ELECTRON TRANSPORT IN METHYLOPHILUS METHYLOTROPHUS

by

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ABSTRACT

Methylophilus methylotrophus is an obligate methylotroph which uses the ribulose monophosphate pathway for the assimilation of its sole carbon and energy source, methanol. Methylophilus methylotrophus was found to contain 2 b-type and 3 c-type cytochromes and 2 alternative cytochrome oxidases, cytochromes a/a_3 and o, by the use of difference spectroscopy. The concentrations of each oxidase were determined by the conditions during growth in continuous culture; during methanol-limited growth cytochrome a/a_3 was present and the concentration of cytochrome o was low, while in methanol-excess conditions of growth cytochrome a/a_3 was absent and the concentration of cytochrome o was increased 10 fold. Cytochromes a/a_3, b, o and some of the cytochromes c were membrane-bound, in addition there was a further quantity of soluble cytochrome c. Redox potentiometry was used to determine the midpoint redox potentials of the membrane-bound cytochromes b, o and c.

3 soluble c-type cytochromes were purified by gel-filtration, ion exchange chromatography and isoelectric focussing; their spectral properties, molecular weights, isoelectric points and midpoint redox potentials have been determined. On the basis of their midpoint redox potentials (which are all above 300mV) they appear identical with the membrane-bound cytochromes c.

Whole bacteria and bacterial membranes were found to oxidize NADH, NADPH and ascorbate/TMPD at good rates; in addition whole bacteria oxidized methanol, formaldehyde and formate. Ascorbate/TMPD was oxidized at a very high rate and it is proposed that this oxidation was probably by way of a direct interaction with cytochrome o. These substrates were used to measure the inhibition of the cytochrome oxidases (cytochromes a/a_3 and o) by cyanide and azide, and the inhibition of the electron transport chain as a whole by antimycin A, 2-heptyl-4-hydroxyquinoline-N-oxide and rotenone.

The results of these investigations are used to propose an electron transport chain for Methylophilus methylotrophus, and to compare it with the electron transport pathways which have been proposed for other methylotrophs.

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CHAPTER 1

INTRODUCTION

1.1 Electron Transport Systems

The function of respiratory chains of bacteria, chloroplasts and mitochondria is to carry out the transfer of reducing equivalents (H or e^-) from reduced substrates to oxygen (or an anaerobic electron acceptor) and to conserve the energy thereby released in a useful form. This enables the organism to carry out the functions necessary to life, in the production of reducing equivalents (NAD(P)H) and energy (ATP) for synthesis of new cellular material, active transport of solutes, motility and maintenance of the cell. For an understanding of the mechanisms involved, a detailed knowledge of the composition of the respiratory chain, the sequence of the components, their physico-chemical properties and their organisation is required.

The most intensively studied of the electron transport systems is that of mitochondria, where the chain can be described as shown in Fig. 1.1, and consists of an arrangement of iron-sulphur proteins, flavoproteins, quinones, cytochromes and a cytochrome oxidase. These same types of redox carriers are to be found in bacteria, but the distribution and properties of the individual components in each system are different, as might be expected because of the different requirements for energy and reducing equivalents demanded by the growth substrate and growth conditions enjoyed by the particular bacterium. In fact, the majority of bacterial electron transport systems are different to the mitochondrial system and show a great variation in their redox components and sensitivity to inhibitors (Jones and Meyer, 1976; Gel'man et al., 1975). The variations include the replacement of one carrier with another, the addition or deletion of a carrier and branched-chain pathways. The latter may branch at the level of any of the carriers and in some cases the branching is extensive (Fig. 1.2). Some of these variations will affect the efficiency of energy conservation in addition to the capacity of the system for electron transport, oxygen affinity and sensitivity to inhibitors (Jones, 1977). In addition to species variation, it is well known that many bacteria alter the composition of their respiratory chain in response to growth conditions, which may include oxygen tension, the age of the culture or growth phase, growth rate, pH, presence of alternate

