and $DADH_2$ both donate electrons to the respiratory chain at the level of cytochrome c (see section 3.6). At physiological pH, for each $2e^-$ donated by TMPD, $1H^+$ is released during the rereduction of TMPD by ascorbate. There is some dispute, however, as to whether $2H^+$ (Moyle & Mitchell, 1978; Mitchell & Moyle, 1979), or substantially less than $2H^+$ (see Wikström & Krab, 1979) are released per $2e^-$ donated by $DADH_2$.

There is little likelihood of interference by endogenous substrate oxidation during measurement of the $\rightarrow H^+/O$ quotient for respiration from the rapidly oxidised substrate ascorbate-TMPD (Fig. 4.7a; K_M = 0.4mM, V_{max} = 1.7µg-atom O/min per mg dry wt. bacteria). However, at concentrations above 100µM, TMPD substantially increases the permeability of the coupling membrane to protons, as shown by a decrease in the $t_{1/2}$ of decay of the ΔpH induced by an oxygen-pulse (Fig. 4.7a). The value of approximately 0.8g-ion H^+/g-atom O for the $\rightarrow H^+/O$ quotient at saturating concentrations of TMPD (Fig. 4.7a) must therefore be regarded as a minimum estimate. As the ascorbate-TMPD oxidase reaction is associated with an overall stoicheiometry of $-1H^+/O$, the ΔpH induced by an oxygen-pulse would be expected to decay below the original baseline; and in the presence of FCCP, an alkalinisation corresponding to $1H^+/O$ would be predicted in response to an oxygen-pulse. No overall alkalinisation was, however, observed either in the presence or absence of uncoupling agent. These findings may be partly explained by the


(a)
$\rightarrow H^+/O$ quotient (g-ionH^+/g-atom O)
Respiration rate (μg-atom O/min per mg)
$t_{1/2}$ (s)
[TMPD] (mM)
0
1
2
3
0
2
4
6
0
0.5
1.0
1.5
0
20
40
60

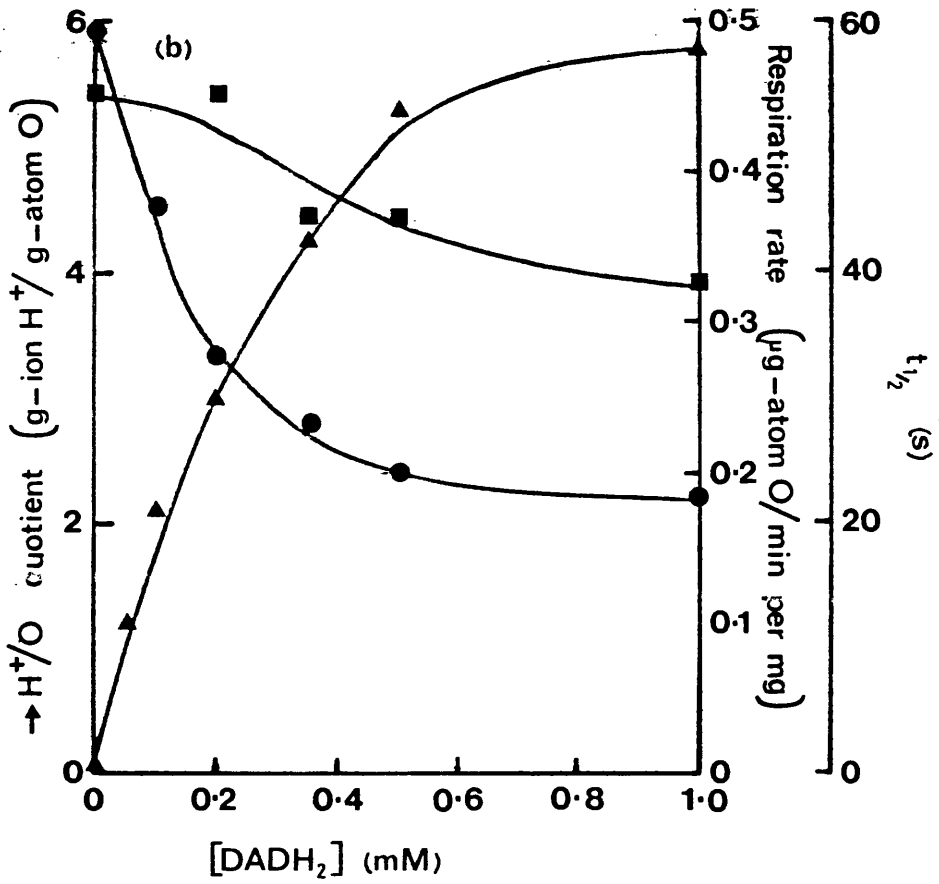


Fig. 4.7 Respiration rates, $\rightarrow H^+/O$ quotients, and half-times of the decay of the ΔpH resulting from an oxygen pulse ($t_{\frac{1}{2}}$), in the presence of ascorbate-TMPD or $DADH_2$. $\rightarrow H^+/O$ quotients and $t_{\frac{1}{2}}$'s were measured as described in section 2.8.1. Respiration rates (corrected for autoxidation) were determined as described in section 2.6 in the same reaction mixture, and after similar incubation periods, as used for the oxygen-pulse method. The substrates used were (a), ascorbate-TMPD (the ascorbate concentration was 3mM throughout); and (b), $DADH_2$. Symbols: ▲, respiration rate; ●, $\rightarrow H^+/O$ quotient; ■, $t_{\frac{1}{2}}$.

decay of dehydroascorbate to 2, 3-diketo-L-gulonate and subsequently to products which are stronger acids than dehydroascorbate (Borsook et al., 1937). However, it also seems likely that at least some of the oxidised TMPD is re-reduced by endogenous substrates, as opposed to by ascorbate. This phenomenon would tend to lead to overestimation of the $\rightarrow H^+/O$ quotient, but may be more than countered by the increased permeability of the membrane to protons, in the presence of TMPD.

Compared to ascorbate-TMPD, $DADH_2$ has the advantage that it does not act as an uncoupling agent, but the disadvantage that it is oxidised less rapidly (Fig. 4.7b; K_M = 0.26mM, V_{max} = 600ng-atom O/min per mg dry wt. bacteria), and hence may allow concomitant oxidation of endogenous substrates. The $\rightarrow H^+/O$ quotient at saturating $DADH_2$ concentrations was found to be approximately 2.2g-ion H^+/g-atom O (Fig. 4.7b).

The $\rightarrow H^+/O$ quotients for the oxidation of ascorbate-TMPD and $DADH_2$ were no greater than could be explained by scalar release of protons into the external medium on the donation of electrons to cytochrome c. There is thus no need, at this stage, to implicate a proton pump mechanism for either of the two cytochrome oxidases present in M.methylotrophus. Indeed the results presented above are consistent with a simple redox arm mechanism in which cytochrome c is situated on the periplasmic face of the membrane, and cytochrome oxidases aa_3 and o have their reaction sites on the cytoplasmic face, the electron-donation and oxygen-reduction reactions being connected via inwardly directed electron flow (Fig. 4.8).

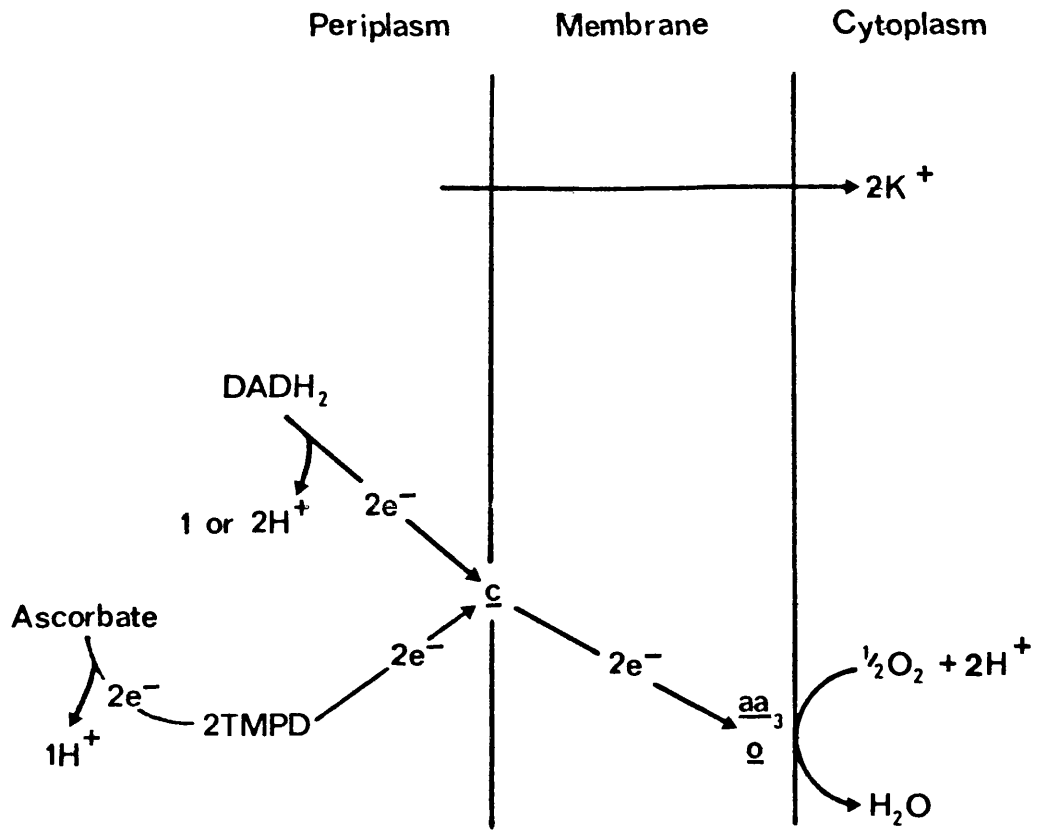


Fig. 4.8 Probable arrangement of the ascorbate-TMPD and $DADH_2$ oxidase systems in M.methylotrophus

4.5.2 Stoicheiometry of proton and charge translocation linked to the oxidation of ascorbate-TMPD, measured by the initial-rate method

Fig. 4.9ab shows typical traces of H^+ and K^+ movements obtained when ascorbate-TMPD was added to an aerobic suspension of M.methylotrophus, in the presence or absence of the uncoupling agent, FCCP. It should be noted that the TMPD concentration in these experiments (100μM) was insufficient to cause substantial uncoupling (see Fig. 4.7a), and indeed similar values for the $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients were obtained using TMPD concentrations as low as 10μM (see Fig. 4.10). The $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients shown in Fig. 4.9ab are fully consistent with the redox arm mechanism proposed for energy conservation at site III of the respiratory chain (Fig. 4.8). The initial uncoupler-sensitive acidification corresponds to the scalar proton released during ascorbate oxidation, and the net alkalinisation, characteristic of the ascorbate-TMPD oxidase reaction, is reflected by the subsequent alkalinisation phase, and by immediate alkalinisation in the presence of uncoupler. (This alkalinisation confirms that, under the aerobic conditions of this experiment, oxidised TMPD is re-reduced by ascorbate as opposed to by endogenous substrates [see section 4.5.1]). The transmembrane electron flow is balanced by the uncoupler-sensitive inward translocation of approximately $2K^+/O$.

4.5.3 Effect of cyanide on the stoicheiometry of proton and charge translocation linked to the oxidation of ascorbate-TMPD

Although the results described in sections 4.5.1 and 4.5.2 are fully consistent with the scheme depicted in Fig. 4.8, they could alternatively reflect a composite situation in which there are contributions from the two major terminal oxidases (cytochromes aa_3 and o) acting dissimilarly in

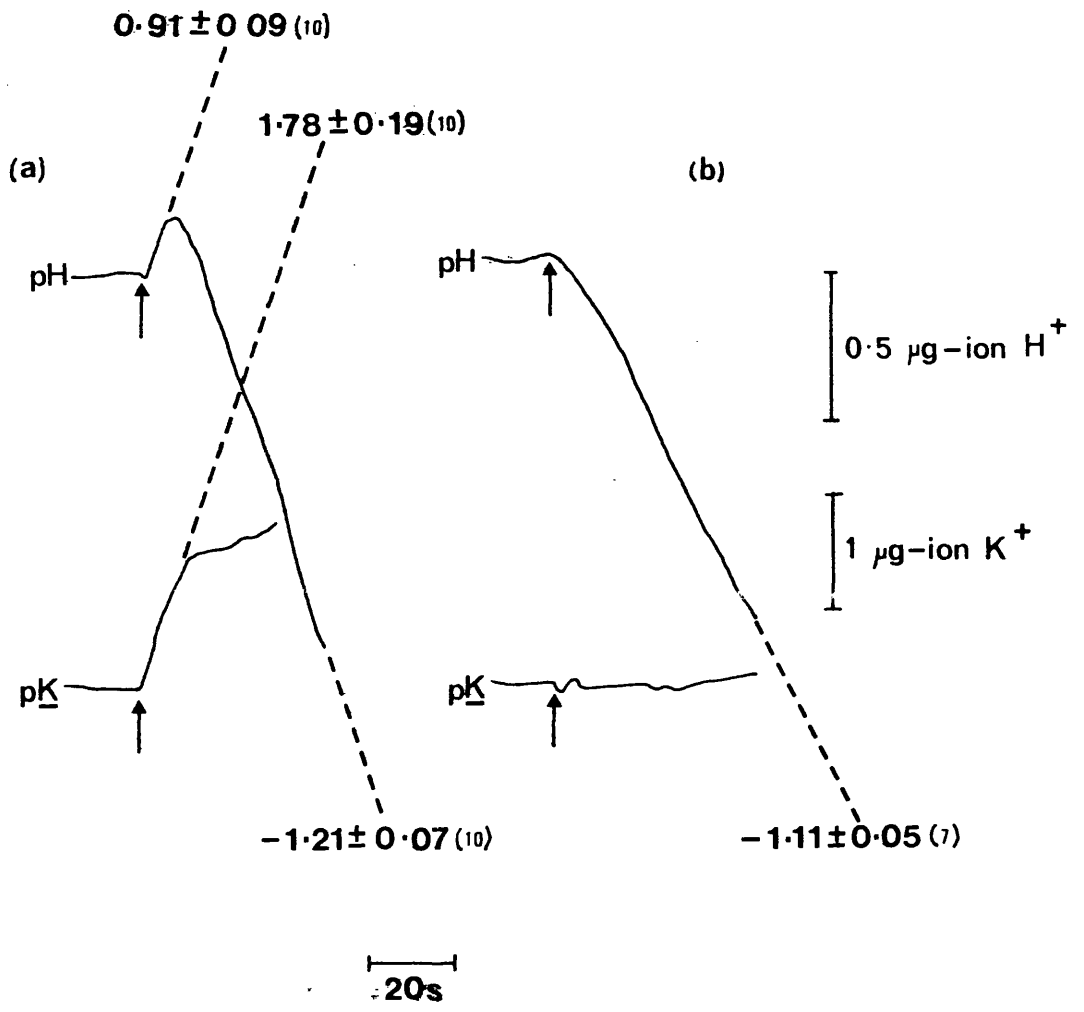


Fig. 4.9 Changes of pH and pK on the addition of ascorbate-TMPD to an aerobic suspension of M.methylotrophus. The pH and pK changes were recorded simultaneously as described in section 2.8.2. The arrows indicate the addition of ascorbate-TMPD to a final concentration of 3mM-ascorbate and 100μM-TMPD, and the numbers on the traces are → H^+/O or → K^+/O quotients (g-ion $H^+(K^+)$/g-atom O) calculated using the simultaneously determined respiration rate (see section 2.6). In (b), the bacteria were preincubated with FCCP (5μM) for 5 min prior to the addition of ascorbate-TMPD.

terms of energy conservation. For example, one oxidase may be situated with its reaction site on the external face of the membrane such that the cytochrome c oxidase reaction is associated with a $\rightarrow$ charge/O quotient of zero ($\rightarrow H^+/O = -2$), whereas the other oxidase may act as a proton pump as well as being associated with transmembrane electron flow (i.e. $\rightarrow$ charge/O > 2, $\rightarrow H^+/O > 0$).

Unfortunately, it was not possible to investigate the possibility of proton pumping via the use of DCCD, which appears to be a potent inhibitor of the cytochrome oxidase proton pump in mitochondria (Casey et al., 1979, 1980; but see Coin & Hinkle, 1979), as this reagent caused lysis of the bacteria, even at low concentrations (25nmoles/mg dry wt. bacteria). It was, however, possible to investigate whether the two oxidases have similar proton and charge translocating properties by using cyanide to selectively inhibit cytochrome oxidase o. Under the conditions of the experiment shown in Fig. 4.10 (10μM-TMPD to yield low respiration rates), it can be predicted from Fig. 3.6c that the I_{50}'s for cytochrome oxidases aa_3 and o will be approximately 40μM and 2.5μM respectively. Fig. 4.10 shows that 10μM-cyanide, which should restrict electron flux almost entirely to cytochrome oxidase aa_3, had no effect on either the $\rightarrow H^+/O$ or the $\rightarrow K^+/O$ quotient for ascorbate-TMPD oxidation. Unfortunately, it was not possible to perform the converse experiment in which cytochrome oxidase aa_3 is selectively inhibited by cyanide, at high rates of ascorbate-TMPD oxidation, due to the uncoupling properties of the high concentrations of TMPD which would be required for this experiment (Fig. 4.7a). Nevertheless, it seems likely that the two cytochrome oxidases, aa_3 and o, are similar in terms of their energy conservation properties, each exhibiting a charge/O quotient of 2 and a $\rightarrow H^+/O$ quotient of zero at site III.

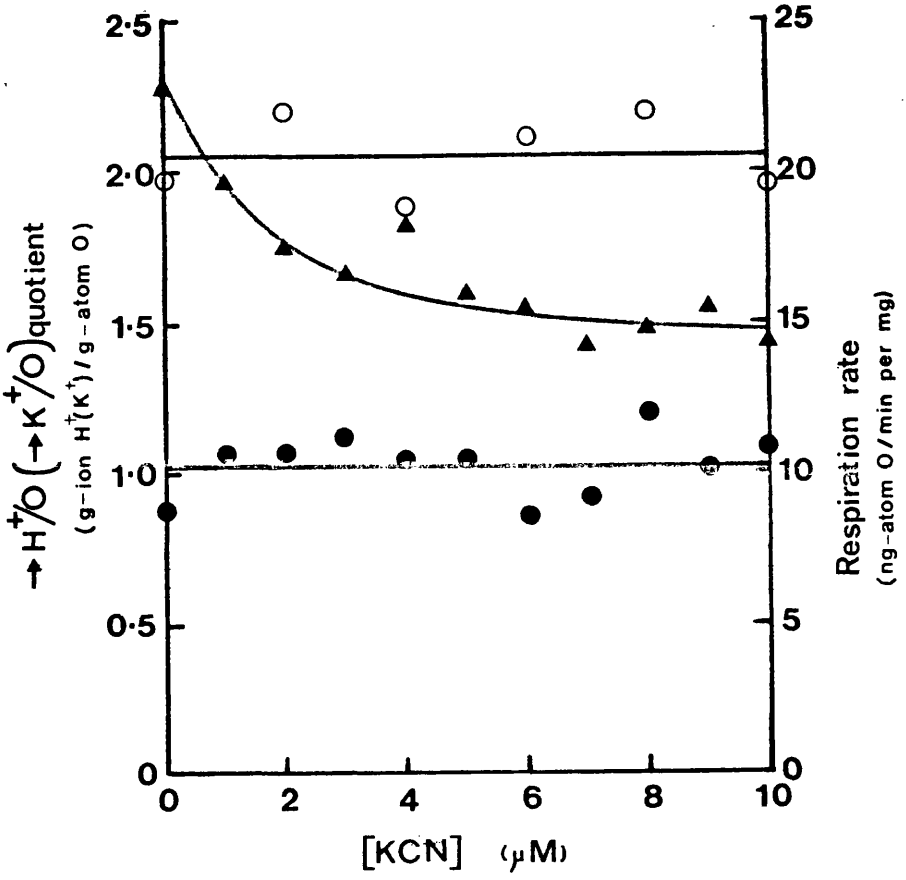


Fig. 4.10 Effect of cyanide on the → H^+/O and → K^+/O quotients for the oxidation of ascorbate-TMPD. The → H^+/O and → K^+/O quotients were determined simultaneously by the initial-rate method as described in section 2.8.2. The ascorbate-TMPD concentration was 3mM-ascorbate, 10μM-TMPD. Symbols: ▲ , respiration rate; ● , → H^+/O quotient; ○ , → K^+/O quotient.

4.6 Discussion and conclusions

The oxygen-pulse method did not prove to be a particularly useful technique for the investigation of respiration-linked proton translocation in M.methylotrophus. Despite the very low rate of oxidation of endogenous substrates, and the substantial increase in respiration rate on addition of substrate, there was clearly interference by endogenous substrate oxidation during the measurement of $\rightarrow H^+/O$ quotients for the oxidation of added substrates. The initial-rate method was found to be a much more useful technique on two counts - firstly, identification of the respiratory substrate was unambiguous, and secondly, it was possible to measure $\rightarrow$ charge/O ($\rightarrow K^+/O$) quotients in addition to $\rightarrow H^+/O$ quotients.

Analysis of the $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients obtained shows that, except in the case of formate oxidation when movement of formic acid into the bacteria caused underestimation of the $\rightarrow H^+/O$ quotient, there was no evidence for interference in the measurement of $\rightarrow H^+/O$ quotients by rapid secondary proton movements in symport with anions (as is possibly the case in mitochondria [see section 1.7.1]). It is unlikely that the $\rightarrow K^+/O$ quotients were affected by K^+/anion symport, since the K^+ movements occurring in these experiments are small compared with the total K^+ present, and hence would not establish a substantial K^+ gradient; furthermore neither the ΔpH nor $\Delta\Psi$ (which is anyway minimised in the presence of K^+/valinomycin) is able to drive K^+/anion symport. N-Ethylmaleimide, at similar concentrations to those used to inhibit phosphate transport in mitochondria, did not cause any increase in either the $\rightarrow H^+/O$ quotient for the oxidation of endogenous substrates (measured by the oxygen-pulse method), or the $\rightarrow H^+/O$ or $\rightarrow K^+/O$ quotient for methanol oxidation (measured by the initial-rate method). It is not known whether the phosphate transport

system(s) of this organism is sensitive to inhibition by N-ethylmaleimide, but no evidence was obtained for an increased $\rightarrow H^+/O$ quotient caused by the inhibition of H^+/phosphate symport.

The oxidation of one molecule of NADH by the respiratory chain of M.methylotrophus is associated with the translocation of $6H^+$ outwards across the coupling membrane. This conclusion is supported by oxygen-pulse experiments which yielded $\rightarrow H^+/O$ quotients of approximately 6 for the oxidation of both endogenous substrates (NAD(P)H; it should be noted here that this organism contains only a soluble energy-independent transhydrogenase [see section 3.3], and hence NADH and NADPH are energetically equivalent as respiratory substrates), and low concentrations of formate (via NAD^+-linked formate dehydrogenase). Furthermore, the $\rightarrow K^+/O$ quotient of approximately 6 for formate oxidation is numerically equal to the true number of protons translocated during the oxidation of one molecule of formate, and is unaffected by movements of the neutral species, formic acid.

Methanol oxidation yielded a $\rightarrow H^+/O$ quotient of close to 2, measured either by the oxygen-pulse method, in the presence of HQNO to inhibit electron flux from endogenous substrates through the respiratory chain prior to cytochrome c, or by the initial-rate method. By this latter method, a $\rightarrow K^+/O$ quotient of approximately 2 was also obtained.

The oxidation of formaldehyde is associated with $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients of 3-3.5 (measured by the initial-rate method) which surely reflects the two modes of oxidation of this substrate in vitro (see section 1.3.3), i.e. via either NAD^+ reduction and reoxidation ($\rightarrow H^+/O = \rightarrow K^+/O = 6$) or directly via methanol oxidase ($\rightarrow H^+/O = \rightarrow K^+/O = 2$).

Respiration from duroquinol yielded an $\rightarrow H^+/O$ quotient of approximately 3.5, measured by both the oxygen-pulse and initial-rate methods. The exact nature of the interaction of duroquinol with the respiratory chain of M.methylotrophus is unknown, but this value is consistent with the translocation of up to $4H^+$ when $2e^-$ pass from the quinone region to oxygen.