Fig. 1.1 The mitochondrial respiratory chain

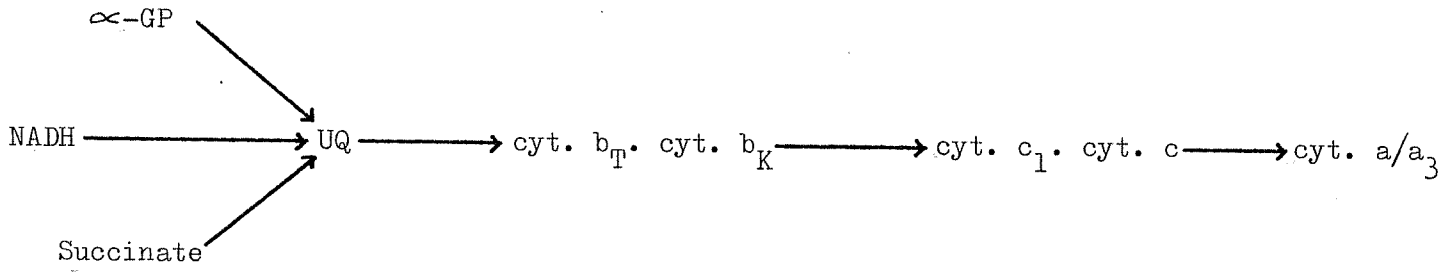
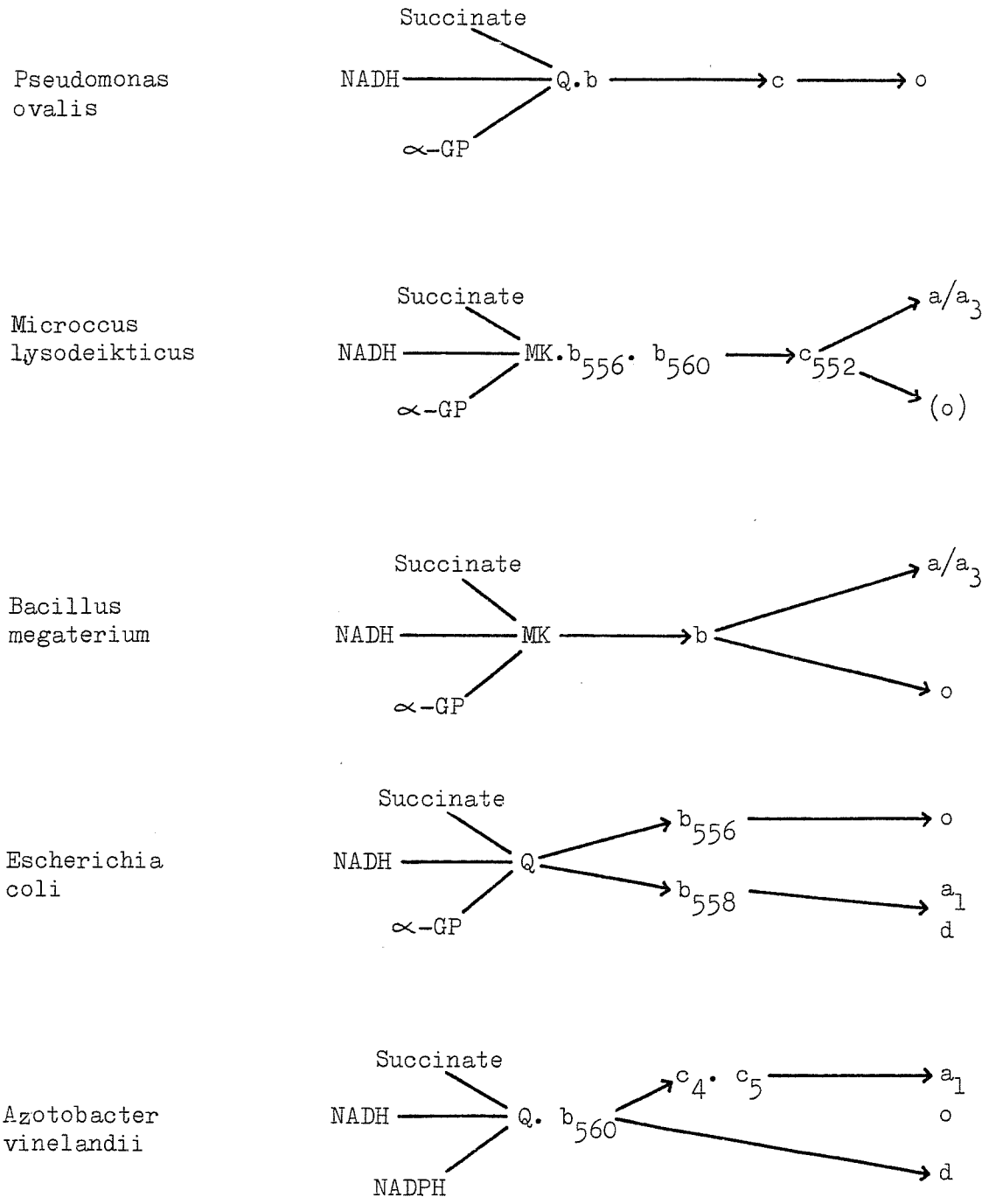