The $\rightarrow H^+/O$ quotient for the oxidation of either ascorbate-TMPD or $DADH_2$ was never greater than could be accounted for by scalar proton release from these substrates. The occurrence of only transmembrane electron flow, and lack of proton pumping, at site III of this organism was also supported by the $\rightarrow K^+/O$ quotient of approaching 2 for ascorbate-TMPD oxidation. The use of cyanide to confine electron flux to cytochrome oxidase aa_3 indicated that this cytochrome certainly does not act as a proton pump; and unless respiration from ascorbate-TMPD (and also methanol) is preferentially routed via cytochrome aa_3 under these conditions, even in the absence of cyanide, one can also infer that cytochrome oxidase o does not act as a proton pump.

Interestingly, the oxidation of externally supplied NADH by M.methylotrophus was associated with very low proton and charge translocation stoicheiometries ($\rightarrow H^+/O = 1.96 \pm 0.11(5)$ g-ion H^+/g-atom O, $\rightarrow K^+/O = 1.19 \pm 0.13(7)$g-ion H^+/g-atom O, as measured by the initial-rate method [Cooke, S.E. and Jones, C.W. unpublished results]). It is, however, extremely difficult to interpret these stoicheiometries in the absence of information as to if and how NADH is taken up into the bacterium.

Fig. 4.11 depicts the simplest scheme for the respiratory system of M.methylotrophus which is consistent with the results presented here. It is proposed that there are three energy conserving sites, each with a

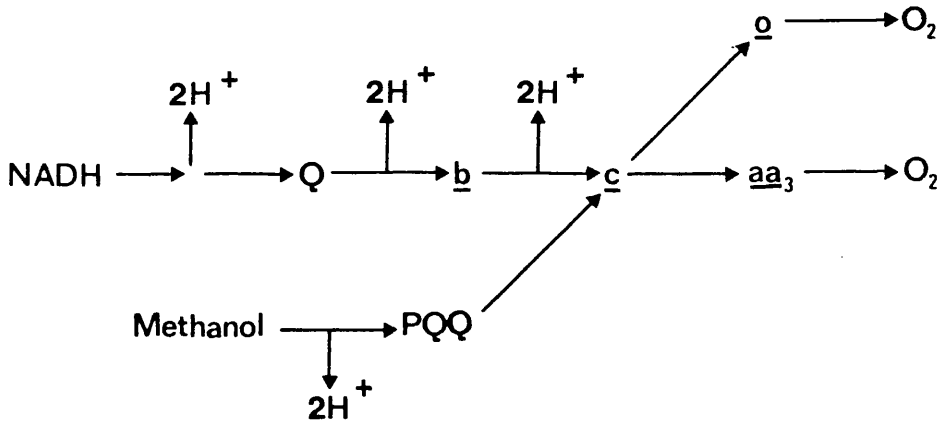


Fig. 4.11 Proposed scheme for respiration linked proton translocation in M.methylotrophus.

→ charge/site quotient of 2. Site I is associated with NADH : ubiquinone oxidoreductase (→ $H^+/2e^-$ = 2, → charge/$2e^-$ = 2), site II is associated with ubiquinol : cytochrome c oxidoreductase (→ $H^+/2e^-$ = 4, → charge/$2e^-$ = 2), and site III is associated with cytochrome c oxidase (→ H^+/O = 0, → charge/O = 2). Only this third site of energy conservation is involved in methanol oxidation. It should be noted that, although the → $H^+/2e^-$ quotient is different at each energy-conserving site, it is the → charge/$2e^-$ quotient which is important in terms of energy conservation, and sites I-III are thus equivalent in this respect.

As discussed in section 1.9, the magnitude of the → $H^+/2e^-$ and → charge/$2e^-$ quotients gives us a limited amount of information about the mechanism of proton translocation at each site of energy conservation. It should be noted, however, that scalar proton release or consumption is associated with reactions such as the oxidation of methanol to formaldehyde, and this must be taken into account when drawing conclusions from measured proton and charge translocation stoicheiometries. On the basis of the stoicheiometries considered in Fig. 1.6, site I may function by either a redox loop or a proton pump mechanism (see section 1.9.2); site II may comprise a protonmotive quinone cycle, or may involve a proton pump in conjunction with outward electron flow, and possibly a redox loop (see section 1.9.3); and site III probably constitutes a redox arm, though the alternative possibility that the site of oxygen consumption is external, and the protons consumed in the oxidase reaction are delivered from the internal phase by a proton pump mechanism, would result in the same proton and charge translocation stoicheiometries (i.e. → H^+/O = 0; → charge/O = 2; see section 1.9.4).

The methanol dehydrogenase of P.denitrificans appears to be located on the periplasmic face of the coupling membrane, as it is readily released during the preparation of sphaeroplasts from this organism (Alefounder & Ferguson, 1981). Comparable experiments have shown that the methanol dehydrogenase of M.methylotrophus is similarly located, along with a substantial proportion of the total cytochrome c (Kingsbury, S.A. and Jones, C.W., unpublished results). In view of these findings, it seems likely that the methanol oxidase system of M.methylotrophus consists of a redox arm, in which methanol dehydrogenase and cytochrome c are situated on the periplasmic side of the membrane and cytochrome oxidases aa_3 and o have their reaction sites on the cytoplasmic side, the two protolytic reactions being connected via inwardly directed electron transfer (Fig. 4.12).

The scheme in Fig. 4.11 has many similarities with that proposed by Keevil & Anthony (1979b) for carbon-limited cultures of Pseudomonas AM1 (Fig. 4.1b). The scheme proposed by Higgins et al. (1976, 1977), however, for respiratory chain energy conservation in M.trichosporium (Fig. 4.1a) is rather difficult to reconcile with the results from other methylotrophs. Unless the interaction of methanol dehydrogenase with the respiratory chain of M.trichosporium is substantially different than in M.methylotrophus, Pseudomonas AM1 or P.denitrificans, then it is not easy to see how there could be energy conservation in a methanol oxidase system terminated by cytochrome c_{co}. Indeed, this would require the cytochrome c_{co} to be located on the internal face of the coupling membrane, or the methanol dehydrogenase of M.trichosporium to act as a proton pump. Furthermore, in view of its extremely high apparent K_M for oxygen (250μM; Tonge et al., 1977b), which

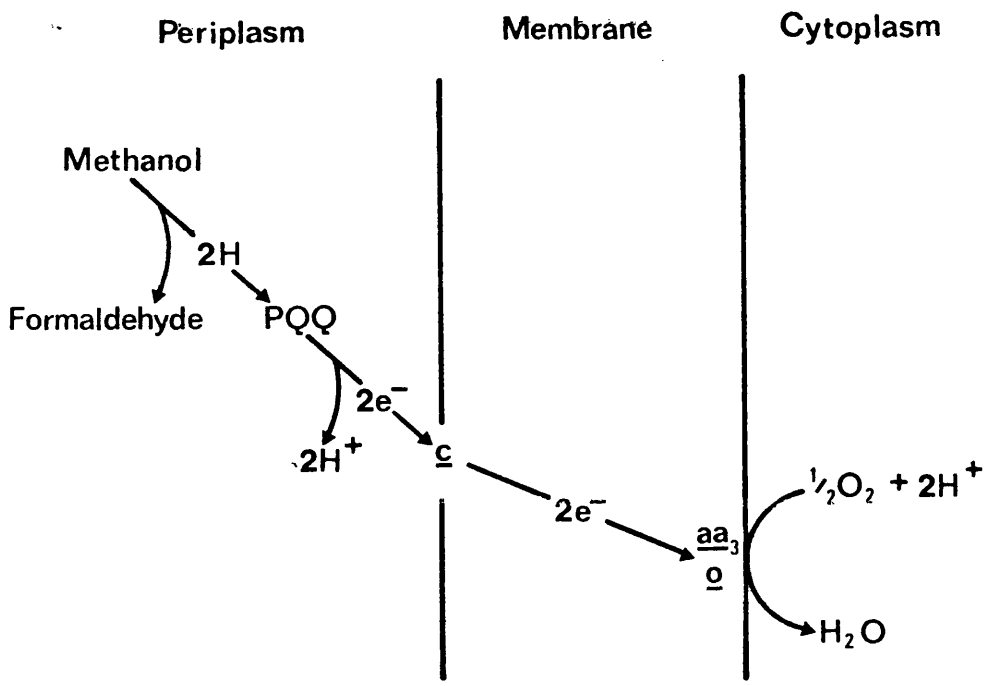


Fig. 4.12 Proposed arrangement of methanol oxidase in M.methylotrophus.

Abbreviation: PQQ, pyrrolo-quinoline quinone (prosthetic group of methanol dehydrogenase).

corresponds to approximately 1.5 times air-saturation, it seems unlikely that the cytochrome c_{co} of this organism will have substantial oxidase activity under physiological conditions; and it is clearly not feasible that this cytochrome is responsible for methanol oxidase activity under the conditions of very low oxygen concentration prevalent during the measurement of proton translocation stoicheiometries by the oxygen-pulse method. The low proton translocation stoicheiometries determined in M.trichosporium (Tonge et al., 1977a) may well reflect a partial localisation of the proton current within the extensive internal membrane system of this organism (see section 1.5.2).

The scheme proposed here (Fig. 4.11) for respiration-linked proton translocation in M.methylotrophus differs from that proposed for P.denitrificans (Fig. 4.1c; van Verseveld et al., 1981) in three respects. Firstly, P.denitrificans is claimed to exhibit an $\rightarrow H^+/O$ quotient of 3 at site I, though this may well reflect the presence of an energy-dependent transhydrogenase (site 0) in the respiratory membrane of this organism (Scholes & Mitchell, 1970b; Edwards et al., 1977). Secondly, the branch-point to cytochrome oxidases aa_3 and o is at the level of cytochrome b in P.denitrificans, as opposed to cytochrome c in M.methylotrophus. Thirdly, the cytochrome aa_3 of P.denitrificans appears to act as a proton pump (see section 1.9.4). The finding that the two terminal oxidases of M.methylotrophus (cytochromes aa_3 and o) are associated with equal efficiencies of energy conservation, although different from the situation found in P.denitrificans, is consistent with the work of Jones and co-workers (Jones et al., 1975; Jones, 1977) who showed that bacterial respiratory systems terminated by one or the other of these oxidases appear to be equivalent in terms of growth yields and proton translocation

stoicheiometries. Furthermore, the finding that the cytochrome $\underline{aa}_3$ of M.methylotrophus probably does not act as a net proton pump suggests that there may be two classes of bacterial cytochrome oxidases $\underline{aa}_3$ - those from organisms such as P.denitrificans, B.stearothermophilus and the thermophile PS3 (see section 1.9.4) which catalyse net proton translocation ($\rightarrow H^+/O > 0$, $\rightarrow$ charge/O > 2), and those from M.methylotrophus, and probably several other less well-characterised bacteria, which act only as transmembrane electron carriers ($\rightarrow H^+/O = 0$, $\rightarrow$ charge/O = 2).

CHAPTER 5

RESPIRATORY CHAIN COMPOSITION AND ENERGY CONSERVATION IN OXYGEN- AND AMMONIUM-LIMITED *METHYLOPHILUS METHYLOTROPHUS*

5.1 Introduction

5.2 Cytochrome content of oxygen- and ammonium-limited *M.methylotrophus*

5.3 Transhydrogenase activity of oxygen-limited *M.methylotrophus*

5.4 Respiratory activity of whole cells of oxygen-limited *M.methylotrophus*

5.5 Effect of inhibitors on the respiratory activity of whole cells of oxygen-limited *M.methylotrophus*

5.6 Stoicheiometry of respiration-linked proton translocation in oxygen-limited *M.methylotrophus*, measured by the oxygen-pulse method

5.7 Stoicheiometry of respiration-linked proton and charge translocation in oxygen-limited *M.methylotrophus*, measured by the initial-rate method

5.8 Discussion and conclusions

CHAPTER 5

RESPIRATORY CHAIN COMPOSITION AND ENERGY CONSERVATION IN OXYGEN- AND AMMONIUM-LIMITED *METHYLOPHILUS METHYLOTROPHUS*

5.1 Introduction

As discussed in section 1.8.2, the ability experimentally to vary the composition of bacterial respiratory chains, both phenotypically and genotypically, is one of the most potent tools available in the study of respiratory chain energy conservation. The study of growth in oxygen-limited continuous culture has proved especially useful in providing an understanding of the function of the terminal region of the respiratory chain, as the composition of this region appears to be particularly variable with respect to the dissolved oxygen tension in the growth medium.

The effects of oxygen-limited growth on the growth yield, respiratory chain composition, and proton translocation stoicheiometry of a number of bacteria have now been studied (see Jurtshuk *et al.*, 1975; Harrison, 1976a; Haddock & Jones, 1977). It should, however, be noted that oxygen-limited and carbon substrate-limited cultures differ in the concentrations of both oxygen and carbon substrate, and that any differences observed in the energy conservation parameters mentioned above may be due to either one or both of these factors. Therefore, in order to gain a fuller insight into the factors controlling respiratory-chain composition and energy conservation, a study of at least one other nutrient-limitation (e.g. ammonium-limitation – excess carbon and oxygen) is required.

The effects of oxygen-limited growth in different species of bacteria appear to be far from uniform but a number of general points can be made:

(1) In most obligate aerobes, oxygen-limitation is accompanied by an increase in the total level of membrane-bound cytochromes, particularly the cytochrome oxidases (see Jurtshuk et al., 1975; Harrison, 1976a).

(2) In many facultative anaerobes, on decreasing the dissolved oxygen tension, there is at first an increase in the total level of membrane-bound cytochromes and cytochrome oxidases, but at still lower oxygen tensions there may be a decrease in the total amount of cytochromes and/or a switch to the components of the anaerobic respiratory chain (e.g. Wimpenny & Necklen, 1971).

(3) In those bacteria capable of synthesising cytochrome d, the level of this cytochrome is increased during oxygen-limited growth (see Castor & Chance, 1959; Jurtshuk *et al.*, 1975; Harrison, 1976a). In those bacteria containing a high-potential membrane-bound cytochrome c, the replacement of cytochrome oxidase aa_3 and/or o by d leads to decreased proton translocation stoicheiometries and growth yields; whereas in the absence of cytochrome c, the induction of cytochrome d does not *per se* affect energy conservation (see Jones, 1977).

(4) In some bacteria containing cytochrome oxidases aa_3 and o, decreased oxygen concentrations lead to the complete or partial replacement of cytochrome oxidase aa_3 by o (e.g. Paracoccus denitrificans; Sapshead & Wimpenny, 1972), whereas in other bacteria the levels of both oxidases are increased (e.g. Bacillus megaterium D440; Downs & Jones, 1975). The energy conserving properties of these two oxidases are discussed in section 4.6.

(5) In some facultative anaerobes, the NADH:ubiquinone oxidoreductase segment of the respiratory chain (site I) loses its capacity to conserve energy during oxygen-limited growth (e.g. Escherichia coli; Haddock & Jones, 1977; but see Farmer & Jones, 1976; Rice & Hempfling, 1978).

The changes described above presumably reflect attempts to counter oxygen insufficiency by increasing the capacity to utilise low concentrations of oxygen. Although the cytochrome content does not generally have a profound influence on the maximum rate of respiration at high oxygen concentrations (see Harrison, 1976a), it is probable that, at the lower oxygen concentrations prevalent in oxygen-limited cultures, the cytochromes, and cytochrome oxidases in particular, may become limiting for respiration (see White, 1963). The mechanism of the increase in oxygen-utilising capacity may be two-fold - there is almost ubiquitously an increase in the total level of respiratory chain components, particularly terminal oxidases, and this is often accompanied by a switch to oxidases with higher affinities (lower K_M) for oxygen. This latter statement is rather difficult to verify

as the measurement of absolute values of the K_M for oxygen of cytochrome oxidase is technically difficult, and conflicting results have been obtained (e.g. White, 1963; Meyer & Jones, 1973; Weston et al., 1974; Linton et al., 1977). However, bacteria containing cytochrome oxidase d invariably outgrow competitors containing cytochrome oxidases aa_3 and/or o in oxygen-limited continuous culture (Jones, C.W., Brice, J.M. & Edwards, C. unpublished results cited in Jones, 1979), thus suggesting that the affinities of bacterial cytochrome oxidases for molecular oxygen are in the order d > o or aa_3.

In facultative anaerobes, an increased capacity to utilise low concentrations of oxygen is often accompanied by a lowering of the efficiency of energy conservation, probably in order to allow rapid reoxidation of reduced coenzymes during fermentative metabolism. In the presence of an alternative electron acceptor, however, there may be a switch to the components of the anaerobic respiratory chain.

The effect of different nutrient limitations on respiratory-chain energy conservation has so far been studied in detail for only one methylotroph, Pseudomonas AM1 (Keevil & Anthony, 1979b). When this organism is grown in methanol- or succinate-limited continuous culture, it contains a high-potential membrane-bound cytochrome c which is associated with a third site of energy conservation (Keevil & Anthony, 1979b; see Jones, 1977). However, during growth in batch culture, or in oxygen- or ammonium-limited continuous-culture, no membrane-bound cytochrome c can be detected, though soluble cytochrome c is present (Keevil & Anthony, 1979b), and loss of the third site of energy conservation is reflected in decreased proton translocation stoicheiometries and growth yields (MacLennan et al., 1971; O'Keefe & Anthony, 1978; Keevil & Anthony, 1979b). This response to

different nutrient limitations is so far unique, and interestingly these changes appear to be controlled by the concentration of carbon substrate rather than of oxygen. Another unusual feature of Pseudomonas AM1 is that, from the single determination reported by Keevil & Anthony (1979b), there appears to be no increase in the total level of membrane-bound cytochromes during oxygen-limited growth, in contrast to the situation found with other obligate aerobes (see above). It remains to be seen whether the response of this organism to different nutrient limitations is indeed unusual, or whether it is for some reason typical of the methylotrophs.

Brooks & Meers (1973) noted that when the pulse frequency of discontinuous methanol addition to 'methanol-limited' continuous cultures of M.methylotrophus was decreased, the growth yield with respect to methanol decreased correspondingly. These authors suggested that the decreased yield might be due to transient conditions of methanol excess and/or oxygen-limitation. In large-scale single-cell protein production it is desirable to maintain the dissolved oxygen concentration at a low level in order to maximise oxygen transfer, as the cost of air-compression may be a substantial fraction of the total production costs (see section 1.11.3). However, in a large fermentation vessel, it is impossible to prevent local fluctuations of the methanol and oxygen concentrations; and a study of the effect of growth conditions on energy conservation in M.methylotrophus is thus particularly apposite.

This chapter describes an investigation of the composition and energy-conserving properties of the respiratory chain of oxygen-limited cultures of M.methylotrophus, with a view to identifying any factors which might lead to a decrease in the growth yield, as compared to methanol-limited

cultures. The composition of the respiratory chain of ammonium-limited M.methylotrophus has also been investigated, in order to determine whether any differences observed between methanol- and oxygen-limited cultures are due to differences in the concentration of oxygen, or of methanol.

After most of this work had been completed, Cross & Anthony (1980b) published a study of the respiratory chain composition of M.methylotrophus grown under various conditions. This work will be discussed in the conclusion to this chapter (section 5.8), as will some further data on the growth yield of this organism in oxygen-limited continuous culture which has recently become available.

5.2 Cytochrome content of oxygen- and ammonium-limited M.methylotrophus

Fig. 5.1a shows the reduced minus oxidised difference spectrum of the membrane fraction from oxygen-limited M.methylotrophus. The presence of membrane-bound cytochromes b and c is clearly indicated by the shoulder at 555nm and peak at 547nm respectively. No peak in the 600-605nm region which might correspond to cytochrome aa_3 was observed, but there was a strong peak at 428nm and trough at 442nm in the reduced plus CO minus reduced difference spectrum (Fig. 5.1b), thus indicating that cytochrome aa_3 was in fact the most abundant CO-binding cytochrome during oxygen-limited growth. It is not easy to explain this peculiar spectral behaviour of the a-type cytochrome during oxygen-limited growth, but Lanyi (1968) observed similar behaviour with the a-type cytochrome from Halobacterium cutirubrum. The peak at 412nm in Fig. 5.1b clearly corresponds to cytochrome c_{co}, and cytochrome o was also present (shoulder 420nm, broad trough 560nm). There was no indication that cytochrome d may be present from either the reduced

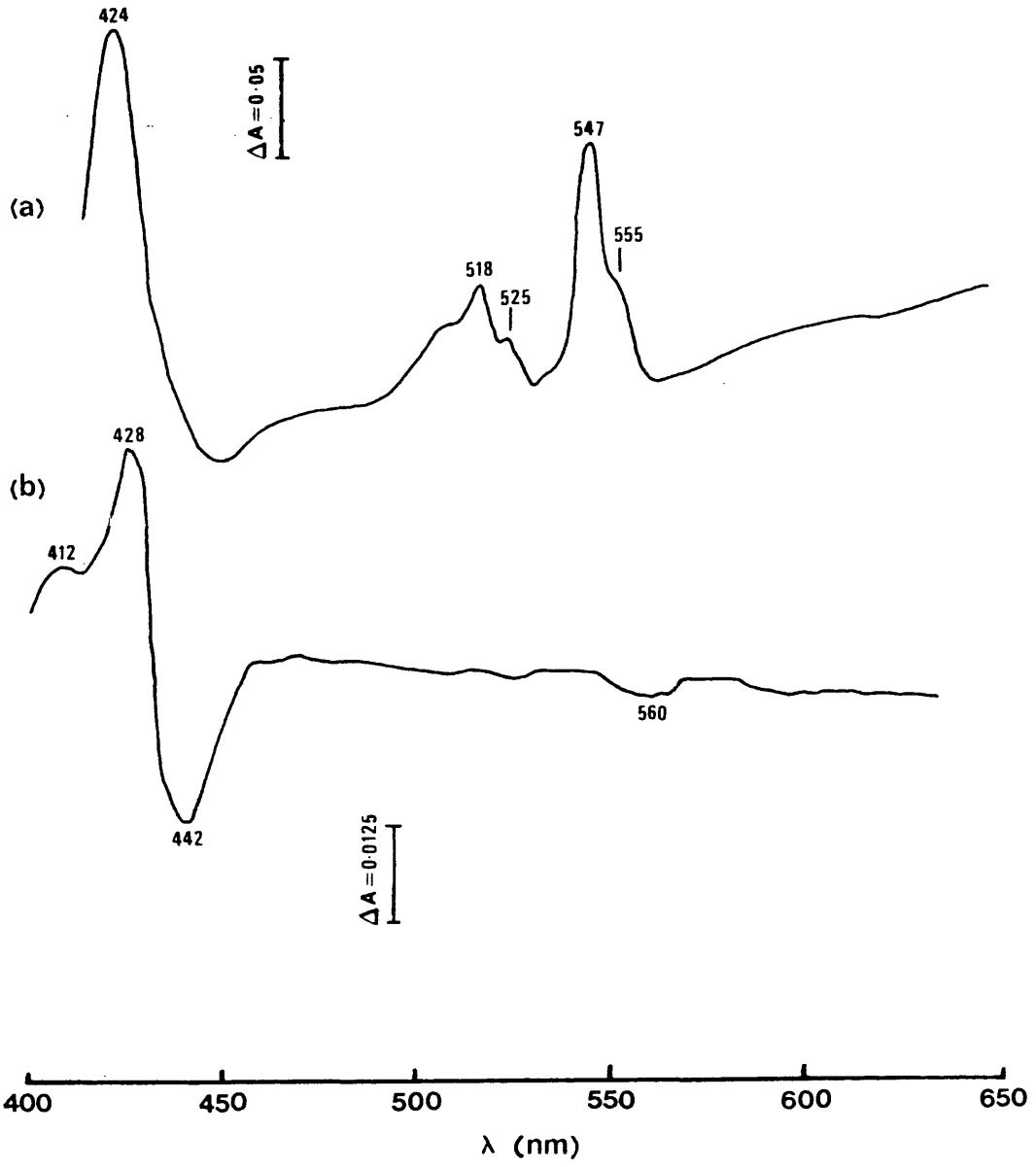


Fig. 5.1 Difference spectra of the membrane fraction of oxygen-limited M.methylotrophus. (a) Low temperature reduced minus oxidised difference spectrum (21.2mg protein/ml); (b) Room temperature reduced plus CO minus reduced difference spectrum (8.2mg protein/ml).

minus oxidised difference spectrum (cytochrome d is characterised by a peak at approximately 430nm), or the reduced plus CO minus reduced difference spectrum (cytochrome d-CO does not have a distinct absorbtion band in the Soret region, but yields a readily observable peak at 647nm with a corresponding trough at 630nm). As with methanol-limited cultures, the soluble fraction from oxygen-limited M.methylotrophus contained only cytochrome c (a small fraction of which bound CO), and fermenter supernatants contained cytochrome c and possibly flavin (see section 3.2).