Fig. 1.2 Some examples of bacterial respiratory chains



electron acceptors, the redox potential, the presence of oxidizable substrate, the nitrogen source, iron content of the medium as well as the presence of respiratory chain inhibitors (Jurtshuk et al., 1975). The best known factor which affects the cytochrome content of the respiratory chain is the oxygen tension, but it should be pointed out that it is not sufficient to grow bacteria in conditions of oxygen-limitation and oxygen-excess and then attribute any changes which may occur in the redox carriers to oxygen concentration (as is often done) since the concentration of another substrate must become limiting during the excess-oxygen condition, which may itself be exerting the effect independently of oxygen supply. Therefore at least three different growth conditions must be established to determine the factor which is causing changes in the composition of the respiratory chain.

As stated above, variations in the composition of the respiratory chain may affect the efficiency of energy conservation and consequently growth yield; for example, in Azotobacter vinelandii under conditions where oxygen tension is high, most respiration is by way of cytochrome d (Fig. 1.2) rather than by way of cytochromes a_1 and o with a reduction in the number of sites of phosphorylation and a consequent reduction in yield (Harrison, 1976). A very similar effect is seen in Escherichia coli where a decreased yield is observed under conditions of low oxygen tension following the loss of a site of ATP synthesis (Harrison and Loveless, 1971). It has been suggested that respiratory chains terminating in cytochrome o and a/a_3 will have different efficiencies of oxidative phosphorylation (chains with cytochrome o being less efficient than those with a/a_3), (Stouthamer, 1977; van Verseveld et al., 1977). Furthermore, a survey of nine aerobic bacteria indicated that the presence of a high-potential membrane-bound cytochrome c was a prerequisite for the presence of proton translocating segment 3 and that molar growth yields of carbon-limited continuous cultures indicated that the presence of the same cytochrome was essential for energy coupling at site 3 (Jones et al., 1977).

1.2 Constraints on the Electron Transport Chains of Methylotrophs

There are 4 major pathways for the assimilation of substrate carbon into cell material in methylotrophic organisms and these each have different requirements for reducing power (in the form of NAD(P)H) and energy (in the form of ATP). It is therefore of interest to see what effect these different demands have on the composition of the electron

transport chain. The pathways of carbon assimilation in methylotrophs were first elucidated by Quayle and his colleagues (Quayle, 1972; Anthony, 1975b; Cox and Quayle, 1975; Quayle and Ferenci, 1978; van Dijken et al., 1978; O'Connor and Quayle, 1979). The pathways are shown in Fig. 1.3 and summarized in Fig. 1.4; with the different variants of the pathway there are at least 6 different routes.

The least efficient pathway is the 'autotrophic' route of the ribulose biphosphate pathway which occurs in Paracoccus denitrificans in which the carbon is fixed at the oxidation level of carbon dioxide, the acceptor molecule being ribulose biphosphate and the product being 2 molecules of 2-phosphoglycerate.

The serine pathway is the assimilation pathway found in the majority of facultative methylotrophs (able to grow on multi-carbon compounds in addition to C_1 compounds). In this pathway two thirds of the methanol is assimilated at the level of formaldehyde by addition to glycine, the product being serine; a cycle of reactions regenerates the glycine and produces phosphoglycerate for biosynthesis. This cycle involves one carboxylation reaction for every two molecules of formaldehyde assimilated, and a route for the oxidation of acetyl-CoA to glyoxylate, the immediate precursor of glycine. Bacteria with the icl^+-serine pathway effect this conversion by way of isocitrate lyase whereas the enzymes for this oxidation are not known in bacteria such as Pseudomonas AM1 which are said to have the icl^--pathway. Many of these bacteria have a complete TCA cycle; they are thus able to grow on a wide range of multi-carbon compounds and they also produce poly β - hydroxybutyrate as a source of carbon and reducing equivalents.

The ribulose monophosphate pathway also involves the assimilation of methanol at the level of formaldehyde, the acceptor molecule being ribulose monophosphate and the product being a 3-hexulose 6-phosphate. Three molecules of fructose phosphate derived from this product are re-arranged to give a C_3 compound and three molecules of 'regenerated' ribulose monophosphate. There are at least two different re-arrangement sequences; the fructose bisphosphate variant yields triose phosphate as the product and the Entner-Douderoff variant yields pyruvate. Methanol-utilizing bacteria with the RMP pathway usually have an incomplete TCA cycle and are unable to use multi-carbon compounds; they are thus obligate methylotrophs.

Fig. 1.3 Assimilation of substrate carbon into cell material by methylotrophus

(a) The ribulose biphosphate pathway

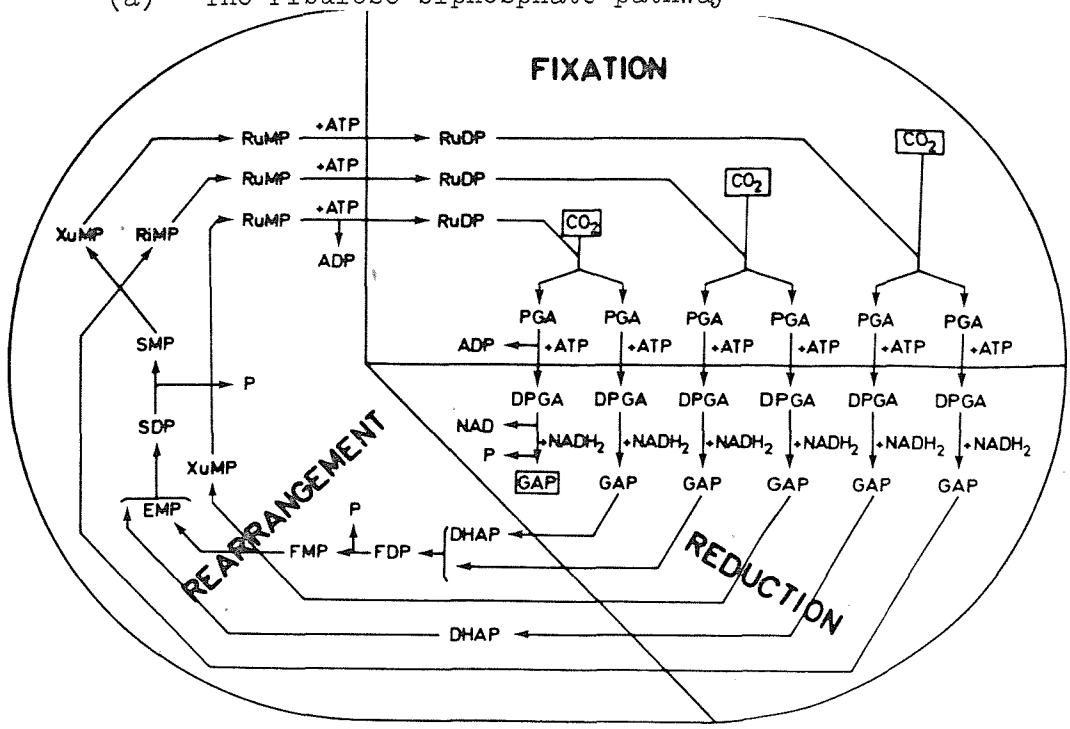


FIG. 1. *RuDP cycle (I). (The cycle involves sedoheptulose diphosphatase but not transaldolase.) Abbreviations throughout figures: DPGA, 1,3-diphosphoglycerate; EMP, erythrose-4-phosphate; SMP, sedoheptulose-7-phosphate; GMP, glucose-6-phosphate; XuMP, xylulose-5-phosphate; NuMP, nucleoside monophosphate; 6-PG, 6-phosphogluconate; Py, pyruvate.*