Ammonium-limited cultures of M.methylotrophus also contained membrane-bound cytochromes b (shoulder 558nm) and c (peaks 550, 520nm) (Fig. 5.2a). The presence of cytochrome aa_3 in ammonium-limited cultures was variable. A small peak was sometimes observed at approximately 600nm in the reduced minus oxidised difference spectrum, and when such a peak did appear, the presence of cytochrome aa_3 was also indicated by the reduced plus CO minus reduced difference spectrum - a shoulder appearing on the peak in the Soret region at approximately 428nm with a corresponding trough at approximately 442nm. However, in other batches of cells no peak was observed at circa 600nm in the reduced minus oxidised difference spectrum (Fig. 5.2a), and the trough in the Soret region of the reduced plus CO minus reduced difference spectrum appeared at approximately 430nm (Fig. 5.2b). On these occasions, the spectrally dominant CO-binding cytochrome was cytochrome c_{co} (peak 412nm), and cytochrome o was also present (shoulder 419nm, trough 430nm). As with methanol- and oxygen-limited cultures, the soluble fraction of ammonium-limited M.methylotrophus contained only cytochrome c.

Quantitative estimates of the cytochrome contents of the membrane and soluble fractions of oxygen- and ammonium-limited cultures are shown in Table 5.1. The cytochrome content of methanol-limited M.methylotrophus (see Table 3.1) is included for comparison.

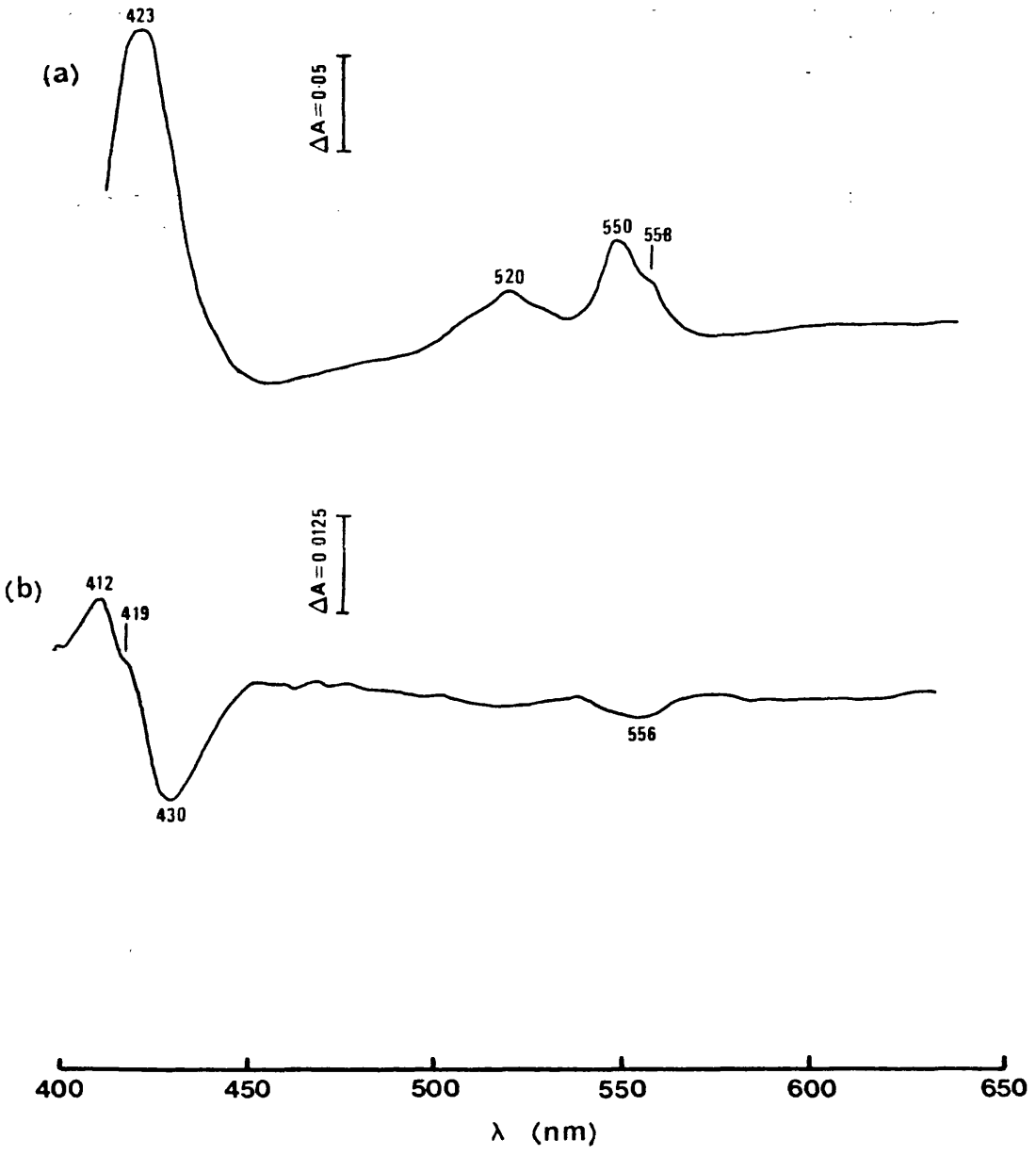


Fig. 5.2 Room temperature spectra of the membrane fraction of ammonium-limited M.methylotrophus. (a) Reduced minus oxidised difference spectrum (6.9 mg protein/ml); (b) reduced plus CO minus reduced difference spectrum (6.9 mg protein/ml).

Growth limitation	Membrane fraction						Soluble fraction	
	aa_3*	b*	c*	a_3†	o†	c_{co}† (nmoles/g protein)	c*	c_{co}†
Methanol-limited	59 ± 9(7)	243 ± 23(7)	444 ± 37(7)	18 ± 3(3)	33 ± 6(3)	38 ± 8(3)	1023 ± 85(7)	18 ± 4(3)
Oxygen-limited	0	516 ± 39(7)	1240 ± 71(7)	71 ± 13(3)	43 ± 9(3)	37 ± 7(3)	426 ± 16(5)	10-15
NH_4^+ - limited	0-25	207 ± 23(3)	471 ± 156(3)	0-34	15-41	23-43	574 ± 153(3)	28 ± 6(3)

Table 5.1 Cytochrome contents of methanol-, oxygen-, and ammonium-limited M.methylotrophus.

The membrane and soluble fractions were prepared as described in section 2.4.1. Cytochromes marked * were estimated from reduced minus oxidised difference spectra, whereas those marked † were estimated from reduced plus CO minus reduced difference spectra. The wavelength pairs and molar extinction coefficients used are listed in section 2.5.3.

5.3 Transhydrogenase activity of oxygen-limited M.methylotrophus

In common with methanol-limited cultures (see section 3.3), oxygen-limited M.methylotrophus contains only a soluble nicotinamide nucleotide transhydrogenase. The specific activity in the soluble fraction (19.3 or 6.6 nmoles NADPH oxidised/min per mg protein, measured by oxygen-consumption or 3-acetylpyridine-NAD^+ reduction respectively) was very close to that observed in methanol-limited cultures.

5.4 Respiratory activity of whole cells of oxygen-limited M.methylotrophus

The respiration rates of oxygen-limited cultures of M.methylotrophus from selected substrates are shown in Table 5.2. The rates of oxidation of methanol, formaldehyde, ascorbate-TMPD and $DADH_2$ were similar in oxygen-limited and methanol-limited bacteria (see Table 3.3), but the rates of respiration from endogenous substrates and duroquinol were approximately 2-fold more rapid in the former than in the latter. The rate of formate oxidation was approximately 3-fold slower in oxygen-limited cultures than in methanol-limited cultures.

5.5 Effect of inhibitors on the respiratory activity of whole cells of oxygen-limited M.methylotrophus

The mid-chain inhibitor, HQNO, had similar relative effects on the oxidation of physiological substrates in oxygen-limited cultures of M.methylotrophus as in methanol-limited cultures (see Fig. 3.5). Respiration from the NAD^+-linked substrate formate was substantially affected by 75μM-HQNO (81% inhibition), whereas respiration from methanol was virtually unaffected (only 14% inhibition); intermediate inhibition of respiration

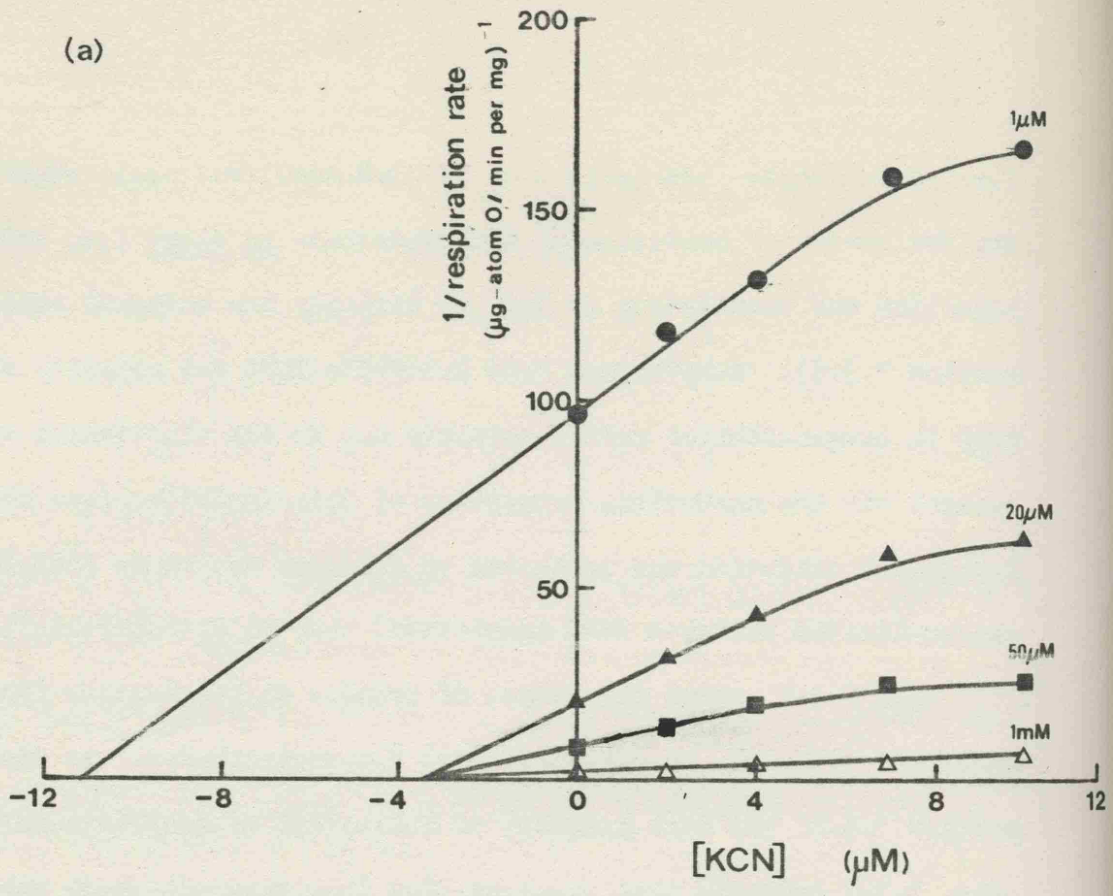
Substrate	Respiration rate (ng-atom O/min per mg dry wt. bacteria)
Endogenous	10 ± 1 (9)
Methanol	310 ± 5 (3)
Formaldehyde	380 ± 21 (5)
Formate	12 ± 1 (6)
Ascorbate-TMPD	1600
$DADH_2$	400
Duroquinol	428 ± 28 (7)

Table 5.2 Respiratory activity of whole cells of oxygen-limited *M.methylotrophus*. Respiration rates were measured as described in section 2.6 in a reaction mixture containing 140mM-KCl, 20mM-glycylglycine (pH7.4) plus sufficient washed cell suspension to give a convenient rate of oxygen consumption. All substrates were used at saturating concentrations (methanol, 10mM; formaldehyde, 2mM; formate, 5mM; ascorbate, 3mM-TMPD, 3mM; $DADH_2$, 1mM; duroquinol, 1mM), and respiration rates were corrected for the endogenous respiration. The values of the respiration rates from ascorbate-TMPD and $DADH_2$ were taken from plots of the respiration rate (corrected for autoxidation) versus the substrate concentration.

from formaldehyde (54% inhibition by 75μM-HQNO) was again observed due to the two routes of oxidation of this substrate in vitro i.e. either via the reduction and reoxidation of NAD^+ or directly via methanol oxidase (see section 1.3.3). Respiration from ascorbate-TMPD was slightly stimulated by HQNO in oxygen-limited cells, possibly due to the alleviation of respiratory control via the uncoupling properties of this inhibitor (see section 4.5.1). Duroquinol oxidation was inhibited to a lesser degree by 75uM-HQNO in oxygen-limited cultures (66% inhibition) than in methanol-limited cultures.

Fig. 5.3ab shows the effect of cyanide on respiration from ascorbate-TMPD, and methanol, in oxygen-limited M.methylotrophus. As discussed in section 3.5.2, the dual kinetics of inhibition of ascorbate-TMPD oxidation (Fig. 5.3a) indicate that electron flux from this substrate may be conducted to oxygen via either cytochrome aa_3 (uncompetitive inhibition, $K_i \leqslant 3\mu M$) or cytochrome o (non-competitive inhibition, $K_i = 3\mu M$). In contrast to the situation in methanol-limited cultures (see Fig. 3.6a), methanol oxidation in oxygen-limited M.methylotrophus was inhibited in an uncompetitive manner ($K_i \leqslant 6\mu M$) by cyanide (Fig. 5.3b); and it would appear, therefore, that this substrate is oxidised predominantly via cytochrome aa_3 in oxygen-limited cultures. This finding was not altogether unexpected as this cytochrome comprises a substantially larger proportion of the total CO-binding cytochrome in oxygen-limited cultures than in methanol-limited cultures (see Table 5.1). The kinetics of inhibition of formate oxidation could not be ascertained due to the very low respiration rate from this substrate during oxygen-limited growth.

No evidence was found for significant cyanide-insensitive respiration which might have occurred via either cytochrome c_{co} (see section 3.5.2) or cytochrome d (see section 5.1; Arima & Oka, 1965; Jones & Redfearn, 1967).


(a)
1/respiration rate
(µg-atom O/ min per mg)$^{-1}$
200
150
100
50
1µM
20µM
50µM
1mM
-12
-8
-4
0
4
8
12
[KCN] (µM)

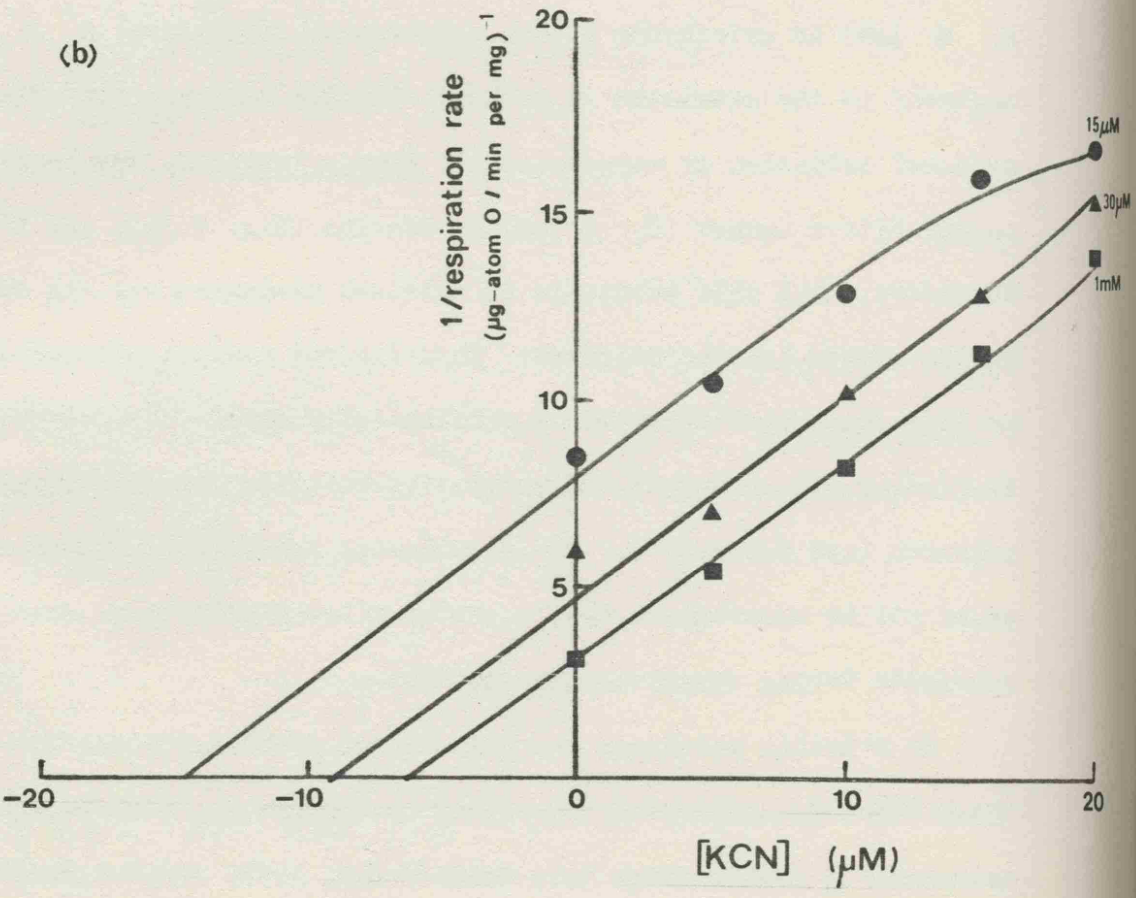

(b)
1/respiration rate
(µg-atom O / min per mg)$^{-1}$
20
15
10
5
15µM
30µM
1mM
-20
-10
0
10
20
[KCN] (µM)

Fig. 5.3 The effect of cyanide on respiration from ascorbate-TMPD and methanol in oxygen-limited *M.methylotrophus* (Dixon plots).

Respiration rates were determined as described in section 2.6, in a mixture containing 140mM-KCl, 20mM-glycylglycine (pH7.4) plus sufficient washed cell suspension to give a convenient rate of oxygen consumption. The cyanide was added 2 min prior to the addition of substrate. The substrates used, at the concentrations shown on the plots, were (a), ascorbate-TMPD (the ascorbate concentration was 3mM throughout, and the TMPD concentration was varied as shown; correction was made for the autoxidation of this substrate); and (b), methanol.

5.6 Stoicheiometry of respiration-linked proton translocation in oxygen-limited M.methylotrophus, measured by the oxygen-pulse method

The optimum pH and valinomycin concentrations for the determination of $\rightarrow H^+/O$ quotients in oxygen-limited M.methylotrophus were found to be the same as for bacteria grown in methanol-limitation i.e. pH6.2, 1μg valinomycin/mg dry wt. bacteria (see section 4.2). The $\rightarrow H^+/O$ quotient for the oxidation of endogenous substrates in oxygen-limited cultures was found to be 5.74 ± 0.09(7), and this value could be increased to 6.21 ± 0.14(5) if bacterial suspensions were washed under anaerobic conditions in order to deplete the cells of any permeant anions which might otherwise cause underestimation of the $\rightarrow H^+/O$ quotient (see section 4.1). The kinetics of decay of the ΔpH following an oxygen pulse were very similar to those observed in methanol-limited cultures (see section 4.2); the first order region of decay had a half-time of 70 ± 9(6)s in the presence of valinomycin.

Table 5.3 shows the values of the $\rightarrow H^+/O$ quotient for the oxidation of various substrates in oxygen-limited M.methylotrophus. The rate of decay of the ΔpH following an oxygen pulse was similar to that observed in the presence of endogenous substrates alone, except in the presence of ascorbate-TMPD (in the presence of 3mM-ascorbate - 1mM-TMPD the $t_{\frac{1}{2}}$ was approximately 17s). As was discussed in some detail in section 4.3, endogenous substrate oxidation seriously interferes with the measurement of $\rightarrow H^+/O$ quotients for the oxidation of added substrates. The true $\rightarrow H^+/O$ quotient for methanol oxidation could only be measured in the presence of HQNO to inhibit endogenous substrate oxidation (see Table 5.3); this inhibitor, however, caused a substantial increase in the rate of decay of the ΔpH following an oxygen pulse.

Respiratory substrate	$\rightarrow H^+/O$ quotient (g-ion H^+/g-atom O)	
	No addition	+ 75μM HQNO
Endogenous	5.74 ± 0.09 (7)	n.d
Formate	5.3	n.d
Duroquinol	3.97 ± 0.14 (5)	n.d
Formaldehyde	5.26 ± 0.26 (6)	2.45 ± 0.25 (8)
Methanol	5.64 ± 0.16 (5)	2.01 ± 0.14 (8)
Ascorbate-TMPD	1.7	n.d
$DADH_2$	2.0	n.d

Table 5.3 $\rightarrow H^+/O$ quotient of oxygen-limited M.methylotrophus, measured by the oxygen-pulse method. $\rightarrow H^+/O$ quotients were determined as described in section 2.8.1. The substrate concentrations were formate, 1mM; duroquinol, 1mM; formaldehyde, 2mM; methanol, 10mM; ascorbate, 3mM - TMPD, 3mM; $DADH_2$, 1mM. The values of the $\rightarrow H^+/O$ quotients for formate, ascorbate-TMPD and $DADH_2$ oxidation were taken from plots of the $\rightarrow H^+/O$ quotient versus the substrate concentration.

Abbreviation : n.d, not determined.

5.7 Stoicheiometry of respiration-linked proton and charge translocation in oxygen-limited M.methylotrophus, measured by the initial-rate method

The addition of methanol, formaldehyde, formate or duroquinol to an aerobic suspension of oxygen-limited M.methylotrophus (in the presence of K^+/valinomycin) elicited respiration and acidification of the external medium (Fig. 5.4). The oxygen-consumption rate became constant after a lag of 2-3s but, in contrast to the situation found with methanol-limited bacteria (see Fig. 4.6), the rate of acidification decreased with time after the rapid increase elicited by the addition of substrate. The non-linearity of the acidification was most pronounced in the case of respiration from formate and duroquinol. $\rightarrow H^+/O$ quotients obtained from the linear phase of oxygen-consumption and the initial-rate of acidification are shown for various substrates in Table 5.4; also shown are $\rightarrow K^+/O$ quotients determined in parallel experiments but in low K^+ medium. The pH response to the addition of ascorbate-TMPD was variable; acidification rarely occurred but there was generally a delay of up to 10s prior to the onset of the alkalinisation phase characteristic of the ascorbate-TMPD oxidase reaction. K^+ uptake ($\rightarrow K^+/O = 1.30 \pm 0.13(7)$) confirmed that there is transmembrane electron flow between cytochrome c and at least one of the cytochrome oxidases, during growth in oxygen-limitation.