(b) The serine pathway

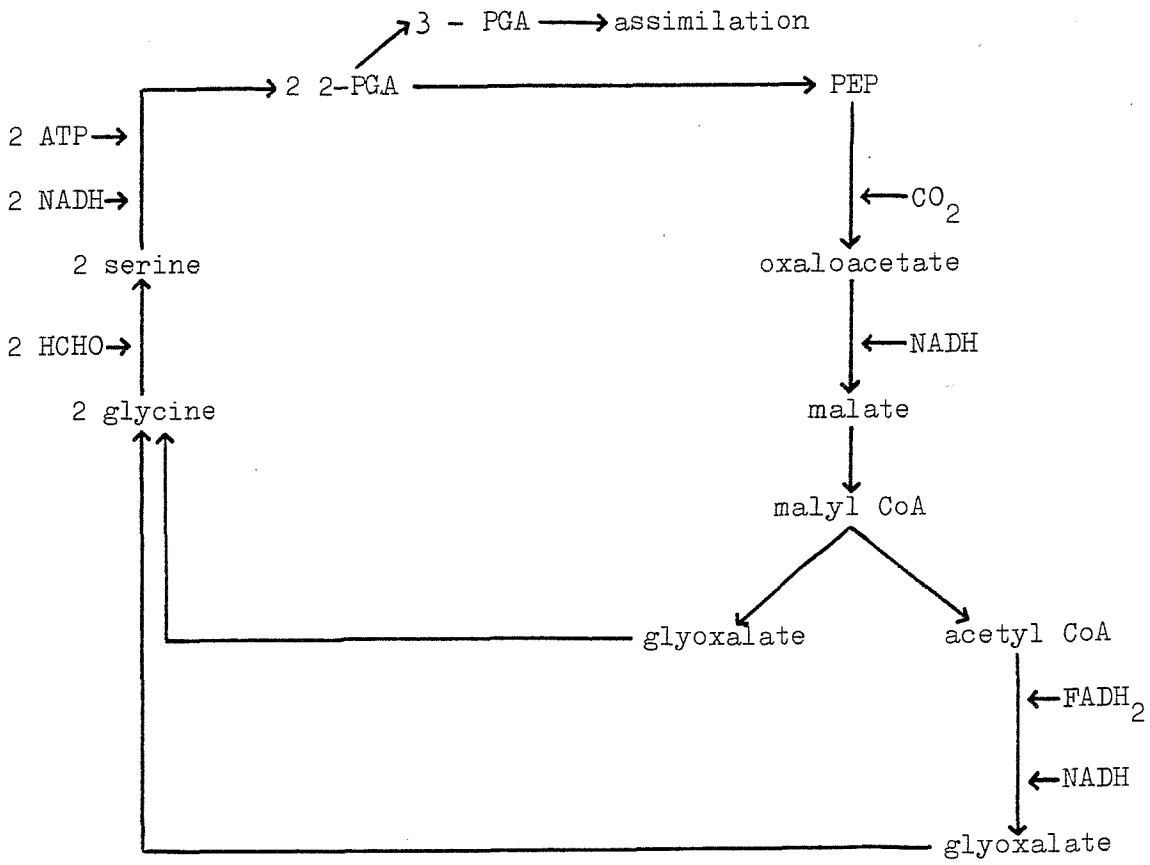


Fig. 1.3 (Cont'd.)

(c) The ribulose monophosphate pathway

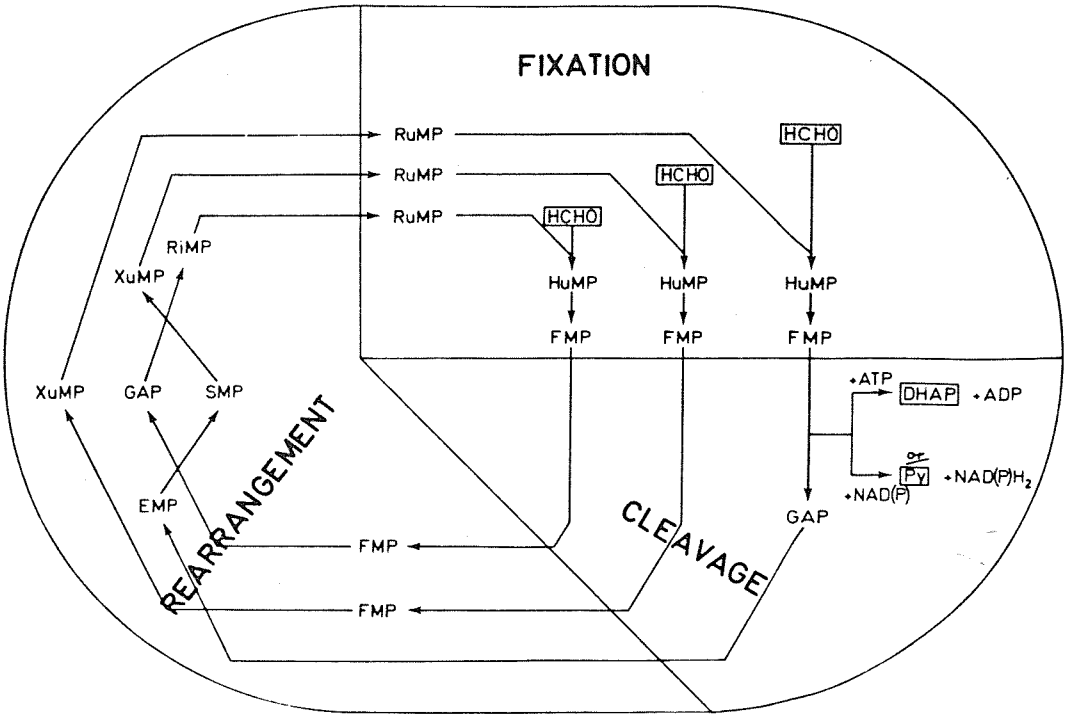


FIG. 4. *RuMP cycle (II). (This rearrangement variant involves transaldolase but not sedoheptulose diphosphatase. Two alternative modes of cleavage are shown involving FDP aldolase and KDPG aldolase, respectively; see Fig. 5.)*

(d) The dihydroxyacetone pathway

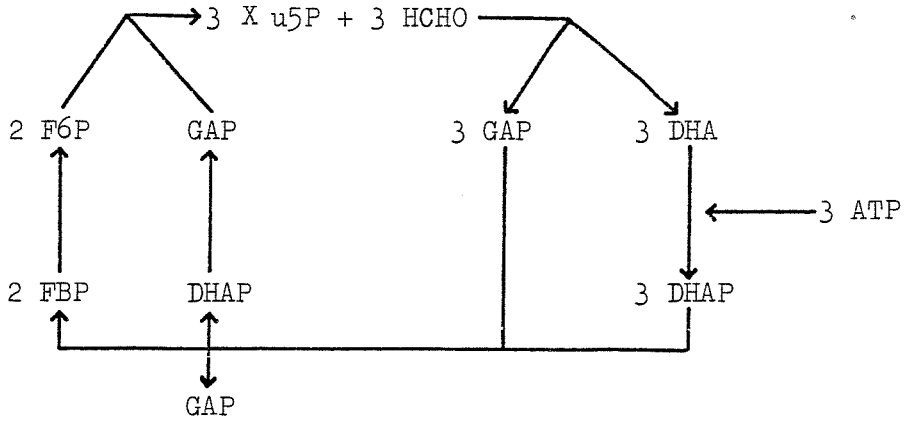


Fig. 1.4 Summary of assimilation pathways.