The values of the $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients (measured by the initial-rate method) for the oxidation of formate, duroquinol and ascorbate-TMPD, were substantially lower in oxygen-limited cultures than in methanol-limited cultures (see Table 4.1, Fig. 4.9), despite the fact that $\rightarrow H^+/O$ quotients determined by the oxygen-pulse method were found to be similar for bacteria grown in these two modes of nutrient-limitation (see sections 4.2, 4.3, 4.5.1, 5.6). This discrepancy, together with the failure to observe a

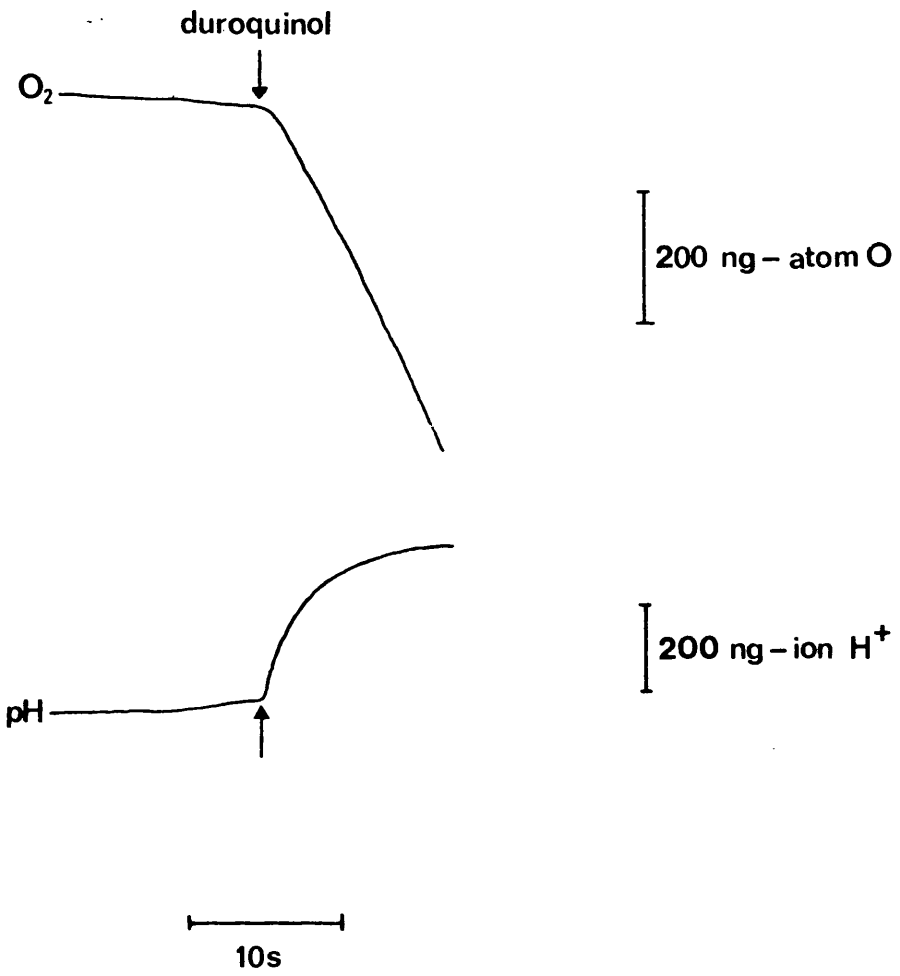


Fig. 5.4 Respiration and proton translocation following the addition of duroquinol to an aerobic suspension of oxygen-limited M.methylotrophus. The changes in pH and oxygen concentration were determined as described in section 2.8.2 in the high K^+ (140mM-KCl) medium. The arrows indicate the addition of duroquinol to a final concentration of 1mM.

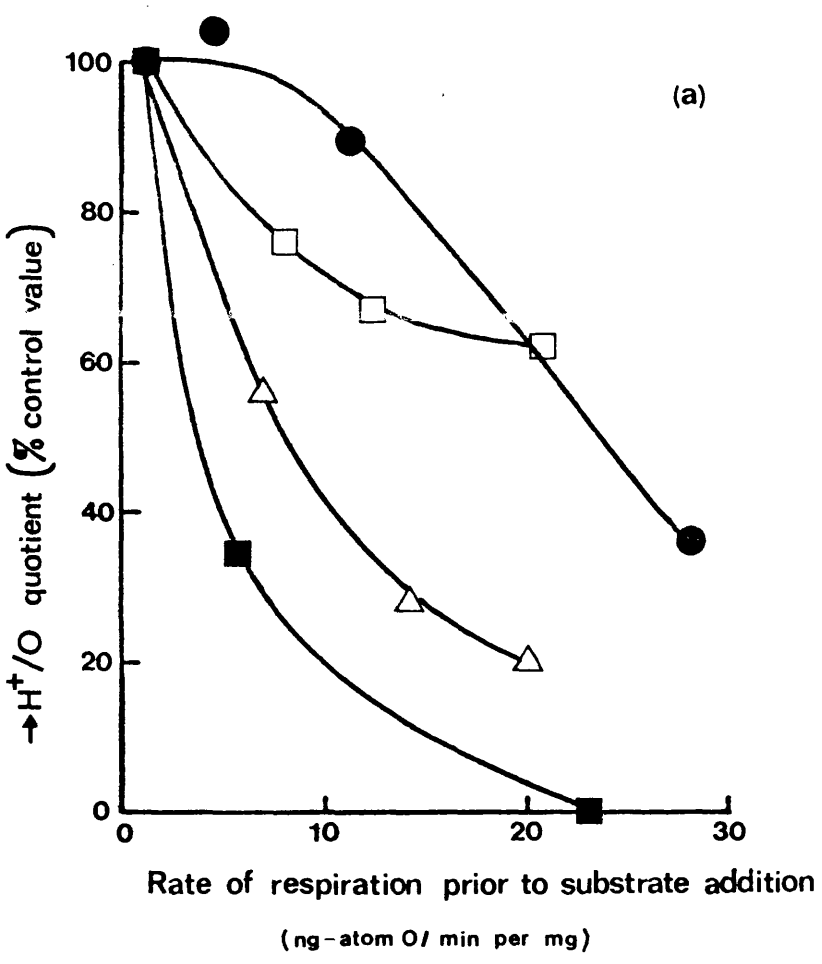
Respiratory substrate	→ H^+/O quotient (g-ion H^+/g-atom O)	→ K^+/O quotient (g-ion K^+/g-atom O)
Formate	1.73 ± 0.17 (6)	3.07 ± 0.19 (6)
Duroquinol	2.38 ± 0.27 (9)	n.d
Formaldehyde	3.46 ± 0.19 (6)	0.97 ± 0.11 (10)
Methanol	1.96 ± 0.15 (5)	1.23 ± 0.15 (10)
Ascorbate-TMPD	approx. 0	1.30 ± 0.13 (7)

Table 5.4 → H^+/O and → K^+/O quotients of oxygen-limited M.methylotrophus, measured by the initial-rate method. → H^+/O and → K^+/O quotients were measured in separate experiments by the initial-rate method, in high K^+ (140mM-KCl) and low K^+ (10mM-KCl, 140mM-LiCl) media, respectively, as described in section 2.8.2. The substrate concentration was 1mM throughout, except ascorbate (3mM)-TMPD (100μM).

Abbreviation : n.d, not determined.

well-defined linear phase of acidification on the addition of substrate, and the finding that the endogenous respiration rate of bacteria grown in oxygen-limitation (5.0 ± 0.7(11) ng-atom O/min per mg dry wt. bacteria, after the 30 min incubation period in the presence of valinomycin required for both the oxygen-pulse and initial-rate methods) was 3- to 4-fold higher than that of methanol-limited bacteria (1.4 ± 0.1(10) ng-atom O/min per mg dry wt. bacteria), suggests that there might be sufficient endogenous reserves in oxygen-limited M.methylotrophus to establish a significant protonmotive force, even in the absence of added substrate. The pre-existence of a protonmotive force would be expected to oppose proton translocation on the addition of substrate, thus leading to non-linear acidification and underestimation of the $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients.

In order to test this hypothesis, an attempt was made to examine the effect of the magnitude of the endogenous respiration rate on the $\rightarrow H^+/O$ quotient measured by the initial-rate method. Endogenous respiration was mimicked by the addition, to methanol-limited bacteria, of either low concentrations of TMPD in the presence of 3mM-ascorbate (Fig. 5.5a), or low concentrations of formate (Fig. 5.5b). It is clear from Fig. 5.5ab, that the imposition of a preexisting respiration rate decreased the values of the $\rightarrow H^+/O$ quotients for the oxidation of all substrates; and the acidification observed on the addition of the assay substrate became non-linear when a pre-existing respiration rate, comparable to the endogenous respiration rate of oxygen-limited bacteria, was imposed. The $\rightarrow H^+/O$ quotients for the oxidation of formate and duroquinol were affected considerably more by 'endogenous' respiration than those for respiration from methanol and formaldehyde. No acidification phase, on adding


(a)
100
80
60
40
20
0
0
10
20
30
→H+/O quotient (% control value)
Rate of respiration prior to substrate addition
(ng-atom O/ min per mg)

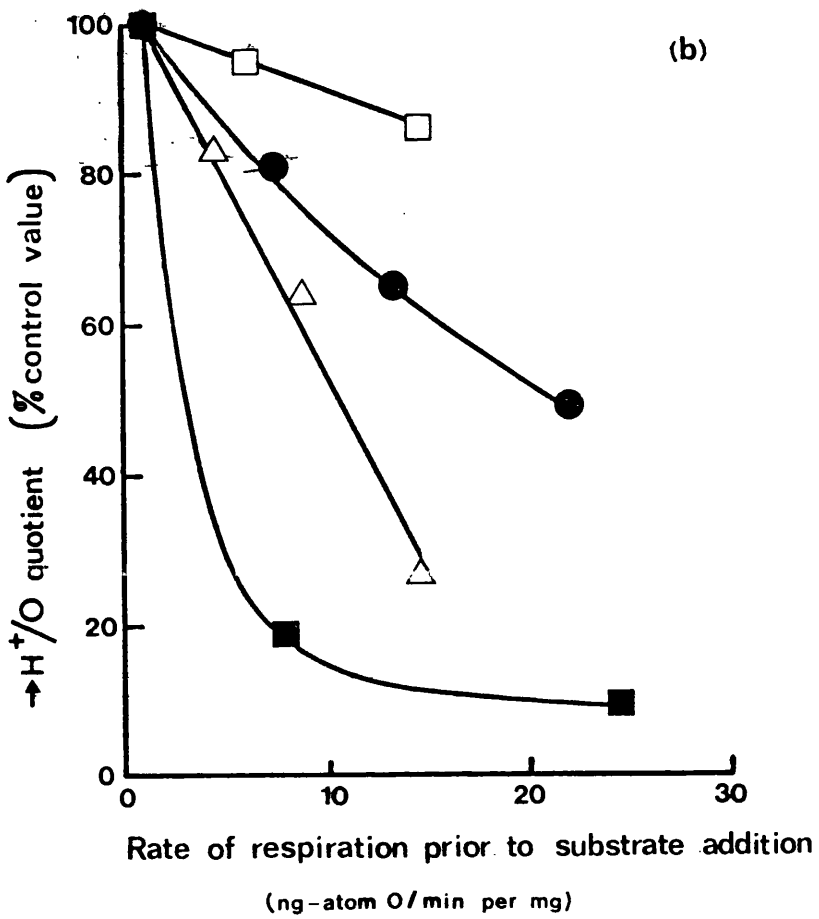


Fig. 5.5 Effect of respiration rate prior to substrate addition on the → H^+/O quotient, measured by the initial-rate method. → H^+/O quotients were determined in methanol-limited bacteria as described in section 2.8.2, in the high K^+ (140mM-KCl) medium. 2 min before the addition of substrate to initiate respiration and proton translocation, low concentrations of either (a), TMPD in the presence of 3mM-ascorbate, or (b), formate, were added to impose a low basal rate of respiration. The assay substrates were ●, methanol; ■, formate; □, formaldehyde; △, duroquinol.

ascorbate-TMPD (as the assay substrate), was observed in the presence of a pre-existing respiration rate greater than the 1.4 ± 0.1(10) ng-atom O/ min per mg dry wt. bacteria which represents the endogenous respiration rate of bacteria grown in methanol-limited continuous culture.

It is clear from these experiments that, unless the endogenous respiration rate is extremely low, endogenous respiration may support a protonmotive force which will oppose proton translocation linked to respiration from added substrates, and hence cause underestimation of the $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients, when measured by the initial-rate method. It is unlikely, however, that the $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients, measured by the initial-rate method in methanol-limited M.methylotrophus (see Table 4.1; Fig. 4.9), were underestimated, as the endogenous respiration rate of these bacteria is extremely low, and a well-defined linear phase of acidification occurred on the addition of substrate under these conditions. It is significant that the $\rightarrow H^+/O$ quotients for formate, duroquinol and ascorbate-TMPD oxidation were the ones most affected by the pre-existence of a protonmotive force, as the stoicheiometry of proton and charge translocation linked to respiration from these substrates, in particular, was lower in oxygen-limited cultures than in methanol-limited cultures; and, furthermore, the acidification elicited by the addition of formate or duroquinol to aerobic suspensions of oxygen-limited M.methylotrophus deviated substantially from linearity.

It follows, from the above discussions, that the values of the $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients shown in Table 5.4 must be regarded as minimum estimates, and there is no convincing evidence for lower proton or charge translocation stoicheiometries in bacteria grown in oxygen- as opposed to methanol-limited continuous culture.

5.8 Discussion and conclusions

Both the total level of membrane-bound cytochromes, and the level of CO-binding cytochromes were 2- to 3-fold higher in M.methylotrophus grown in oxygen-limitation than in methanol- or ammonium-limited cultures. These results are consistent with the almost ubiquitous finding that, in obligate aerobes, decreased oxygen tensions lead to increased synthesis of membrane-bound cytochromes and cytochrome oxidases (see section 5.1). In contrast to the situation found in some bacteria (see section 5.1), the level of cytochrome aa_3 was particularly increased in oxygen-limited cultures, the level of cytochrome o being approximately similar under all growth conditions. It is far from clear why no peak occurred at approximately 600nm in the reduced minus oxidised difference spectrum of oxygen-limited bacteria, but the presence of cytochrome aa_3 is surely indicated by the reduced plus CO minus reduced difference spectrum, by the kinetics of inhibition by cyanide, and by the very different reduced plus CO minus reduced difference spectrum observed in ammonium-limited cultures when cytochrome aa_3 is truly absent. There was no evidence for the production of cytochrome d in M.methylotrophus under any growth conditions.

In contrast to the situation found with the membrane-bound cytochromes, the level of the soluble cytochrome c was 2- to 3-fold lower in oxygen- and ammonium-limited cultures than during methanol-limited growth. It seems probable that the level of this soluble cytochrome is controlled by the methanol concentration, rather than by the oxygen or ammonium concentration.

After most of this work had been completed, Cross & Anthony (1980b) published a report on the respiratory chain of M.methylotrophus grown under various conditions. There are, however, a number of discrepancies

between that work and the work reported here. Cross & Anthony (1980b) reported:-

(i) no increase in the total level of membrane-bound cytochromes in oxygen-limited growth,

(ii) no difference in the level of the soluble cytochrome c under different growth conditions, and

(iii) that cytochrome aa_3 was completely replaced by cytochrome o during growth in methanol-excess cultures.

The finding of similar cytochrome contents in all methanol-excess cultures, regardless of the nutrient-limitation, led Cross & Anthony (1980b) to speculate that the methanol concentration may be a major controlling factor with respect to the content of membrane-bound cytochromes in M.methylotrophus. Keevil & Anthony (1979b) reported a similar situation in Pseudomonas AM1, but the results of this present investigation are not compatible with a controlling-role for the methanol concentration in M.methylotrophus. Indeed, as has been found almost ubiquitously amongst obligate aerobes (see section 5.1), the concentration of oxygen was found here to exert the most profound influence over the membrane-bound cytochrome content.

The reason for the discrepancies between the results presented here and those reported by Cross & Anthony (1980b) probably lies in minor differences in the growth conditions. In particular, the concentration of iron in the growth medium is known to exert a powerful controlling influence over the cytochrome content of bacteria (see Rainnie & Bragg, 1973; Jurtshuk et al., 1975). The concentration of iron in the growth medium used at Billingham for the production of bacteria used for the determination of the cytochrome contents reported here, was 160-210μmoles/g

dry wt. bacteria, whereas that employed for the remainder of the experiments described here (and carried out at Leicester) was 14-22μmoles/g dry wt. bacteria (see sections 2.2.3-2.2.5). Control experiments showed that the cytochrome contents of bacteria grown under the slightly different conditions employed at Leicester and at Billingham were similar. However, if the concentration of iron in the growth medium used at Leicester was further decreased to 7μmoles/g dry wt. bacteria, the level of membrane-bound cytochromes in oxygen-limited cultures fell substantially, whereas that in methanol-limited cultures was virtually unaffected. The concentrations of iron in the media employed by Cross & Anthony (1980b) and Keevil & Anthony (1979b) may be calculated to be only 9 and 4.5μmoles/g dry wt. bacteria, respectively. In view of the critical effect of the iron concentration on the cytochrome content of M.methylotrophus reported here, we can perhaps rationalise the failures of Cross & Anthony (1980b) and Keevil & Anthony (1979b) to observe higher cytochrome contents in oxygen-limited, than in carbon-limited, continuous cultures of M.methylotrophus and Pseudomonas AM1, respectively, as being due to the low concentrations of iron employed for bacterial growth, by these workers. It is not clear whether the discrepancy between the work reported here, and by Cross & Anthony (1980b), as regards the effect of growth conditions on the relative levels of cytochrome oxidases aa_3 and o in M.methylotrophus, may be similarly rationalised. Neither in this investigation, nor in the work of Cross & Anthony (1980b) was there any evidence for the loss of membrane-bound cytochrome c (as was found by Keevil & Anthony (1979b) in methanol-excess cultures of Pseudomonas AM1) under any growth conditions.

Results using respiratory chain inhibitors suggest that the sequential organisation of the respiratory chain components, and the levels at which the various substrates donate their reducing equivalents, are the same in oxygen-limited as in methanol-limited cultures of M.methylotrophus (see section 3.6). Measurement of the stoicheiometry of proton and charge translocation, in oxygen-limited M.methylotrophus, was complicated by the 3- to 4-fold higher endogenous rate found in oxygen-limited as compared to methanol-limited cultures, under the conditions of these experiments. Nevertheless, evidence was obtained, from oxygen-limited cultures, for $\rightarrow H^+/O$ quotients of approximately 6 and 2 for the oxidation of NADH and methanol respectively, in agreement with the results from methanol-limited cultures (see section 4.6).

It would appear, therefore, that the decreased growth yields measured by Brooks & Meers (1973) under conditions of transient methanol excess, and probably oxygen-limitation, cannot be explained in terms of a reduced efficiency of respiratory chain energy conservation. Indeed, more recent growth studies have shown that, although the yield with respect to methanol ($Y_{methanol}$) is lower in oxygen-limited than in methanol-limited continuous culture, the yield with respect to oxygen (Y_{O_2}) is similar under these two growth limitations, or even higher during oxygen-limited growth (Vasey, R.B. unpublished results). It is the Y_{O_2} rather than the $Y_{methanol}$ which more simply reflects the efficiency of respiratory-chain energy conservation, and hence the near equality of the Y_{O_2} during oxygen- and methanol-limited growth is in agreement with the present finding that the value of the $\rightarrow H^+/O$ quotient is similar during growth under these two nutrient-limitations. The decreased $Y_{methanol}$ in oxygen-limited cultures was accompanied by an increase in the level of non-methanol carbon in the

fermenter supernatant. It is interesting in this context that formate is released into the suspending medium during the oxidation of methanol *in vitro* in washed cell suspensions of *M.methylotrophus* (see section 6.4.2). Indeed, Häggström & Dostálek (1981) have recently obtained evidence for formate production during growth of *Methylomonas methanica* in methanol-excess conditions, or during pulsed methanol addition to 'methanol-limited' continuous cultures. Clearly further investigation of the nature of the carbon compounds released into the growth medium during oxygen-limited growth of *M.methylotrophus*, and the mechanism of the fall in $Y_{methanol}$ under these conditions, is required.

CHAPTER 6

MAGNITUDES OF THE PROTONMOTIVE FORCE ($\Delta\bar{\mu}H^+$) AND PHOSPHORYLATION POTENTIAL (ΔG_p) IN METHANOL-LIMITED METHYLOPHILUS METHYLOTROPHUS

6.1 Introduction

6.2 Transmembrane potential difference ($\Delta\Psi$) in M.methylotrophus

6.3 Effect of ionophores on the $\Delta\Psi$

6.4 Transmembrane pH difference (ΔpH) in M.methylotrophus

6.4.1 Intracellular buffering capacity of M.methylotrophus

6.4.2 Internal pH of M.methylotrophus

6.5 Effect of the external pH on the $\Delta\Psi$, ΔpH and $\Delta\bar{\mu}H^+$

6.6 Energy charge and ΔG_p sustained by M.methylotrophus

6.7 Effect of the external pH and ionophores on the ΔG_p

6.8 ΔG_p sustained by respiration from various substrates

6.9 Discussion and conclusions

CHAPTER 6

MAGNITUDES OF THE PROTONMOTIVE FORCE ($\Delta\bar{\mu}H^+$) AND PHOSPHORYLATION POTENTIAL (ΔG_p) IN METHANOL-LIMITED *METHYLOPHILUS METHYLOTROPHUS*

6.1 Introduction

In Chapter 4, the determination, in methanol-limited *M.methylotrophus*, of two important kinetic parameters of respiratory chain energy conservation - the $\rightarrow H^+/O$ and $\rightarrow$ charge/O quotients - was reported. This chapter describes the determination of two thermodynamic parameters of energy conservation - the protonmotive force ($\Delta\bar{\mu}H^+$) and the phosphorylation potential (ΔG_p. the free energy of hydrolysis of ATP).

From a thermodynamic viewpoint, the Chemiosmotic hypothesis (see section 1.5.1) states that the energy released during substrate oxidation is converted to a transmembrane electrochemical gradient of protons, and the latter exerts a protonmotive force which drives ATP synthesis to establish a phosphorylation potential. Therefore, in a perfectly coupled system at equilibrium:

$$-\frac{2\,\Delta E_h}{\rightarrow H^+/2e^-} = -\Delta\bar{\mu}H^+ = -\frac{\Delta G_p}{F.\rightarrow H^+/ATP} \qquad (6.1)$$

where ΔE_h is equal to the redox potential of the substrate couple less that of the $\frac{1}{2}O_2/H_2O$ couple, $\rightarrow H^+/2e^-$ is the stoicheiometry of respiration-linked proton translocation (see Chapter 4), and $\rightarrow H^+/ATP$ is the number of protons translocated via the ATP phosphohydrolase per molecule of ATP synthesised (or hydrolised).

Equation (6.1) describes an equilibrium or 'static-head' situation in which there is no net flux through the system. Clearly oxidative phosphorylation will only occur when the driving force exceeds the opposing force, i.e.:

$$- \frac{2\ \Delta E_h}{\rightarrow H^+/2e^-} > - \Delta\bar{\mu}H^+ > - \frac{\Delta G_p}{F. \rightarrow H^+/ATP} \qquad (6.2)$$

When the driving force greatly exceeds the opposing force, the only limit on the rate of flux through the system is the maximum catalytic activity (i.e. the maximum activity of the respiratory chain or the ATP phosphohydrolase), and this state is known as 'level-flow'. In vivo, under normal respiring conditions, the energy-conserving apparatus of both bacteria and mitochondria is poised somewhere between 'static-head' and 'level-flow', such that the difference between the driving and opposing forces controls the rate of flux through the system, and this regulation is known as respiratory control.

Only at equilibrium, however, can kinetic and thermodynamic parameters be equated; and, therefore, when measuring the $\Delta\bar{\mu}H^+$ and ΔG_p, it is usual to chose experimental conditions which allow the system to approach a 'static-head'. This is best achieved by suspending washed, energised bacteria in non-permeant buffers of simple ionic composition, so as to minimise ATP utilisation and dissipation of the $\Delta\bar{\mu}H^+$ by ion-cycling. In practice, it may well be impossible to achieve near-equilibrium between the ΔE_h and $\Delta\bar{\mu}H^+$, as the proton permeability of the coupling membrane is known to increase rapidly, and become non-ohmic, above a threshold or breakdown potential (Nicholls, 1974). On the other hand, there is good evidence that near-equilibrium can be achieved between the

$\Delta\bar{\mu}H^+$ and ΔG_p (Michel & Oesterhelt, 1980; Lemasters, 1980), and this allows estimation of the $\rightarrow H^+/ATP$ quotient from the ratio $\Delta G_p/\Delta\bar{\mu}H^+$.

The redox potentials of the substrate couples involved in the work reported here, and the redox potential differences for the oxidation of these substrates are shown in Table 6.1.

Measurement of the $\Delta\bar{\mu}H^+$ usually involves separate determinations of the component parts, $\Delta\Psi$ and ΔpH, which may be summed as follows:

$$\Delta\bar{\mu}H^+_{(in-out)} = \Delta\Psi_{(in-out)} - 2.303\,\frac{RT}{F}\,\Delta pH_{(in-out)} \qquad (6.3)$$

The four principal methods which have been employed for the determination of the $\Delta\Psi$ (see Rottenberg, 1975, 1979) are discussed below:

(1) Microelectrodes - the use of microelectrodes to measure the $\Delta\Psi$ has generally yielded very low values, the polarity often being the reverse of that predicted by the Chemiosmotic hypothesis (see Tedeschi, 1980). Most workers have attributed this to membrane damage, caused by the insertion of the electrodes, but others have used these negative results to deny the existence of a $\Delta\Psi$ across the coupling membrane (Tedeschi, 1980). Felle *et al*. (1980), however, have recently determined a value for the $\Delta\Psi$ in giant cells of *Escherichia coli* (grown in the presence of mecillinam), using microelectrodes, which was similar to that obtained using other methods.

(2) Spectral shifts of intrinsic and extrinsic chromophores - Witt and coworkers (see Witt, 1971) have shown that the application of an electrical potential difference across a membrane can cause a shift in the absorbtion spectrum of chromophores within the membrane. This electrochromic shift has been used extensively to monitor the $\Delta\Psi$ in

Substrate couple	E_o' (V)	$\Delta E_o'$ (V)
formaldehyde/methanol	-0.19	-1.01
formate/formaldehyde	-0.45	-1.27
carbon dioxide/formate	-0.42	-1.24
dehydroascorbate/ascorbate	+0.06	-0.76
$NAD^+/NADH + H^+$	-0.32	-1.14

Table 6.1 Standard redox potentials (E_o') and redox potential differences ($\Delta E_o'$) for the oxidation of some substrates utilised by M.methylotrophus.

The data were taken from Ribbons et al. (1970) and Mahler & Cordes (1971).

photosynthetic bacteria where there are intrinsic chromophores such as the carotenoids. However, in order to obtain an absolute value for the $\Delta\Psi$, the spectral shifts must be externally calibrated, usually by the imposition of a potassium diffusion potential, and there is some dispute as to the validity of this calibration procedure (see Rottenberg, 1979; Symons et al., 1979; Tedeschi, 1980).

When using extrinsic chromophores, there is the additional problem that the location of the chromophore within the membrane/interface/bulk-phase system is difficult to ascertain, and this may lead to problems of interpretation (see Rottenberg, 1979). Nevertheless, values for the $\Delta\Psi$, obtained using both intrinsic and extrinsic chromophores, are consistently higher than those obtained using other methods (see Ferguson et al., 1979), and a possible reason for this discrepancy is discussed in the conclusion to this chapter.

(3) Distribution of permeant ions - assuming that they are neither bound nor acted on by energy-linked transport systems, permeant ions should distribute across the coupling membrane in accordance with the Nernst equation:

$$\Delta\Psi_{(in-out)} = 2.303 \frac{RT}{zF} \log \frac{(\text{cation}^{z+})_{out}}{(\text{cation}^{z+})_{in}}$$

$$, \text{ or } = 2.303 \frac{RT}{zF} \log \frac{(\text{anion}^{z-})_{in}}{(\text{anion}^{z-})_{out}} \qquad (6.4)$$

where $(ion)_{in}$ and $(ion)_{out}$ are the activities of the permeant ion inside and outside the bacteria, or vesicles. Provided that the activity co-efficient is the same in the internal and external phases, the $\Delta\Psi$ may be

determined from the ion accumulation ratio. Clearly, in order to observe ion-uptake, cations must be used when the internal phase is negative (whole cells, right-side out vesicles), and anions when the internal phase is positive (inverted membrane vesicles). The permeant ion must be present at very low concentration so that ion movements do not perturb the $\Delta\Psi$.

A number of methods have been used for the determination of the ion distribution including centrifugation, filtration, flow dialysis and monitoring with ion-specific electrodes. With the first three methods, it is normal to use radiolabelled permeant ions in order to allow simple and sensitive determination. The ion-specific electrode and flow dialysis methods have the advantage that they avoid the possibility of redistribution of ions during cell separation, but these methods are not as sensitive as centrifugation or filtration. Flow dialysis has been employed in this study for determination of the $\Delta\Psi$ in M.methylotrophus (see section 6.2).

(4) Changes in ion distribution on de-energisation - in the presence of K^+/valinomycin, the $\Delta\Psi$ and ΔpH may be determined from the changes in the extracellular pK ($-\log_{10}[K^+]$) and pH, respectively, when a bacterial suspension becomes de-energised (see Mitchell & Moyle, 1969; Collins & Hamilton, 1976). It should be noted that, unless the concentration of K^+ is very low (< 100μM), the relative contributions of the $\Delta\Psi$ and ΔpH to the $\Delta\bar{\mu}H^+$ are likely to be perturbed by K^+ movements. This method may alternatively be used, in the absence of valinomycin, for measurement of the ΔpH alone, and this latter procedure has been used here to determine the ΔpH in M.methylotrophus (see section 6.4).

In methods (2-4), above, the internal volume of the bacteria/vesicles must be known in order to calculate the $\Delta\Psi$. The internal volume is

usually determined by comparing the uptake of two differently-radio-labelled species, one of which is freely permeant throughout the bacteria or vesicles (e.g. [^{3}H] H_2O), and the other of which can permeate only as far as the bacterial inner membrane (or the vesicular membrane; e.g. [^{14}C] mannitol, [^{14}C] sucrose [see Rottenberg, 1979]). In method (4), above, a value for the intracellular buffering capacity is required for the calculation of the ΔpH - the determination of the intracellular buffering capacity of M.methylotrophus is described in section 6.4.1.

The majority of determinations of the $\Delta\Psi$ have been carried out in membrane vesicles (of both right-side out and inverted orientations), but some measurements have been made in whole cells, and sphaeroplasts. A representative selection of the values which have been obtained is shown in Table 6.2.

In addition to measuring the change in pH on de-energisation of a cell suspension, three other methods have been employed for the determination of the ΔpH in bacteria, and these are discussed below:

(1) ^{31}P-NMR - this method is based on the fact that the position of the peak due to inorganic phosphate, or phosphate-containing compounds, in the ^{31}P-NMR spectrum, depends on the pH - the ΔpH may thus be estimated from the difference between the peaks corresponding to phosphorus-containing species in the internal and external phases (see Ogawa et al., 1978). A major drawback of this method, however, is that thick suspensions of bacteria are required in order to overcome the relatively low sensitivity of the apparatus, thus making it difficult to maintain the energised state. Nevertheless, values for the ΔpH, which are comparable with those determined by other methods, have been recently obtained by ^{31}P-NMR (see Nicolay et al., 1981).

Organism	Whole cells/ vesicles	Method	$\Delta\Psi$ (mV)	References
Escherichia coli	giant cells	Microelectrodes	-133	Felle *et al*. (1980)
Rhodospirillum rubrum	chromatophores	Carotenoid band shift	+258	Bashford *et al*. (1979)
E.coli	whole cells	DDA^+ distribution (centrifugation)	-140	Griniuviene *et al*. (1974)
E.coli	RSV	DDA^+ distribution (filtration)	-100	Hirata *et al*. (1973)
E.coli	RSV	$TPMP^+$ distribution	-75	Ramos *et al*. (1976)
Thermus thermophilus	whole cells	(flow dialysis)	-197	McKay *et al*. (1981)
Paracoccus denitrificans	IMV	SCN^- distribution (flow dialysis)	+145	Kell *et al*. (1978a)
P. denitrificans	whole cells	$BTPP^+$ distribution (electrode)	-160	McCarthy *et al*. (1981)
E.coli	sphaeroplasts	changes in K^+ distribution	-132	Collins & Hamilton (1976)
Staphylococcus aureus	whole cells	on de-energisation	-134	Collins & Hamilton (1976)

Table 6.2 Values of the $\Delta\Psi$ in bacteria. Abbreviations: RSV, right-side out vesicles; IMV, inverted membrane vesicles; DDA^+, dibenzyldimethylammonium$^+$; $TPMP^+$, trimethylphenylphosphomium$^+$; $BTPP^+$, butyltriphenylphosphonium$^+$. All the determinations reported here were carried out at close to pH7.0.

(2) Distribution of weak acids/bases - this method is analogous to the permeant ion distribution method for the determination of the $\Delta\Psi$, and similar measuring techniques may be used. The basis of this method is that only the uncharged species (i.e. undissociated acid or deprotonated base) is able to cross the cell membrane, and the charged species will thus partition across the membrane in accordance with the difference between the pK and the pH in each compartment. When the internal pH is greater than the external pH (usually whole cells, right-side out vesicles), weak acids may be used, and the internal pH may be calculated from the equation:

$$pH_{in} = \log_{10}\left[\frac{(A^T)_{in}}{(A^T)_{out}}\left(10^{pK} + 10^{pH_{out}}\right) - 10^{pK}\right] \qquad (6.5)$$

where $(A^T)_{in}$ and $(A^T)_{out}$ are the total activities of all species of the acid inside and outside the bacteria/vesicles. Again the assumptions must be made that there is no energy-linked transport system, and that the activity coefficient is similar in the internal and external phases.

Where the internal pH is less than the external pH (usually inverted membrane vesicles), an analogous approach may be used with weak bases.

(3) Indicator dyes - indicator dyes may have applications for the study of rapid changes in the internal pH of well-buffered systems, but they are of limited use for the determination of absolute values of the ΔpH. In most cases, it is difficult to determine the location of the dye in the internal or external phases, or within the membrane itself, and an unambiguous calibration of the dye response is difficult to obtain (see Rottenberg, 1979).

Some of the values of the ΔpH which have been determined by these various methods are shown in Table 6.3.

In order to determine the ΔG_p it is necessary to determine both the standard free energy of ATP hydrolysis ($\Delta G^{o'}$), and the concentrations of ATP, ADP and inorganic phosphate:

$$\Delta G_p = \Delta G^{o'} - 2.303 \frac{RT}{F} \log_{10} \frac{[ATP]}{[ADP][Pi]} \qquad (6.6)$$

The magnitude of the $\Delta G^{o'}$ depends on the internal pH, and to a lesser extent on the temperature, ionic strength and concentration of Mg^{2+} (see Rosing & Slater, 1972). The ΔG_p sustained by inverted membrane vesicles from a number of bacteria has been determined, but there have been relatively few determinations of the ΔG_p in whole cells (e.g . Erecińska et al., 1979). A selection of values for the ΔG_p in various bacteria is shown in Table 6.4, and these are compared with the $\Delta\bar{\mu}H^+$ to yield the $\rightarrow H^+/ATP$ quotient. The values obtained for the latter vary from 2.1 to 13 g-ion H^+/mol ATP, and a possible reason for this wide variation is discussed in the conclusion to this chapter. As yet, neither the $\Delta\bar{\mu}H^+$ nor the ΔG_p has been determined in a methylotroph.

As well as under 'static-head' conditions, it would be interesting to obtain a value for the ΔG_p in growing bacteria, as this would allow estimation of the thermodynamic efficiency of oxidative phosphorylation during growth. Unfortunately, it is difficult to determine the intracellular concentration of inorganic phosphate, in the presence of relatively high concentrations of phosphate in the growth medium; however, the adenylate energy charge (EC, Atkinson & Walton, 1967; Atkinson, 1968) of a number of growing bacteria has been determined (Knowles, 1977):

Organism	Whole cells/ vesicles	Method	External pH	ΔpH	References
Escherichia coli	sphaeroplasts	change in pH on de-energisation	6.45 to 6.75	+1.65	Collins & Hamilton (1976)
Staphylococcus aureus	whole cells		6.25 to 6.45	+1.3	
E.coli	whole cells	^{31}P-NMR	6.8	+0.7	Ogawa *et al.* (1978)
E.coli	whole cells	methylamine, DMO distribution	6.0	+2.2	Padan *et al.* (1976)
		(centrifugation)	8.0	-0.5	
S.aureus	growing cells	benzoate distribution	6.0	+1.5	Kashket (1981)
		(filtration)	7.0	+0.5	
Micrococcus lysodeikticus	whole cells/	benzoate, DMO, acetate	5.5	+1.4	Friedberg & Kaback
	RSV	distribution (flow dialysis)	7.8	0	(1980)
E.coli	RSV	acetate, DMO distribution	5.5	+2.2	Ramos *et al.* (1976)
		(flow dialysis)	7.5	0	
E.coli	IMV	methylamine distribution	6.0	-0.7 to -1.3	Reenstra *et al.*
		(flow dialysis)	8.0	-0 to -0.7	(1980)

Table 6.3 Values of the ΔpH in bacteria. Abbreviations: RSV, right-side out vesicles; IMV, inverted membrane vesicles; DMO, 5,5'-dimethyloxazolidine-2,4-dione.

Organism	Whole cells/ vesicles	$-\Delta G_p/F$ (mV)	$\Delta\bar{\mu}H^+$ (mV)	$\rightarrow H^+/ATP$ quotient (g-ionH^+/mol ATP)	References
Rhodospirillum rubrum	chromatophores	609	+100	6.1	Kell *et al*. (1978b)
Rh. rubrum	chromatophores	564	+270	2.1	Bashford *et al*. (1979)
Rh. rubrum	chromatophores	530 to 580	+170 to +220	2.4 to 3.4	Cirillo & Gromet-Elhanan (1981)
Halobacterium halobium	whole cells	350 to 510	-120 to -140	2.5 to 4.3	Michel & Oesterhelt (1980)
Paracoccus denitrificans	IMV	559	+145 to +175	3.2 to 3.9	Kell *et al*. (1978a)
P. denitrificans	whole cells	522	-40 to -58	9 to 13	Deutsch & Kula (1978)
Bacillus alcalophilus	RSV	462 to 504	-40 to -125	4.0 to 11.6	Guffanti *et al*. (1978)
Thermus thermophilus	whole cells	449	-197	2.3	McKay *et al*. (1981)

Table 6.4 Comparison of the phosphorylation potential (ΔG_p) and protonmotive force ($\Delta\bar{\mu}H^+$) in bacteria.

Abbreviations: IMV, inverted membrane vesicles; RSV, right-side out vesicles.

$$EC = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]} \quad (6.7)$$

The determination of the energy charge in growing M.methylotrophus is reported in section 6.6.

6.2 Transmembrane electrical potential difference ($\Delta\Psi$) in M.methylotrophus

Fig. 6.1 depicts a typical flow dialysis experiment. Shortly after the addition of $[^{14}C]$ $TPMP^+$ to the upper chamber of the flow dialysis cell, the radioactivity appearing in the dialysate increased, reaching a maximum value after approximately 4 min. If no further additions were made, the level of radioactivity in the dialysate decreased at a constant slow rate (Fig. 6.1a). In the experiment depicted in Fig. 6.1b, however, the addition of 200µl of a cell suspension, to the reaction chamber, resulted in a rapid fall in the radioactivity in the dialysate. This fall reflects both the dilution of the $[^{14}C]$ $TPMP^+$ due to the addition of cell suspension, and the uptake of $[^{14}C]$ $TPMP^+$ into the bacteria. After approximately 3 min, a steady level was achieved, and the rate of decrease of radioactivity in the dialysate became comparable with that in the control experiment (Fig. 6.1a).

The addition of FCCP to the reaction chamber, caused the level of radioactivity in the dialysate to rise back to that which would be expected simply due to the dilution effect of the added cell suspension (dashed line). The return of the radioactivity to this level confirms that there was negligible binding of $[^{14}C]$ $TPMP^+$ to the bacteria.

The relationship between the concentration of free $[^{14}C]$ $TPMP^+$ in the reaction chamber, and the level of radioactivity in the dialysate,

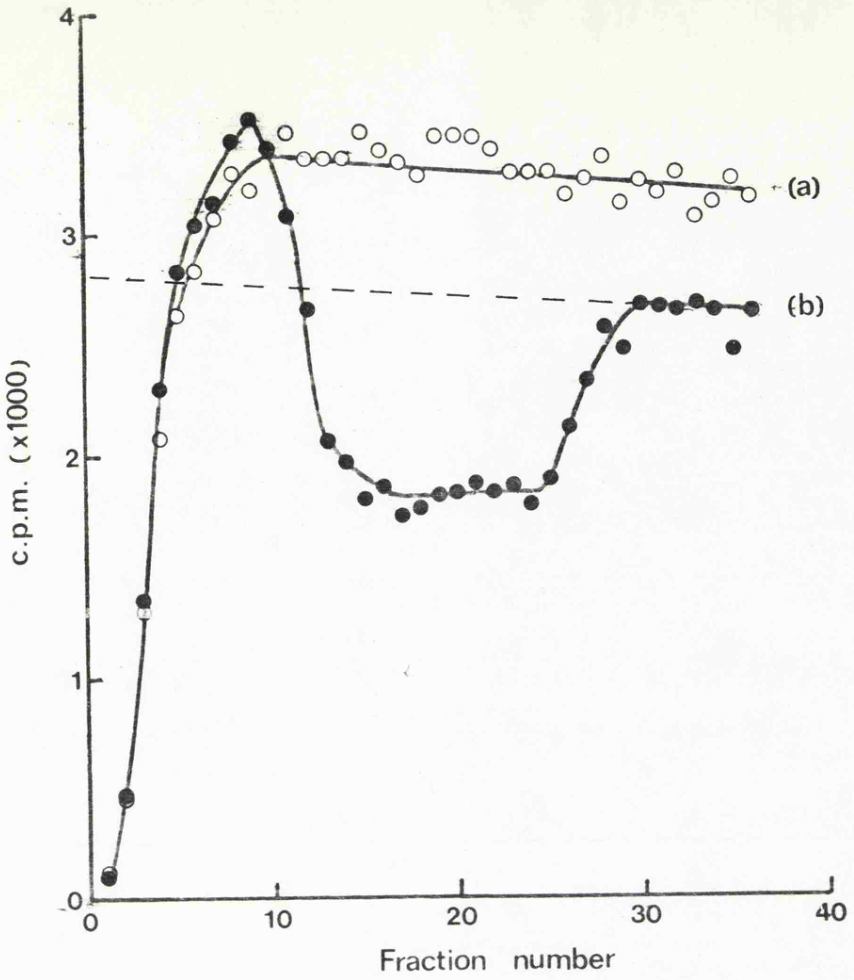


Fig. 6.1 Determination of [^{14}C] TPMP$^+$ uptake by flow dialysis. The flow dialysis experiments were carried out as described in section 2.9, 1ml samples being collected every 30s. The 0.8ml reaction mixture contained 140mM-KCl, 50mM-glycylglycine (pH7.0) and 25mM-methanol. At sample 0, 4µl of [^{14}C] TPMP$^+$ (11.25mM in ethanol) was added. In experiment (a), no further additions were made, but in experiment (b), 200µl of a suspension of M.methylotrophus (12.5mg dry wt./ml in 50mM-glycylglycine, 140mM-KCl), and 25 nmoles of FCCP were added at samples 9 and 24, respectively. The dashed line indicates the predicted level of radioactivity due solely to the dilution caused by the added cell suspension.

was determined from the maximum level of radioactivity achieved, extrapolated back to the point of addition of the [^{14}C] TPMP$^+$. The concentrations of free [^{14}C] TPMP$^+$, in the energised and de-energised cell suspensions, were determined from the level of radioactivity prior to FCCP addition, and the dashed line, respectively; and the amount of [^{14}C] TPMP$^+$ inside the energised bacteria was then calculated from the difference between these two concentrations. The intracellular [^{14}C] TPMP$^+$ concentration, and hence the accumulation ratio, were calculated using the cell density and the known internal volume of M.methylotrophus (1.06µl/mg dry wt. bacteria; see section 2.9), and a value for the $\Delta\Psi$ was then obtained using the Nernst equation (6.4); i.e. from Fig. 6.1:

$$56.3\mu M \ [^{14}C] \ TPMP^+ \equiv 3550 \text{ c.p.m.}$$

Therefore, the concentration of free [^{14}C] TPMP$^+$ in the energised cell suspension, at fraction 23 $= \frac{1800}{3550} \times 56.3\times10^{-6} = 28.55\mu M$

And the concentration of free [^{14}C] TPMP$^+$ in the de-energised cell suspension, extrapolated back to fraction 23 (dashed line)

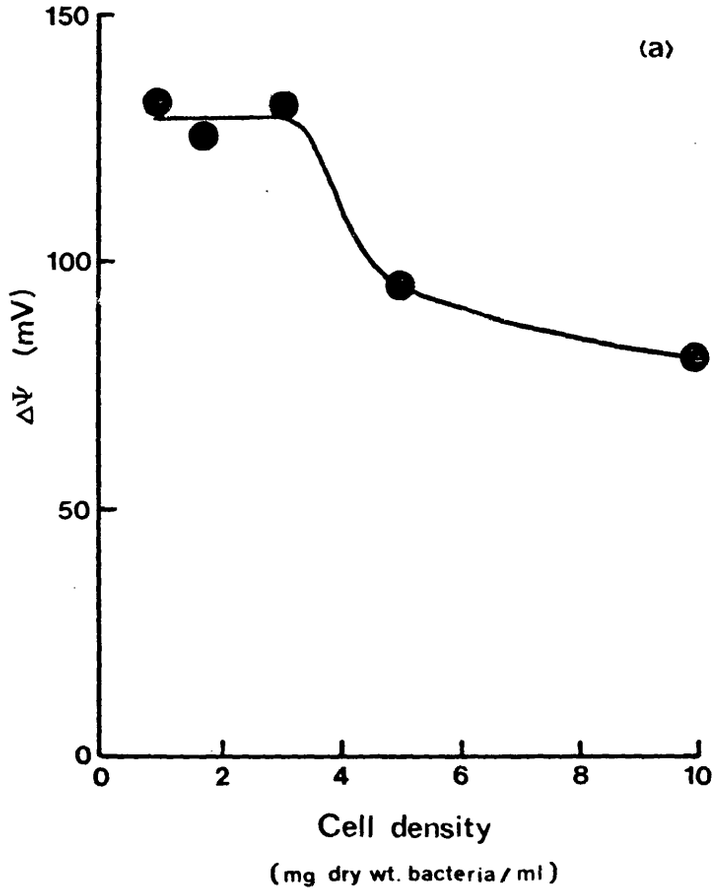
$$= \frac{2725}{3550} \times 56.3\times10^{-6} = 43.22\mu M$$

Therefore, the intracellular concentration of [^{14}C] TPMP$^+$, at fraction 23

$$= \frac{(43.22 - 28.55) \times 10^{-6} \times 10^{-3}}{2.5 \times 1.06 \times 10^{-6}} = 5.536mM$$

And $$\Delta\Psi = 2.303 \frac{RT}{F} \log_{10}\left(\frac{5.536\times10^{-3}}{28.55 \times 10^{-6}}\right) = 142mV$$

Fig. 6.2ab shows the effects of the cell density and [^{14}C] TPMP$^+$ concentration on the $\Delta\Psi$. The $\Delta\Psi$ was found to be constant at cell


150
100
50
0
ΔΨ (mV)
(a)
0
2
4
6
8
10
Cell density
(mg dry wt. bacteria / ml)

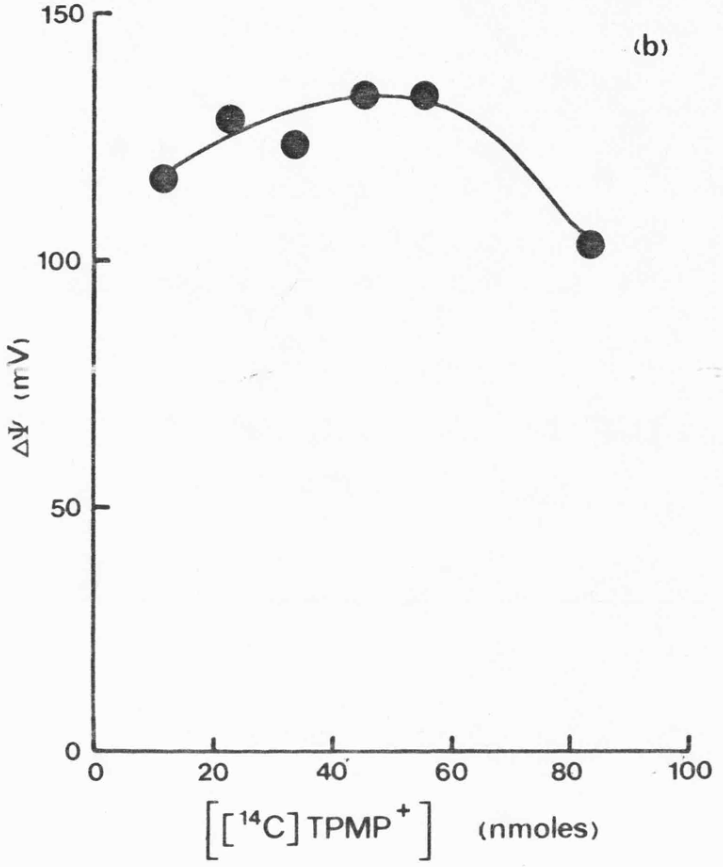


Fig. 6.2 Effect of cell density and $[^{14}C]$ $TPMP^+$ concentration on the $\Delta\Psi$. The flow dialysis experiments were carried out in 140mM-KCl, 50mM-glycylglycine buffer (pH7.0), using $[^{14}C]$ $TPMP^+$, as described in section 2.9, except that in (a), the cell density was varied, and in (b), the amount of $[^{14}C]$ $TPMP^+$ used was varied.

densities in the range 1 to 3mg dry wt. bacteria/ml, but the value obtained decreased at higher cell densities, probably due to the difficulty of maintaining aerobiosis in the reaction chamber. Maximum values of the $\Delta\Psi$ were obtained at $[^{14}C]TPMP^+$ concentrations of 45 to 55μM, whereas, above and below this concentration range, lower values were measured. The decrease in the magnitude of the $\Delta\Psi$ at low concentrations of $[^{14}C]TPMP^+$ is not readily rationalised (unless the proton current is partially localised, and the $[^{14}C]$ $TPMP^+$ is responsible for causing partial delocalisation, as well as for monitoring the delocalised $\Delta\bar{\mu}H^+$ [see section 6.9]), but, at higher concentrations, $[^{14}C]$ $TPMP^+$ uptake may itself cause partial collapse of the $\Delta\Psi$. A cell density of 2.5 to 3.0 mg dry wt. bacteria/ml, and $[^{14}C]$ $TPMP^+$ concentration of 45μM were adopted for all further determinations.

A second permeant cation, $[^3H]TPP^+$, was also used for the determination of the $\Delta\Psi$ in M.methylotrophus. The results of flow dialysis experiments using this cation were very similar to those depicted in Fig. 6.1. Fig. 6.3 shows that the effects of the $[^3H]$ TPP^+ concentration on the value of the $\Delta\Psi$ were qualitatively similar to those of $[^{14}C]$ $TPMP^+$. 160pmoles of $[^3H]TPP^+$ were used in all further experiments.

Table 6.5 shows values of the $\Delta\Psi$ sustained by respiration from methanol, in M.methylotrophus, at pH7.0. There was no significant difference between the values of the $\Delta\Psi$ obtained using $[^{14}C]$ $TPMP^+$ and $[^3H]TPP^+$. The magnitude of the $\Delta\Psi$ obtained in different buffers, however, varied substantially, and possible reasons for this variation are discussed in the conclusion to this chapter.

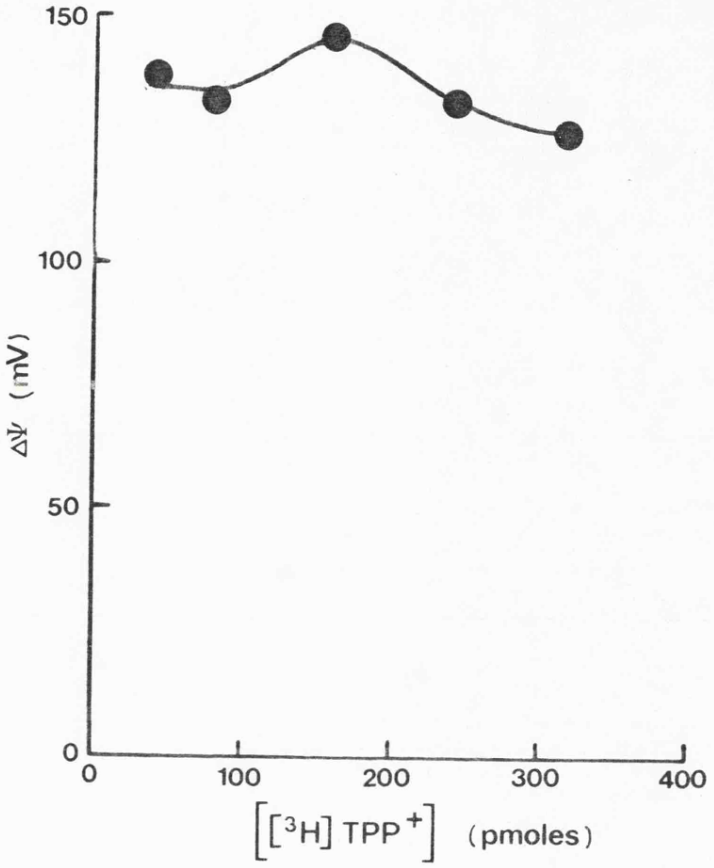


Fig. 6.3 Effect of the $[^3H]$ TPP^+ concentration on the $\Delta\Psi$. The flow dialysis experiments were carried out in 140mM-KCl, 50mM-glycylglycine buffer (pH7.0), using $[^3H]$ TPP^+, as described in section 2.9, except that the amount of $[^3H]$ TPP^+ used was varied.

Reaction medium	$-\Delta\Psi$ (mV)		
	$[^{14}C]TPMP^+$		$[^3H]TPP^+$
	No additions	+ nigericin	
50mM-glycylglycine, 140mM-KCl (pH7.0)	143 ± 2 (3)	125 ± 7 (3)	144 ± 3 (2)
50mM-Pipes, 140mM-KCl (pH7.0)	133 ± 4 (5)	103 - 120	137 ± 3 (4)
50mM-glycylglycine/ LiOH (pH7.0)	165 ± 2 (4)	n.d.	n.d.

Table 6.5 $\Delta\Psi$ sustained by respiration from methanol in M.methylotrophus. The $\Delta\Psi$ was determined as described in section 2.9, in the media shown in the table. When used, nigericin (1μg/mg dry wt. bacteria) was incubated with the cell suspensions for 5 min, at 40°C, before addition of the bacteria to the reaction mixture. Abbreviation: n.d. not determined.

6.3 Effect of ionophores on the $\Delta\Psi$

In the presence of either FCCP (25μM), or K^+/valinomycin (1μg/mg dry wt. bacteria), there was no measureable uptake of permeant cation into the bacteria (it should be noted, however, that the limit of detection of the flow dialysis method corresponds to an accumulation ratio of approximately 10 i.e. ≡ 62mV). These results are consistent with the known effects of FCCP and valinomycin which render the coupling membrane permeable to H^+ and K^+, respectively.

Nigericin catalyses H^+/K^+ antiport, and would hence be expected to dissipate the ΔpH component of the $\Delta\bar{\mu}H^+$, thus allowing the total $\Delta\bar{\mu}H^+$ to be expressed as a $\Delta\Psi$. At pH7.0, the $\Delta\Psi$, measured in the presence of K^+/nigericin, was approximately 20mV lower than in the absence of this ionophore (see Table 6.5). This may indicate that the internal pH of energised cells (in the absence of nigericin) is less than 7.0, i.e. approximately 6.7; but, alternatively, it is possible that nigericin is causing partial uncoupling, in addition to its well-known H^+/K^+ exchange properties.

6.4 Transmembrane pH difference (ΔpH) in M.methylotrophus

It did not prove possible to estimate the ΔpH, in M.methylotrophus, from the transmembrane distribution of [1-^{14}C] acetate, [1-^{14}C] phenylacetate or [2-^{14}C] DMO. Although [1-^{14}C] acetate was taken up into the cells, there was no release on adding FCCP, thus indicating that the [1-^{14}C] acetate was probably metabolised (though 10mM-acetate did not support respiration in oxygen-uptake experiments). No accumulation of either [1-^{14}C] phenylacetate or [2-^{14}C] DMO was observed, even at low pH (6.2) and in the presence of valinomycin, when

the ΔpH should be maximal. Under these conditions, a ΔpH of approximately 1.0 and 1.3 should have been readily detectable using [1-^{14}C] phenylacetate and [^{3}H]-DMO, respectively.

Owing to the lack of success of the weak acid distribution experiments, the internal pH of M.methylotrophus was estimated from the change in external pH on de-energisation of a cell suspension. In order to determine the internal pH in these experiments, it was first necessary to obtain a value for the intracellular buffering capacity.

6.4.1 Intracellular buffering capacity of M.methylotrophus

Fig. 6.4 depicts a typical determination of the intracellular buffering capacity of M.methylotrophus. The total buffering capacity, corrected for added reagents ($B^{T'} = B^{T} - B^{R}$), was similar regardless of whether FCCP plus valinomycin, or FCCP plus valinomycin plus Triton X-100, was used to permeabilise the bacteria. It is unlikely, therefore, that the addition of Triton X-100 caused substantial artifactual changes in the buffering capacity due to the solubilisation of membrane proteins, as noted elsewhere (see Mitchell & Moyle, 1969; Rottenberg, 1975).

In some experiments, the extracellular buffering capacity (B^{E}) was determined in an aerobic cell suspension (in the absence of substrate). The aerobic, extracellular buffering capacity was found to be considerably less than that of an anaerobic cell suspension, and hence the intracellular buffering capacity (B^{I}) was found to be substantially greater in aerobic, than in anaerobic cells. It should be noted, however, that the difference between the total and extracellular buffering capacities ($B^{T'} - B^{E}$) is only equal to the intracellular buffering capacity under

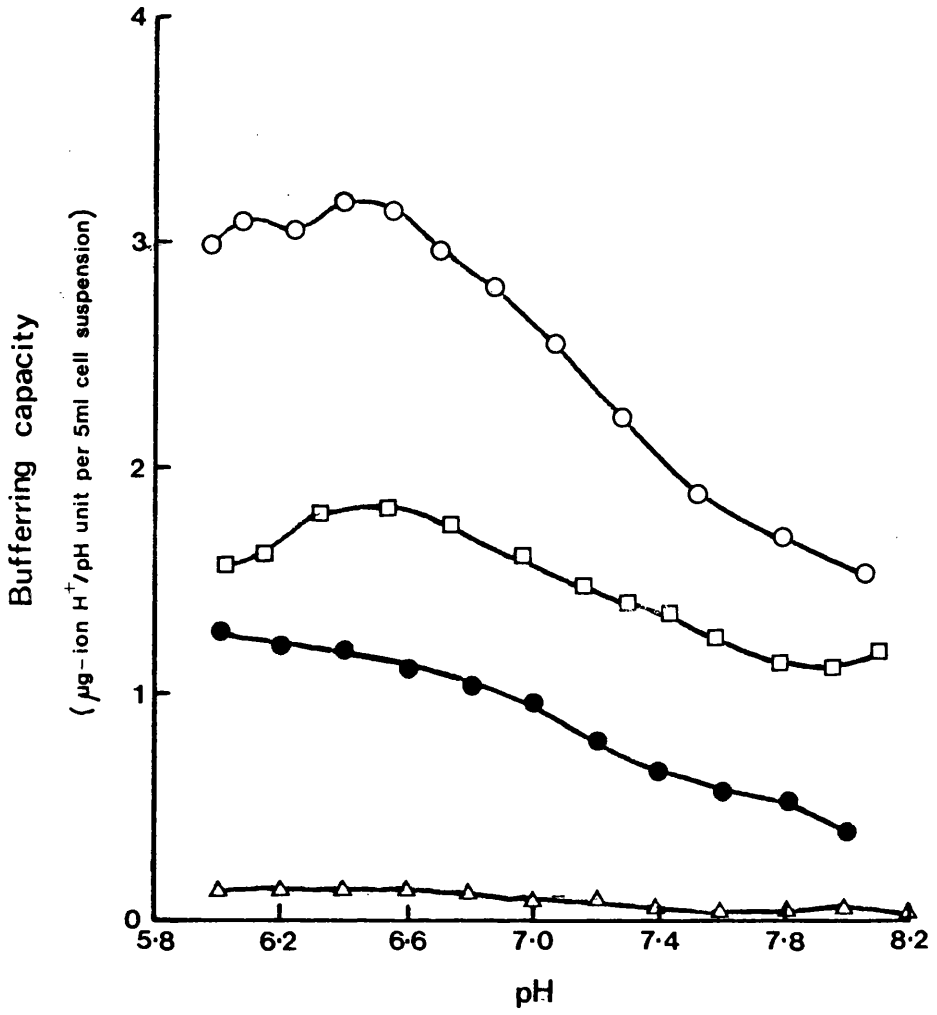


Fig. 6.4 Intracellular buffering capacity of M.methylotrophus. The extracellular (B^E, □) and total (B^T, ○) buffering capacities of an anaerobic suspension of M.methylotrophus (3.0mg dry wt./ml), and the buffering capacity of the reagents used to permeabilise the bacteria (10µM-FCCP plus 1µg of valinomycin/mg dry wt. bacteria plus 0.4% (v/v) Triton X-100 ; B^R, △), were measured as described in section 2.10.1. The intracellular buffering capacity (B^I, ●) was determined from the difference between the total and extracellular buffering capacities, corrected for the added reagents i.e. $B^I = (B^T - B^R) - B^E$.

conditions where there is no transmembrane ΔpH, or when the ΔpH does not vary with the external pH. When neither of these requirements is met, the true internal buffering capacity is given by:

$$B^{T'} - B^{E} = B^{I}_{true} \left[1 - \left({}^{d\,\Delta pH}/_{dpH^{E}} \right) \right] \qquad (6.8)$$

(see section 2.10.1). Under anaerobic conditions, there was no ΔpH in M.methylotrophus, regardless of the external pH (see section 6.4.2), but it is quite probable that aerobic cells will maintain a ΔpH, even in the absence of added substrate. Indeed, if the $d\,\Delta pH/dpH^{E}$ term were as high (negative) as in the presence of methanol (-1.0; see section 6.5), the internal buffering capacity of aerobic cells would be over-estimated by as much as 100%.

A second possible reason for the apparently higher intracellular buffering capacity ($B^{T'} - B^{E}$) of aerobic cells, as compared to anaerobic cells, is that endogenous respiration might be sufficient to retain many buffering ions within the cell, and these ions may then leak from the bacteria on anaerobiosis. When a cell suspension was made anaerobic by respiration from methanol, the apparent external buffering capacity changed from thattypical of an aerobic cell suspension, to that typical of an anaerobic cell suspension, within 2 to 3 min, thus indicating that the release of buffering ions, and/or the collapse of the ΔpH, occurs rapidly after anaerobiosis is reached. In view of this, the internal buffering capacity of anaerobic cells was used exclusively in the determination of the internal pH of M.methylotrophus, as elsewhere (Mitchell & Moyle, 1969; Collins & Hamilton, 1976).

6.4.2 Internal pH of M.methylotrophus

The addition of H_2O_2 to an anaerobic suspension of M.methylotrophus, in the presence of catalase and methanol, caused rapid acidification of the external medium (Fig. 6.5abc). The pH response to the exhaustion of oxygen depended on the external pH. At low pH (5.9 to 6.8; Fig. 6.5a), the acidification of the external medium changed to alkalinisation on anaerobiosis. If no further additions were made, the alkalinisation continued for 10 to 20 min until a final anaerobic pH level (pH_N^E) was reached. The simplest interpretation of this behaviour is that, during respiration, the cell interior was alkaline with respect to the external medium, so that when anaerobiosis was reached, protons moved into the bacteria to collapse the ΔpH. Indeed, if FCCP (10μM) was added, at any stage during the 20 min period after the cessation of respiration, the external pH changed rapidly to the same final pH (pH_N^E) as was observed in the absence of uncoupling agent. As the pH_N^E was not affected by a combination of FCCP (10μM), valinomycin (1μg/mg dry wt. bacteria) and Triton X-100 (0.4% (v/v)), it can be assumed that there was no ΔpH in this final anaerobic state.

When the external pH, on the point of anaerobiosis (pH_0^E), was close to 7.0 (Fig. 6.5b), the pH became constant after the cessation of respiration, thus indicating that the internal pH was also close to 7.0; and furthermore, at higher external pH (Fig. 6.5c), the medium continued to acidify after anaerobiosis was reached, thus indicating that, under these conditions, the internal pH was less than the external pH.

The internal pH of M.methylotrophus was calculated using the internal buffering capacity, determined in the same batch of cells (see section

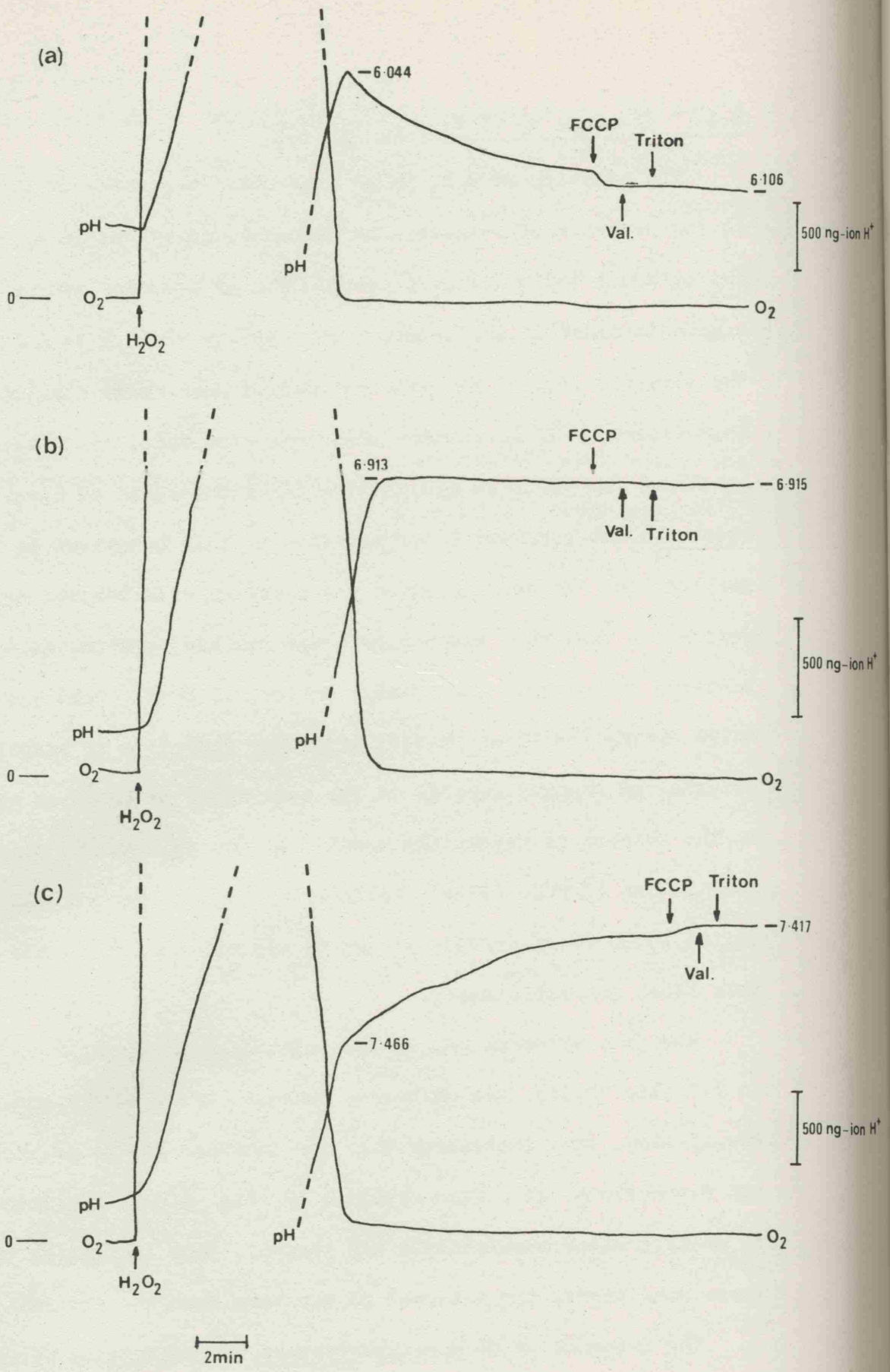

(a)
6·044
FCCP
Triton
6·106
Val.
500 ng-ion H+
pH
0
O2
H2O2
O2
(b)
FCCP
6·913
6·915
Val.
Triton
500 ng-ion H+
pH
0
O2
H2O2
O2
(c)
FCCP
Triton
7·417
Val.
7·466
500 ng-ion H+
pH
0
O2
H2O2
O2
2min

Fig. 6.5 Changes in external pH following an aerobic - anaerobic transition in a suspension of M.methylotrophus. These experiments were carried out, as described in section 2.10.2 in (a), 140mM-KCl, 50mM-glycylglycine buffer, or (b, c), 140mM-KCl, 10mM-glycylglycine buffer. 50µl of 2 vol H_2O_2 were added to initiate respiration, and two or three further 50µl additions were made in order to maintain aerobiosis for 7 to 8 min. FCCP, valinomycin and Triton X-100 were added, as indicated, to final concentrations of 10µM, 1µg/mg dry wt. bacteria and 0.4% (v/v), respectively.

6.4.1). For example, from Fig. 6.5a, 956ng-ion H^+ were taken up into the bacteria during the change in external pH from 6.044 to 6.106. Calculating the area under the plot of the intracellular buffering capacity versus pH, in Fig. 6.4, from pH6.106 upwards, we can see that:

$$\int_{6.106}^{6.950} B^I dpH \quad = \quad 956\text{ng-ion } H^+$$

and therefore the intracellular pH, just before the cell suspension became anaerobic, was 6.95. It did not prove possible to reliably estimate the internal pH at external pH values of greater than 6.9 as, when the medium continues to acidify after anaerobiosis is reached, it is difficult to identify the pH from which this continued acidification should be measured (i.e. pH_0^E). Nevertheless, the internal pH of M.methylotrophus was found to be constant over a range of external pH from 5.9 to 6.9, and to have a value of 7.00 ± 0.03 (8).

In all of the experiments described above, the final anaerobic pH (pH_N^E) was considerably lower than the pH at the beginning of the experiment, thus indicating that the products of methanol oxidation were acidic. This overall acidification, which was largely FCCP-independent, may have been partly due to the solution of carbon dioxide, but the level of formate in the reaction mixture (assayed as per section 2.7.4, after removal of the bacteria by filtration) also increased throughout the aerobic incubation period to a level of approximately 0.60mM after 7.5 min. It is quite probable that this relatively high concentration of the weak acid, formate, will have a bearing on the magnitude of the ΔpH sustained by M.methylotrophus, under these conditions (c.f. Kell et al., 1981).

The conditions for the determination of the ΔpH were chosen to allow direct comparison with the values of the $\Delta\Psi$ determined from the distribution of permeant cations (see Table 6.5); however, in some cases, it proved necessary to use lower buffer concentrations in order to allow accurate measurement of the pH changes following the cessation of respiration. The experiments described above were performed in 10mM or 50mM-glycylglycine buffer, in the presence of 140mM-KCl, and the internal pH was found to be similar when these two buffer concentrations were used. The exclusion of K^+ from the reaction medium (i.e. 10mM-glycylglycine/LiOH) did not significantly affect the internal pH (7.04 $\pm$ 0.06 (5); constant over the pH range 6.0 to 6.8); however, in 140mM-KCl, 10mM-Pipes buffer, the internal pH was significantly lowered (6.58 $\pm$ 0.02 (4); constant over the pH range 6.1 to 6.4). It is not obvious why the internal pH of M.methylotrophus should be substantially lower when the buffer used is Pipes rather than glycylglycine.

6.5 Effect of the external pH on the $\Delta\Psi$, ΔpH and $\Delta\bar{\mu}H^+$

Fig. 6.6 shows the effect of the external pH on the $\Delta\Psi$, ΔpH and $\Delta\bar{\mu}H^+$, in M.methylotrophus. The contribution of the ΔpH to the $\Delta\bar{\mu}H^+$ fell with increasing external pH from approximately 50mV, at pH6.2, to only 12mV, at pH6.8. On the other hand, the $\Delta\Psi$ was found to increase with the external pH over the pH range 6.2 to 7.8. Between pH6.2 and 6.8, the fall in the ΔpH was almost matched by the increase in the $\Delta\Psi$, such that the $\Delta\bar{\mu}H^+$ decreased only slightly with the external pH from 155mV, at pH6.2, to 143mV at pH6.8. Between pH6.8 and 7.8, however, the $\Delta\Psi$ increased at a rate of only 14mV/pH unit, and, if it is assumed that the internal pH was constant at pH7.0, then the $\Delta\bar{\mu}H^+$ decreased from 143mV at pH6.8 to 106mV at pH7.8.

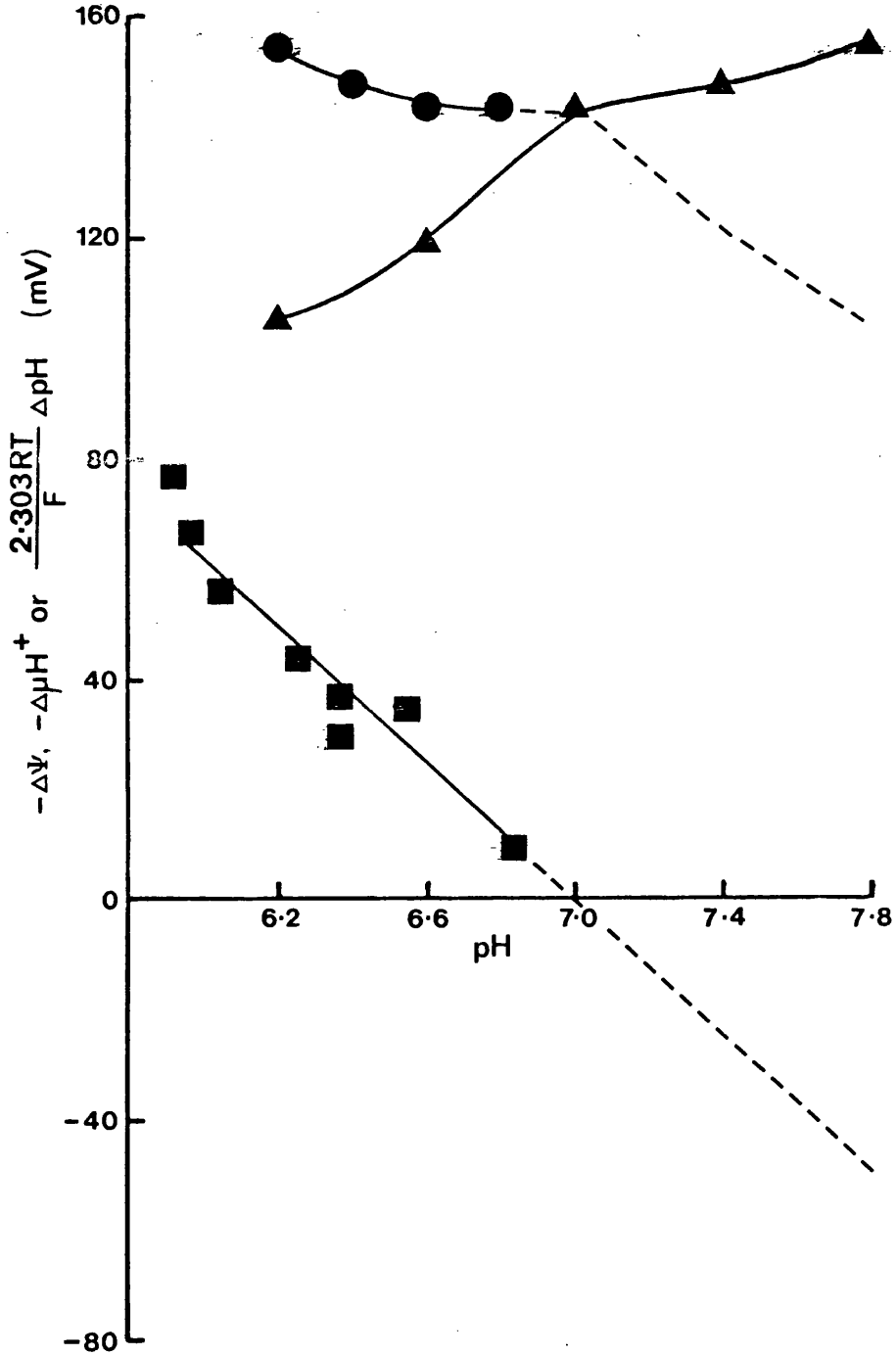

160
120
80
40
0
-40
-80
$-\Delta\Psi$, $-\Delta\mu H^+$ or $\frac{2{\cdot}303RT}{F}\Delta pH$ (mV)
6·2
6·6
7·0
7·4
7·8
pH

Fig. 6.6 Effect of the external pH on the $\Delta\Psi$, ΔpH and $\Delta\bar{\mu}H^+$.

The $\Delta\Psi$ (▲) and ΔpH (■) were determined from the transmembrane distribution of [^{14}C] $TPMP^+$ (see section 2.9), and from the pH changes following an aerobic-anaerobic transition (see section 2.10), respectively. All determinations were carried out in 140mM-KCl, 50mM-glycylglycine buffer, except that the concentration of glycylglycine was reduced to 10mM for the determination of the ΔpH at external pH values of greater than 6.6. The $\Delta\bar{\mu}H^+$ (●) was calculated from the relationship $\Delta\bar{\mu}H^+ = \Delta\Psi - 62.1\ \Delta pH$ (see equation 6.3). The dashed lines indicate projected values of the ΔpH, and $\Delta\bar{\mu}H^+$, assuming that the internal pH is constant at 7.0 over the external pH range 6.8 to 7.8.

6.6 Energy charge and ΔG_p sustained by M.methylotrophus

Table 6.6 shows the intracellular concentrations of adenine nucleotides and inorganic phosphate, in M.methylotrophus, either growing in methanol-limited continuous culture, or harvested and incubated under various conditions. It was assumed, for the purpose of these determinations, that all of the adenine nucleotides and phosphate, in the cell suspensions, was located intracellularly. Typically, however, approximately 5% of the total adenine nucleotides was located extracellularly after centrifugation or filtration; the extracellular concentration of phosphate was too low to be measured accurately. Not more than 3% of the bacteria, in cell suspensions, were lysed at the end of the incubation period, as assessed by the activity of the intracellular marker enzyme, glucose-6-phosphate dehydrogenase, in filtrates, or in the supernatant after centrifugation.

It can be seen, from Table 6.6, that the total concentration of adenine nucleotides was not significantly affected by harvesting of the bacteria. However, slightly lower total adenine nucleotide levels were found in aerobic washed cell suspensions than under anaerobic conditions, or in the presence of FCCP. In aerobic cells, the adenine nucleotides were predominantly in the form of ATP (75% of total), whereas AMP formed up to 74% of the total adenine nucleotides in anaerobic or FCCP-treated cells. Under these last two conditions, the concentration of inorganic phosphate was substantially higher than in aerobic, energised cells, the difference being only partly accountable to the change in the amount of phosphate esterified in the adenine nucleotides.

Conditions	[AMP] (mM)	[ADP] (mM)	[ATP] (mM)	[Pi] (mM)	Energy charge	$-\Delta G_p$ (kJ/mol)
Methanol-limited continuous culture	0.632 ± 0.254 (5)	1.49 ± 0.11 (5)	2.67 ± 0.15 (5)	n.d	0.713	-
Aerobic + methanol	0.155 ± 0.059 (9)	0.633 ± 0.068 (9)	2.38 ± 0.11 (9)	4.9 ± 0.5 (9)	0.851	45.8
Anaerobic + methanol	3.09 ± 0.29 (5)	1.06 ± 0.05 (5)	0.214 ± 0.03 (5)	15.8 ± 1.9 (5)	0.170	35.1
Aerobic + methanol + FCCP	3.34 ± 0.23 (5)	1.05 ± 0.03 (5)	0.125 ± 0.011 (5)	11.2 ± 0.7 (5)	0.144	34.7

Table 6.6 Intracellular adenine nucleotide and phosphate concentrations, energy charge and ΔG_p in M.methylotrophus.

The experiments were performed on either methanol-limited continuous cultures (row 1), or washed cell suspensions in 140mM-KCl, 10mM-glycylglycine buffer (pH7.0) (rows 2 to 4). The latter were incubated with 20mM-methanol for 5 min, at 40°C, either aerobically, or anaerobically, as described in section 2.11. Where indicated, FCCP was present throughout the incubation period, at a concentration of 5μM. The reaction mixtures were quenched, and the adenine nucleotides and phosphate were extracted and assayed, as described in section 2.11. The energy charge and ΔG_p were calculated as detailed in sections 6.1 and 6.6. Abbreviation: n.d., not determined.

Also shown in Table 6.6 are the adenylate energy charge (see section 6.1), and the phosphorylation potential (ΔG_p). Values of the standard free energy of hydrolysis of ATP ($\Delta G^{o'}$), under a wide range of conditions, are available in the literature, but the values quoted by different workers show quite a wide variation (e.g. Phillips et al., 1969; Rosing & Slater, 1972; Guynn & Veech, 1973). The data of Rosing & Slater (1972) have been used most frequently, in recent work (e.g. Kell, 1978ab; Guffanti et al., 1981; McKay et al., 1981; see Slater, 1979), and hence these values are used here for comparative purposes; though it should be noted that the values of the $\Delta G^{o'}$, quoted by these workers, are towards the lower end of the range of values in the literature.

In deriving a value of the $\Delta G^{o'}$, under the conditions used here, from the extensive tabulation of Rosing & Slater (1972), it was assumed that the internal pH, Mg^{2+} concentration and ionic strength were 7.0 (see section 6.4.2), 10mM and 0.10, respectively; the experiments were carried out at 40°C. The value of the $\Delta G^{o'}$ is not critically dependent on the concentration of Mg^{2+}, between 1 and 25mM, or on the ionic strength, between 0.05 and 0.20, so it is unlikely that inaccuracies in the values assumed for these parameters will introduce major error into the value of the $\Delta G^{o'}$ chosen. Taking the above assumptions into account, the best estimate of the $\Delta G^{o'}$, under the conditions of these experiments, is -28.5 kJ/mol.

The ΔG_p and energy charge sustained by M.methylotrophus, during respiration from methanol, were found to be -45.8 kJ/mol and 0.851, respectively. As expected, both of these parameters were considerably decreased under anaerobic conditions, or in the presence of FCCP. Further-

more, the near equality of the values obtained under these last two conditions suggests that there is little or no substrate-level phosphorylation, during respiration from methanol, in this organism. The energy charge in growing cells was found to be significantly lower than that in energised washed cell suspensions. This is probably due to the fact that the rate of utilisation of ATP is substantially more rapid in the former than in the latter, and oxidative phosphorylation is thus operating closer to static-head conditions in washed cell suspensions than in growing cells.

6.7 Effect of the external pH and ionophores on the ΔG_p

Fig. 6.7 shows the effect of the external pH on the ΔG_p in the presence of various ionophores. In deriving a value for the $\Delta G^{o'}$, it has again been assumed that the intracellular ionic strength and Mg^{2+} concentration were 0.10 and 10mM, respectively (see section 6.6). The internal pH has a profound effect on the $\Delta G^{o'}$. In the absence of ionophores, it has been assumed that the internal pH was 7.0, regardless of the external pH (see section 6.4.2), whereas, in the presence of either nigericin or FCCP, the internal and external pH have been assumed to be equal. In the presence of valinomycin, the $\Delta\bar{\mu}H^+$ of -140 to -150mV might be expected to be composed entirely of a ΔpH of approximately 2.5pH units. However, the pH/activity profiles of the respiratory enzymes will presumably impose an upper limit on the internal pH, and thus, in deriving a value for the $\Delta G^{o'}$ in the presence of valinomycin, the internal pH has been assumed to be 8.0, regardless of the external pH.

In the absence of added ionophores, the ΔG_p increased to a maximum

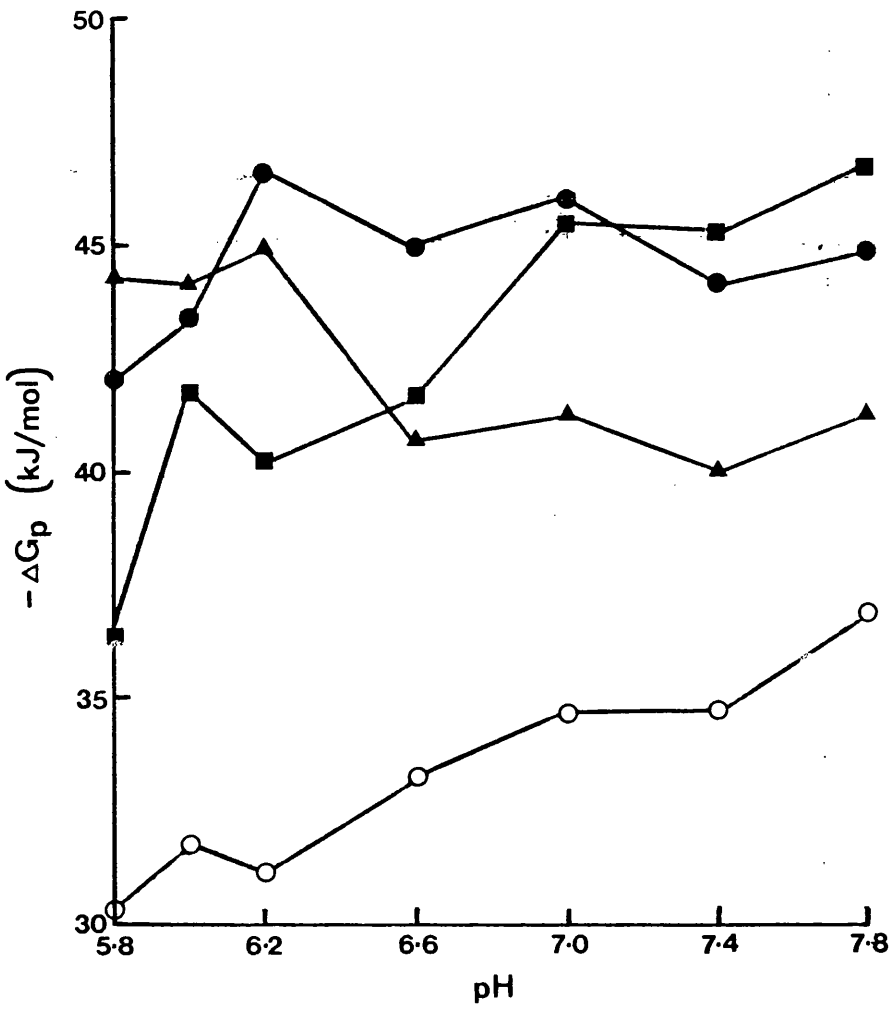


Fig. 6.7 Effect of the external pH and ionophores on the ΔG_p. Washed cell suspensions (3mg dry wt. bacteria/ml in 140mM-KCl, 10mM-glycylglycine, pH7.0) were incubated aerobically, with rapid stirring, in the presence of 20mM-methanol (●). Some of the suspensions also contained nigericin (1μg/mg dry wt. bacteria; ■), valinomycin (1μg/mg dry wt. bacteria; ▲) or FCCP (5μM; ○). After 5 min incubation, the cells were quenched, and the adenine nucleotides were extracted and assayed, as described in section 2.11. The assumptions involved in the calculation of the ΔG_p values are discussed in section 6.7.

value of -46.6 kJ/mol at pH6.2, and then fell slightly with increasing external pH; this behaviour quite closely matches that of the $\Delta\bar{\mu}H^+$ (see Fig. 6.6).

Valinomycin, had little effect on the value of the ΔG_p at pH5.8 to 6.2, but this K^+ ionophore caused a significant decrease in the ΔG_p at higher pH. These results indicate that a ΔpH alone is fully competent to drive ATP synthesis, but, at external pH values of greater than 6.2, the restrictions on the internal pH discussed above limit the magnitude of the ΔpH, and hence of the ΔG_p.

Nigericin did not affect the magnitude of the ΔG_p over the pH range 7.0 to 7.8, thus indicating that a $\Delta\Psi$ alone is fully competent to drive ATP synthesis; however, at lower external pH, this ionophore caused a significant decrease in the ΔG_p, thus suggesting that ATP synthesis is less efficient at low internal pH.

In the presence of the uncoupling agent, FCCP, the magnitude of the ΔG_p was less than 40 kJ/mol, regardless of the external pH. Under these conditions, the ΔG_p presumably reflects the minimum level at which the ATP utilising reactions can operate. The variation of the ΔG_p with the external pH, in uncoupled cells, suggests that the ATP utilisation reactions are sensitive to the adenine nucleotide concentrations, rather than to the ΔG_p.

6.8 ΔG_p sustained by respiration from various substrates

Table 6.7 shows the values of the ΔG_p sustained by respiration from various substrates, in M.methylotrophus. The optimum concentration and period of incubation for each substrate were first determined and then used in all further experiments (see legend to Table 6.7). Also shown are

Substrate	J_{K^+} (ng-ion K^+/min per mg)	$-2\Delta E_0'/\rightarrow K^+/0$ (V.g-atom O/g-ion K^+)	$-\Delta G_p$ (kJ/mol)	
			No addition	+ FCCP
Endogenous	35	n.d.	36.8	35.5
Formate	222	0.413	40.2	34.3
Ascorbate-TMPD	430	0.760	42.6	35.6
Formaldehyde	716	0.553	42.2	32.0
Methanol	1362	0.501	45.8	34.7
NADH	n.d.	n.d.	43.0	35.0

Table 6.7 ΔG_p sustained by respiration from various substrates, and comparison with kinetic and thermodynamic parameters. For the measurement of the ΔG_p, cell suspensions were incubated aerobically in 140mM-KCl, 50mM-glycylglycine buffer (pH7.0), as described in section 2.11. The substrate concentrations, and periods of incubation used, were, endogenous substrates, 5 min; formate (5mM), 10 min; ascorbate (20mM) - TMPD (50μM), 5 min; formaldehyde (10mM), 5 min; methanol (20mM), 5 min; and NADH (2mM), 3 min. Where indicated, FCCP was present throughout the incubation period at a concentration of 5μM.

The adenine nucleotides and phosphate were extracted and assayed as described in section 2.11; and the ΔG_p was calculated, according to equation 6.6, using a $\Delta G^{o'}$ of -28.5kJ/mol. The rate of respiration-linked charge translocation (J_{K^+}) was determined from the product of the respiration rate, measured under the above conditions (see section 2.6), and the $\rightarrow K^+/O$ quotient which was taken to be 6, for endogenous substrates and formate, 3.12 for formaldehyde, and 2 for methanol and ascorbate-TMPD (see Chapter 4). Both the $\Delta E_o'$ and $\rightarrow K^+/O$ values, used for methanol and formaldehyde, were average values assuming equal contributions from each stage in the complete oxidation of these substrates to carbon dioxide.

the rate of respiration-linked charge translocation (J_K+), and the redox potential difference of substrate oxidation per translocated charge ($-2\,\Delta E_o'/\rightarrow K^+/0$; this is equal in magnitude to the $\Delta\bar{\mu}H^+$, at equilibrium, assuming that $\Delta E_o' = \Delta E_h$; see equation 6.1).

The ΔG_p sustained during the oxidation of various substrates showed little correlation ($r = +0.16$) with the value of $-2\,\Delta E_o'/\rightarrow K^+/0$, thus indicating that, even when conditions are chosen to minimise both the dissipation of the $\Delta\bar{\mu}H^+$ and ATP utilisation, ATP synthesis does not come into equilibrium with respiration. Furthermore, if a $\rightarrow H^+/ATP$ quotient of 2g-ion H^+/mol ATP is assumed, the overall thermodynamic efficiency of oxidative phosphorylation ($\rightarrow ATP/0 \times \Delta G_p/2\,\Delta E_o'$), in this organism, was only 29 to 50%. That the value of the ΔG_p is, indeed, subject to kinetic control is confirmed by the good correlation ($r = +0.84$) between the ΔG_p and the J_K+. Much of the energy loss would seem to occur between respiration and proton translocation as, at equilibrium, thermodynamic considerations would predict a $\Delta\bar{\mu}H^+$ ($= 2\,\Delta E_o'/\rightarrow K^+/0$) of -413 to -760mV. Indeed, these results do not rule out the possibility that the $\Delta\bar{\mu}H^+$ is in near-equilibrium with ATP synthesis, as reported elsewhere (Michel & Oesterhelt, 1980; Lemasters, 1980).

The ubiquitously low value for the ΔG_p, obtained in the presence of FCCP, indicates that there is little or no substrate-level phosphorylation during respiration from any of these substrates.

6.9 Discussion and conclusions

M.methylotrophus was found to sustain a $\Delta\bar{\mu}H^+$, during respiration from methanol, of up to -155mV. The relative contributions of the $\Delta\Psi$ and ΔpH to the $\Delta\bar{\mu}H^+$ varied with the external pH - at pH6.2 (in 50mM-glycylglycine, 140mM-KCl), the $\Delta\bar{\mu}H^+$ was composed of a $\Delta\Psi$ of -105mV, and a ΔpH of 0.8 ($-2.303 \frac{RT}{F} \Delta pH = -50mV$), whereas at pH7.0 the $\Delta\bar{\mu}H^+$ was composed entirely of a $\Delta\Psi$ of -143mV. The magnitude of the $\Delta\Psi$, in M.methylotrophus, was well within the range of values determined in other bacteria (see Table 6.2), but the internal pH of this organism (7.0) was found to be lower than that of most respiring neutrophiles (see Table 6.3). A possible reason for the low value of the internal pH, in M.methylotrophus, is that the weak acid, formate - a product of the incomplete oxidation of methanol - may tend to collapse the transmembrane ΔpH (c.f. Kell et al., 1981).

The uncoupling agent, FCCP, caused the collapse of both the $\Delta\Psi$ and ΔpH components of the $\Delta\bar{\mu}H^+$. Furthermore, valinomycin, but not nigericin, caused the collapse of the $\Delta\Psi$; the effect of these ionophores on the ΔpH was not investigated due to lack of time. The slight decrease, caused by nigericin, in the value of the $\Delta\Psi$ at pH7.0, was probably due to a secondary uncoupling property of this ionophore, as the $\Delta\bar{\mu}H^+$ was found to be composed entirely of a $\Delta\Psi$, under these conditions.

M.methylotrophus sustained a ΔG_p, during respiration from methanol, of up to -45.8 KJ/mol, at pH7.0. This value is somewhat lower than has been found in most bacteria (see Table 6.4), but the majority of these determinations have been carried out in vesicular preparations rather

than in whole cells. The low value of the ΔG_p sustained by whole cells of M.methylotrophus thus probably reflects the fact that 'static-head' conditions may be more closely reached in vesicular preparations than in whole bacteria. Either the $\Delta\Psi$ or the ΔpH, alone, was fully competent to drive ATP synthesis in this organism.

Comparison of the ΔG_p sustained by respiration from various substrates with the $\Delta E_o'$ for substrate oxidation, showed that ATP synthesis was not in equilibrium with respiration, in M.methylotrophus. Much of the 'inefficiency' appeared to occur between respiration and proton translocation i.e.

$$\frac{-2\ \Delta E_h}{\rightarrow H^+/2e^-} > -\Delta\bar{\mu}H^+ = \frac{-\Delta G_p}{\rightarrow H^+/ATP} \qquad (6.8)$$

In Table 6.8, the $\Delta\bar{\mu}H^+$ and ΔG_p sustained by respiration from methanol, in different reaction media, are compared to yield the $\rightarrow H^+/ATP$ quotient. Although the values of the ΔG_p sustained in different reaction media were quite similar, there was a large variation in the $\Delta\bar{\mu}H^+$, and hence in the $\rightarrow H^+/ATP$ quotient. This behaviour, which has also been noted in submitochondrial particles (Sorgato et al., 1978), is difficult to reconcile with the Chemiosmotic hypothesis. The independent variation of the ΔG_p and $\Delta\bar{\mu}H^+$ is, however, readily explainable by the Localised Proton hypothesis if the degree of interaction between the 'localised protons' and the bulk aqueous phases depends on the reaction conditions (see Kell, 1979). The $\Delta\bar{\mu}H^+$ measured across the bulk aqueous phases is thus likely to be a variable underestimate of the true driving force for ATP synthesis; and it is interesting, in this context, that values of the $\Delta\Psi$, measured using intramembrane probes (e.g. by the carotenoid band

Reaction medium	$-\Delta G_p/_F$ (mV)	$-\Delta\bar{\mu}H^+$ (mV)	$\rightarrow H^+/ATP$ (g-ion H^+/mol ATP)
50mM-glycylglycine, 140mM-KCl	461	144	3.20
50mM-Pipes, 140mM-KCl	442	109	4.06
50mM-glycylglycine/LiOH	440	167	2.63

Table 6.8 Comparison of the ΔG_p and the $\Delta\bar{\mu}H^+$. The ΔG_p and $\Delta\bar{\mu}H^+$ were both determined, at pH7.0, in the media shown in the table. For the determination of the ΔG_p, washed cell suspensions were incubated, with 50mM-methanol, for 5 min, before extraction and assay of the adenine nucleotides and phosphate (see section 2.11). The ΔG_p was calculated, according to equation 6.6, using a $\Delta G^{o'}$ of -28.5 kJ/mol. The $\Delta\bar{\mu}H^+$ was determined from the $\Delta\Psi$ and ΔpH according to equation 6.3 - the $\Delta\Psi$ was taken from Table 6.5, as the average of the values determined using $[^{14}C]TPMP^+$ and $[^3H]$ TPP^+; and the internal pH, of M.methylotrophus, was taken to be 7.0, in the glycylglycine-buffered media, and 6.6 in Pipes, KCl (see section 6.4.2). The $\rightarrow H^+/ATP$ quotient was calculated from the ratio $\Delta G_p/_{F.\Delta\bar{\mu}H^+}$.

shift), are routinely higher than those measured between the bulk aqueous phases (e.g. by the distribution of permeant ions [see Table 6.2; Ferguson et al., 1979]). The Localised Proton hypothesis is given further support by the wide range of $\rightarrow H^+$/ATP quotients which have been determined by comparison of the ΔG_p and the $\Delta\bar{\mu}H^+$ in various bacteria (see Table 6.4).

If the bulk transmembrane $\Delta\bar{\mu}H^+$ is indeed an underestimate of the true driving force for ATP synthesis, then the values of the $\rightarrow H^+$/ATP quotients, shown in Table 6.8, are likely to be overestimated. The true mechanistic $\rightarrow H^+$/ATP quotient is therefore likely to be 2g-ion H^+/mol ATP. This value has some support from kinetic determinations in both bacteria and mitochondria (see section 1.10.2).

The observation of respiration-linked proton translocation, in M.methylotrophus (see Chapters 4, 5), is not incompatible with the Localised Proton hypothesis. Kell (1979) has suggested that K^+, in the presence of valinomycin, will effectively displace the proton current from localised channels on the membrane surface into the bulk aqueous phases. Two questions would seem to be of major importance in considering the Localised Proton hypothesis vis a vis the Chemiosmotic hypothesis:

(1) To what extent is the proton current localised in vivo?

(2) To what extent can the proton current be forced into the bulk aqueous phases, in vitro, such that the Chemiosmotic hypothesis provides a useful quantitative model for the study of energy conservation?

The first question would appear to be somewhat recalcitrant to investigation, but some observations on the second question follow. The stoicheiometries of respiration-linked proton translocation, measured in

various bacteria, under conditions which would be expected to displace the proton current into the bulk aqueous phases (see Kell, 1979), vary with respiratory chain composition and growth yield in a logical manner (see Jones, 1977). This suggests that the measured stoicheiometries are, at the very least, proportional to the true stoicheiometries of charge separation, and that the measurement of respiration-linked proton translocation stoicheiometries is, thus, a valid quantitative approach to the study of energy conservation. On the other hand, the conditions used for the measurement of the $\Delta\bar{\mu}H^+$ appear to be less effective in delocalisation of the proton current.

I would like to thank Miss Sarah E. Cooke and Miss Jacquie A. Quilter for performing some of the experiments described in this chapter.

CHAPTER 7

SUMMARY AND DISCUSSION

7.1 Summary of results

7.2 Growth energetics of *M.methylotrophus*

7.3 Reasons for the low growth yields of the methanol-utilisers

7.4 Potential for improvement of the respiratory chain energy conservation system of *M.methylotrophus*

7.4.1 Methanol oxidase

7.4.2 Cytochrome *c* oxidase

7.5 Possibilities for future work

CHAPTER 7

SUMMARY AND DISCUSSION

7.1 Summary of results

(1) Methanol-limited M.methylotrophus contains membrane-bound cytochromes aa_3, b and c, and soluble cytochrome c. The membrane-bound cytochromes aa_3, o and c_{co}, are able to bind carbon monoxide.

(2) As in other methylotrophs, methanol is oxidised via a methanol dehydrogenase which donates reducing equivalents to the respiratory chain at the level of cytochrome c.

(3) The respiratory chain of M.methylotrophus branches at the level of cytochrome c to two terminal oxidases, viz cytochromes aa_3 and o. Cytochrome c_{co} probably does not function as a terminal oxidase.

(4) The respiratory chain of M.methylotrophus contains three energy conserving sites. Site I, NADH : ubiquinone oxidoreductase, is associated with $\rightarrow H^+/2e^-$ and $\rightarrow$ charge/$2e^-$ quotients of 2; site II, ubiquinol : cytochrome c oxidoreductase, is associated with $\rightarrow H^+/2e^-$ and $\rightarrow$ charge/$2e^-$ quotients of 4 and 2, respectively; and site III, cytochrome c oxidase, is associated with $\rightarrow H^+/0$ and $\rightarrow$ charge/0 quotients of 0 and 2, respectively.

(5) Respiration via the two terminal oxidases, cytochromes $\underline{aa}_3$ and $\underline{o}$, appears to be associated with equal efficiencies of energy conservation. Neither oxidase catalyses net proton translocation.

(6) Only the third site of energy conservation is involved in the oxidation of methanol, which is associated with $\rightarrow H^+/O$ and $\rightarrow$ charge/O quotients of 2. Methanol oxidase, therefore, probably forms a redox arm in which methanol dehydrogenase and cytochrome $\underline{c}$ are situated externally, and the proton consumption sites of cytochrome oxidases $\underline{aa}_3$ and/or $\underline{o}$ are situated internally; the two protolytic reactions being connected by inward transmembrane electron flow.

(7) Growth in oxygen-limitation causes a 2 to 3-fold increase in the level of membrane-bound cytochromes, and cytochrome oxidases, as compared to methanol-limited continuous culture; whereas growth under conditions of methanol-excess (oxygen-limitation or nitrogen-limitation) is accompanied by a 2 to 3-fold decrease in the level of the soluble cytochrome $\underline{c}$. Cytochrome $\underline{aa}_3$ is sometimes absent in ammonium-limited continuous culture.

(8) The low growth yields obtained in oxygen-limitation as compared to methanol-limitation (Brooks & Meers, 1973) cannot be explained in terms of a decreased efficiency of respiratory-chain energy conservation.

(9) M.methylotrophus sustains a $\Delta\bar{\mu}H^+$ of up to -165mV during respiration from methanol. The magnitude of the $\Delta\bar{\mu}H^+$ depends on the composition of the reaction medium, and on the external pH. The relative contributions of the $\Delta\Psi$ and ΔpH to the $\Delta\bar{\mu}H^+$ also vary with the external pH, the internal pH being maintained at 7.0 (in glycylglycine-buffered media).

(10) M.methylotrophus sustains a ΔG_p during respiration from methanol of -42.5 to -45.8kJ/mol. ATP synthesis is not in equilibrium with respiration.

(11) The value of the $\Delta\bar{\mu}H^+$ varies with the composition of the reaction medium independently of the ΔG_p which is relatively constant. This behaviour is incompatible with the Chemiosmotic hypothesis. Values for the $\rightarrow H^+$/ATP quotient ranging from 2.6 to 4.1 g-ion H^+/mol ATP, obtained from comparison of the ΔG_p and $\Delta\bar{\mu}H^+$, represent overestimates of the true mechanistic stoicheiometry which is probably 2g-ion H^+/mol ATP.

(12) If it is assumed that the $\rightarrow H^+$/ATP quotient is 2g-ion H^+/mol ATP, then M.methylotrophus exhibits ATP/O quotients of 3 and 1 mol ATP/g-atom O during respiration from NADH and methanol, respectively.

7.2 Growth energetics of M.methylotrophus

Assuming that NAD(P)H is the reductant for biosynthesis, that NADH and NADPH are energetically equivalent (M.methylotrophus has only a soluble transhydrogenase), and that the formula for cell biomass is $C_4H_8O_2N$ (MW 102; see Anthony, 1978), the following equation can be constructed to describe the growth of M.methylotrophus:

$$4CH_3OH + NH_3 + (102/Y_{ATP})\ ATP + 0.5\ NADH \qquad (7.1)$$

$$\longrightarrow C_4H_8O_2N\ (102g) + 2H_2O + 4PQQH_2$$

where Y_{ATP} is the molar growth yield with respect to ATP (g cells/mol ATP). (Only the reduced forms of the coenzymes are shown for simplicity). By applying the $\rightarrow H^+/O$ quotients of approximately 6 and 2 g-ion H^+/g-atom O, determined in this study, for the oxidation of NADH and methanol, respectively, it is possible to calculate the amount of methanol that must be completely oxidised to carbon dioxide to provide the 0.5 moles of NADH and $(102/Y_{ATP})$ moles of ATP required for assimilation, as a function of the $\rightarrow H^+/ATP$ quotient. Assuming that the complete oxidation of methanol to carbon dioxide yields one mole of $PQQH_2$ and 2 moles of NADH, 0.25 moles of methanol must be fully oxidised to produce the 0.5 moles of NADH required for assimilation. Omitting the NH_3 and H_2O for simplicity:

$$4.25\ CH_3OH + (102/Y_{ATP})\ ATP \qquad (7.2)$$

$$\longrightarrow C_4H_8O_2N\ (102g) + 4.25\ PQQH_2 + 0.25\ CO_2$$

The 4.25 moles of $PQQH_2$ on the right hand side of equation (7.2) will yield ($4.25 \times 2/ \rightarrow H^+/ATP$) moles of ATP on oxidation. Therefore, ($102/Y_{ATP} - 8.5/ \rightarrow H^+/ATP$) moles of ATP must be produced from further complete oxidation of methanol, in order to supply the energy requirement for assimilation. The complete oxidation of one mole of methanol yields a total of $(2+6+6)/ \rightarrow H^+/ATP$ moles of ATP, therefore

$$\frac{(102/Y_{ATP} - 8.5/ \rightarrow H^+/ATP)}{14/ \rightarrow H^+/ATP}$$

moles of methanol must be fully oxidised to supply the ATP requirement for assimilation. Taking into account the 0.25 moles of methanol which must be oxidised to supply the NADH requirement, and the 4 moles of methanol directly required for assimilation, and omitting the carbon dioxide produced for simplicity:

$$\left[4{\cdot}25 + \frac{(102/Y_{ATP} - 8.5/ \rightarrow H^+/ATP)}{14/ \rightarrow H^+/ATP} \right] CH_3OH \qquad (7.3)$$

$$\longrightarrow \quad C_4H_8O_2N \quad (102g)$$

Table 7.1 shows values for the $Y_{methanol}$ of <u>M.methylotrophus</u>, as predicted from equation (7.3), assuming various values for the Y_{ATP}, and the $\rightarrow H^+/ATP$ quotient. Under similar growth conditions to those used in this study, this organism exhibits a growth yield of 0.53g cells/g methanol (Vasey, R.B, unpublished result). Furthermore, by comparing the growth yield of wild-type <u>M.methylotrophus</u> with that of a mutant requiring one less mole of ATP per mole of ammonia fixed, Senior & Windass (unpublished result) have obtained a value of 6.0g cells/mol ATP for the

Y_{ATP} (g cells/mol ATP)	→ H^+/ATP quotient (g-ion H^+/mol ATP)		
	2	3	4
4	0.438	0.350	0.292
5	0.486	0.398	0.337
6	0.525	0.437	0.375
7	0.557	0.471	0.408
8	0.583	0.500	0.438
9	0.606	0.525	0.465
10	0.625	0.547	0.486

Table 7.1 Predicted growth yield of M.methylotrophus. The figures in the table are values of the molar growth yield on methanol ($Y_{methanol}$; g cells/g methanol) calculated from equation (7.3) using various values for the Y_{ATP} and the → H^+/ATP quotient.

Y_{ATP}. It can be seen from Table 7.1, that these values strongly favour a $\rightarrow H^+$/ATP quotient of 2g-ion H^+/mol ATP, in this organism. Indeed, if the $\rightarrow H^+$/ATP quotient were to be 3g-ion H^+/mol ATP, then the Y_{ATP} would have to be as high as 9g cells/mol ATP.

7.5 Reasons for the low growth yields of the methanol-utilisers

Linton & Stephenson (1977) pointed out that the growth yields of the methylotrophs are lower than those of most heterotrophs when compared to the heats of combustion of their substrates. In the case of the methanol-utilisers, most attempts to explain this discrepancy have been based on the contention that methanol oxidase is thermodynamically inefficient. Indeed, the fourth column of Table 7.2 compares the thermodynamic efficiencies of the first stages of oxidation of a number of carbon substrates, based on standard free energy changes, and it is clear that, on this basis, methanol compares unfavourably with most heterotrophic substrates.

In order to rationalise this apparent inefficiency of methanol oxidase, several authors (e.g. Drozd & Wren, 1980) have pointed out that there may be little selection pressure for higher growth yields in a natural enviroment, though this argument does not differentiate between growth on different substrates. It has also been suggested that growth on methanol may be limited by the supply of NAD(P)H rather than by that of ATP (particularly when the serine pathway is used for carbon assimilation; e.g. Anthony, 1978), and hence an increase in the ATP yield from methanol oxidase would be of little advantage under these conditions. This argument, however, is not applicable to M.methylotrophus, the growth

First stage of substrate oxidation	$-\Delta E'_o$ (V)	ATP yield	Thermodynamic efficiency	
			-	Corrected for transport
Methanol ⟶ formaldehyde	1.01	1	25%	25%
Succinate ⟶ fumarate	0.79	1-2	32-63%	0-32%
Glucose ⟶ 6-phosphogluconate	1.05	1-2	24-48%	12-36%
Glucose ⟶ 2 x 1, 3-diphosphoglycerate	1.83	2-4	27-55%	20-48%
Malate ⟶ oxaloacetate	0.99	2-3	51-76%	25-51%
$H_2 \longrightarrow H_2O$	1.24	2-3	40-61%	40-61%
Formate ⟶ CO_2	1.24	2-3	40-61%	30-50%
Lactate ⟶ pyruvate	1.01	2-3	50-74%	37-62%
Glycerol ⟶ dihydroxyacetone	0.81	2-3	62-93%	62-93%
Glycerol ⟶ dihydroxyacetone-P	0.96	0-1	0-26%	0-26%

Table 7.2 Thermodynamic efficiencies of the first stages of oxidation of various carbon substrates. The thermodynamic data were obtained from Rauen (1969), Sober (1970), Mahler & Cordes (1971) and Lehninger (1975). In calculating the thermodynamic efficiencies of substrate oxidation $\left(\frac{\Delta G_p \cdot ATP/O}{2F.\Delta E'_o}\right)$ it has been assumed that the value of the ΔG_p is - 48.2 kJ/mol (i.e. $\Delta G_p/F = -500$ mV), and that the ATP/O quotients for the oxidation of NADH, succinate ($FADH_2$) and methanol, are 2-3, 1-2 and 1 mol ATP/g-atom O, respectively. In correcting the thermodynamic efficiencies for energy expenditure on substrate transport, it has been assumed that the uptake of methanol, H_2 and glycerol does not require energy, that the uptake of one mole of glucose, formate and lactate requires the equivalent of $\frac{1}{2}$ mole of ATP, and that the uptake of one mole of succinate and malate requires the equivalent of one mole of ATP.

of which is known to be energy-limited as a mutant which utilises one less mole of ATP per mole of ammonia assimilated exhibits a significantly higher growth yield than does the wild-type organism (Senior & Windass 1980).

It should be noted here that the thermodynamic inefficiency of methanol oxidase, as illustrated by the fourth column of Table 7.2, is somewhat overstated. The ability to compete for low concentrations of substrate, which is thought to be a major selective factor for micro-organisms in natural environments (Jannasch & Mateles, 1974; Harder *et al.*, 1977), depends on two factors - *viz* a high affinity for the growth substrate, and the thermodynamic capacity to utilise low concentrations of substrate (i.e. when the ΔE for substrate oxidation is considerably less than under standard conditions). Two basic strategies exist for fulfilling these requirements:

(1) A high affinity (low K_M), energy-linked uptake system concentrates the substrate within the cell thereby increasing its chemical potential. The substrate is then metabolised via a high K_M system which is thermodynamically efficient in terms of standard free energy changes.

(2) The substrate is directly metabolised, without energy-linked uptake, by an enzyme system, with a low K_M, which will appear to be thermodynamically inefficient, when standard free energy changes are considered, because it must be able to operate when its substrate is present at a low chemical potential.

The majority of growth substrates (e.g. succinate, glucose) are utilised via the first strategy. Methanol, however, as a small uncharged molecule which is freely permeant across the cell membrane, cannot be accumulated, and methanol oxidation must, therefore, follow the second strategy. (Methanol dehydrogenase is, in fact, situated on the outer face of the coupling membrane).

It is clearly unreasonable to compare directly thermodynamic efficiencies of energy conservation, based on standard free energy changes, for substrates which are oxidised via these two different strategies, without taking into account the energy expended on transport in strategy (1). In the fifth column of Table 7.2, the efficiencies of the first stages of oxidation of various substrates have been corrected for the expenditure of energy on substrate uptake. Once this correction has been made, it can be seen that the thermodynamic efficiency of methanol oxidase falls within - albeit towards the lower end - the wide range of efficiencies of the first stages of utilisation of other carbon substrates.

A major reason for the low growth yields of the methanol-utilisers may well lie in the one-carbon nature of the methanol molecule. As discussed above, it must be considered that there is an energy expenditure for the securing of a substrate from its environment, whether this energy is expended directly on energy-linked uptake, or indirectly via an apparently inefficient first stage of utilisation. At low substrate concentrations, this energy expenditure is absolutely necessary to compensate for the low chemical potential at which the substrate is present; furthermore this energy expenditure generally persists at higher substrate concentrations, and the majority of microorganisms do not adjust the bioenergetic yields of their substrate utilisation pathways to changes

in the chemical potential at which the substrate is supplied. (A notable exception is provided by Klebsiella aerogenes, growing on glycerol, which uses a low bioenergetic yield pathway during glycerol-limited growth, but switches to a higher yield pathway under conditions of glycerol-excess [Neijssel et al., 1975]). Returning to the case in point, the energy expenditure for securing from the environment a one-carbon compound such as methanol, is the same as for a multicarbon compound, such as succinate or glucose (though the energy may be expended in different ways). It follows that the methanol-utilisers will use a disproportionate amount of their available energy in this way.

7.4 Potential for improvement of the respiratory chain energy conservation system of M.methylotrophus

7.4.1 Methanol oxidase

As discussed in the previous section, methanol oxidase is thermodynamically inefficient, and this has a particularly deleterious effect on the growth yield because this inefficiency must be borne for every atom of carbon utilised. There is no thermodynamic reason why methanol, when present in excess, should not donate reducing equivalents to the respiratory chain via a methanol dehydrogenase which interacts with the latter at the level of the endogenous quinone (rather than at the level of cytochrome c), or possibly even via an $NAD(P)^+$-linked dehydrogenase.

7.4.2 Cytochrome *c* oxidase

Neither cytochrome oxidase aa_3 nor *o*, of *M.methylotrophus*, appears to catalyse net proton translocation, in contrast to the terminal oxidases of mitochondria (see Wikström & Krab, 1979) and of some other bacteria (e.g. *Paracoccus denitrificans* [van Verseveld *et al*., 1981]; *Bacillus stearothermophilus* [Chicken *et al*., 1981]). The third site of energy conservation, in *M.methylotrophus*, may thus be operating at less than its maximum potential efficiency.

7.5 Possibilities for future work

The subject of respiratory-chain energy conservation is a controversial one in which different experimental approaches often yield conflicting results. Therefore, in order to obtain useful information, it is necessary to investigate each facet of the subject by as many approaches as possible. This philosophy has been followed in this study, as far as time has allowed, but inevitably a number of experimental approaches remain to be tried, and some of the more pertinent ones are discussed below.

(1) The different sites of energy conservation, in *M.methylotrophus*, have been studied here by functional separation, using a variety of reducing equivalent donors and specific respiratory inhibitors. An alternative approach is to physically separate these sites, as has been attempted with some success in mitochondria (see Coin & Hinkle, 1979). This approach may be particularly useful in further investigations of the branched terminal oxidase system of this organism.

(2) The ATP/O quotient has been determined here from the $\rightarrow H^+/O$ and $\rightarrow$ charge/O quotients, measured directly, and the $\rightarrow H^+/ATP$ quotient derived from a comparison of the ΔG_p and $\Delta\bar{\mu}H^+$. The ATP/O quotient has also been estimated from theoretical growth calculations. A further approach would be to determine the ATP/O quotient by direct measurement of ATP synthesis and oxygen consumption, in either whole cells or inverted membrane vesicles.

(3) This study has indicated that ATP synthesis, in M.methylotrophus, is not in equilibrium with respiration. Furthermore, some doubt has been cast on the role of the bulk transmembrane $\Delta\bar{\mu}H^+$ as an obligatory intermediate between respiration and ATP synthesis. This point is clearly worthy of further study, and it would be of interest to measure the bulk transmembrane $\Delta\bar{\mu}H^+$ sustained by respiration from different substrates, and to see whether this correlates with the ΔG_p which has already been measured.

The results of the investigations reported in this thesis have raised a number of points which are also worthy of further study.

(1) Methanol oxidase appears to be a relatively simple energy conserving system, and its further resolution and reconstitution would be of great interest.

(2) Cross & Anthony (1980b) first reported that M.methylotrophus will oxidise externally-supplied NADH. It has been shown here that the oxidation of externally-supplied NADH will drive ATP synthesis in an uncoupler-dependent fashion. Furthermore, respiration from externally-supplied NADH does not appear to involve the cytochrome b, c region of the respiratory chain, and is linked to low

stoicheiometries of proton and charge translocation (Cooke, S.E. unpublished results). It seems, therefore, that NADH supplied to whole cells may be oxidised by a different route from internally-generated NADH, and a further investigation of both the mechanism and physiological role of this system may be rewarding.

(3) There are at least three possible fates for formaldehyde in M.methylotrophus - this compound may be oxidised to formate by methanol dehydrogenase, or by an NAD^+-linked formaldehyde dehydrogenase, or may alternatively be condensed with ribulose-5-phosphate to yield hexulose-6-phosphate in a reaction catalysed by hexulose phosphate synthase. The regulation of this branchpoint is complicated by the fact that, at least in vitro, methanol dehydrogenase catalyses the four equivalent oxidation of methanol to formate without detectable formaldehyde production (Duine et al., 1978; see Quayle, 1980). Formaldehyde, however, is only a substrate for methanol dehydrogenase when hydrated (Sperl et al., 1974), and thus, if the active site of this enzyme were located in a hydrophobic environment in vivo, then methanol oxidation would be expected to stop at formaldehyde. It is interesting to speculate that the highly-reactive formaldehyde molecule might then be channelled safely through the membrane to the hexulose phosphate synthase located on the internal face.

The oxidation of formaldehyde, in M.methylotrophus, is thought to proceed primarily via the cyclic RMP pathway rather than by linear oxidation via formate (Beardsmore & Quayle, 1978). However, the production of substantial amounts of formate during the oxidation of

methanol, *in vitro*, suggests that the regulation of formaldehyde utilisation may break down under conditions of methanol-excess. As this may be a contributory factor to the low growth yields obtained in methanol-excess cultures, the regulation of this important branchpoint is clearly worthy of further investigation.

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ABSTRACT

RESPIRATORY CHAIN ENERGY CONSERVATION IN THE METHYLOTROPHIC BACTERIUM

METHYLOPHILUS METHYLOTROPHUS

Michael J. Dawson

M.methylotrophus is an obligately aerobic, Gram negative methylotrophic bacterium which grows preferentially on methanol as the carbon and energy source, and uses the ribulose monophosphate pathway for carbon assimilation. This organism is used for single-cell protein production in the I.C.I. 'PRUTEEN' process. The composition and sequential organisation of the respiratory chain of M.methylotrophus have been studied. and both kinetic ($\rightarrow H^+/O$, $\rightarrow K^+/O$ quotients) and thermodynamic ($\Delta\bar{\mu}_p H^+$; ΔG_p) parameters of energy conservation have been determined. In addition, the effect of the growth conditions on some of these parameters has been investigated.

The respiratory chain of M.methylotrophus was found to branch at the level of cytochrome c to two terminal oxidases, cytochromes aa_3 and o. Methanol is oxidised via a methanol dehydrogenase which donates reducing equivalents to the respiratory chain at the level of cytochrome c, as in other methylotrophs. Proton and charge translocation stoicheiometries indicate the presence of three energy conserving sites between NADH and oxygen, each of which translocates two charges; only the third coupling site, which appears to function by a redox arm mechanism, is involved in respiration from methanol.

M.methylotrophus was found to sustain a ΔG_p, during respiration from methanol, of approximately -45 kJ/mol, but the $\Delta\bar{\mu}H^+$ varied with the reaction conditions such that apparent values of the $\rightarrow H^+$/ATP quotient ranging from 2.6 to 4.1 g-ion H^+/mol ATP were obtained. It was concluded that the proton current, in this organism, is at least partially localised, and theoretical growth calculations suggest that the true value of the $\rightarrow H^+$/ATP quotient is probably 2 g-ion H^+/mol ATP. On this basis, the ATP/O quotients for respiration from NADH and methanol are likely to be 3 and 1 mol ATP/g-atom O, respectively.

There was no evidence that the low growth yields of methanol-excess cultures could be explained by a reduced efficiency of respiratory chain energy conservation.