Ribulose biphosphate pathway	$3CO_2 + 8ATP + 5NADH \longrightarrow PGA$
Serine pathway	$2HCHO + CO_2 + 3ATP + 2NADH \longrightarrow PGA + FPH_2$
Ribulose monophosphate pathway :	
ED variant	$3HCHO + 2ATP \longrightarrow PGA + NADH$
FBP variant	$3HCHO \longrightarrow PGA + NADH$
Dihydroxyacetone pathway	$3HCHO + 2ATP \longrightarrow PGA + NADH$

The final pathway, the dihydroxyacetone pathway also involves assimilation at the level of formaldehyde, the acceptor probably being xylulose 5-phosphate and the products being dihydroxyacetone and phosphoglyceraldehyde. One of the six triose molecules formed this way is assimilated while the others are rearranged to regenerate 3 more xylulose 5-phosphate molecules. This pathway has been proposed as the pathway for the assimilation of methanol in yeasts (van Dijken et al., 1978; O'Connor and Quayle, 1979).

In addition to the differences in assimilation pathways there are also differences in the oxidation pathways of methylotrophs which also give rise to different amounts of energy and reducing equivalents. In bacteria methanol is oxidized to formaldehyde by an unusual dehydrogenase that is neither flavoprotein nor NAD-linked; its prosthetic group, thought for many years to be a pteridine (Anthony and Zatman, 1967) may be a completely novel compound (Westerling et al., 1979; Salisbury et al., 1979). Because all carbon metabolism is by way of this enzyme its nature has far reaching effects. In particular, the reductant produced during the first oxidation step of methanol cannot be used for biosynthesis, but only for ATP synthesis by oxidative phosphorylation, since it is not NAD or FAD-linked.

In yeasts methanol is oxidized to formaldehyde by a flavoprotein oxidase yielding hydrogen peroxide that is decomposed by catalase (Tani et al., 1978), thus neither usable reductant nor energy is available from this first oxidation step in yeasts and hence lower yields are produced by yeasts than by bacteria.

There are two know routes for the further oxidation of formaldehyde to carbon dioxide in bacteria. The first is a direct route by way of formate which gives either two molecules of NADH, or one molecule of NADH from formate dehydrogenase plus a reduced flavoprotein; this is similar to the route in yeasts. The alternative cyclic route giving two molecules of NADH can occur in bacteria with the ribulose monophosphate pathway, many of the enzymes in the oxidative cycle are shared by the assimilation pathway.

In consequence of the above, the growth yields of some methylotrophs are determined partly or exclusively by the supply of NAD(P)H and not predominantly by the ATP supply as is the case in most heterotrophs (Anthony, 1978). The growth yield of bacteria growing on a

single source of carbon plus energy is mainly determined by the efficiency of assimilation of the carbon precursors for biosynthesis and by the supply of the necessary ATP and NAD(P)H required for this assimilation. The conversion of most growth substrates to precursors for biosynthesis (e.g. PGA) requires very little (if any) NAD(P)H. Very high proportions of most growth substrates are converted to cell carbon or oxidized to CO_2 for the production of ATP, and a very low proportion is oxidized to CO_2 for the production of NAD(P)H for biosynthesis. The molar growth yields of most typical heterotrophs are thus predominantly determined by the ATP supply which, in turn, is governed by the P/O ratio. When, by contrast, there is a high NAD(P)H requirement for assimilation of growth substrate into cell material, then growth will be either exclusively NAD(P)H limited or it will be determined by both NAD(P)H and ATP supply; this high NAD(P)H requirement occurs in autotrophs and most methylotrophs, but in very few heterotrophs.

If the oxidation of growth substrate produces reducing equivalents predominantly in the form of NAD(P)H then the high requirement for reducing power may be expressed as a carbon or ATP requirement in equations for the prediction of cell yields, and the usual relationship between P/O ratio, Y_{O_2} and Y_{ATP} values will be valid. A measure of the NAD(P)H requirement for biosynthesis is given by the ratio of substrate oxidized exclusively for NAD(P)H production to the amount of substrate assimilated into cell carbon (Fig. 1.5 a/b). This has been estimated by Anthony (1978). For methane utilizing organisms the ratio is always high if they require NAD(P)H for the initial methane to methanol hydroxylation, as it appears they do (Stirling and Dalton, 1979), but otherwise the ratio is always low for bacteria using the RMP pathway (such as Methylophilus methylotrophus). The ratio for bacteria using the serine pathway or the RBP pathway depends on the yield of NAD(P)H produced during the oxidation of formaldehyde to CO_2. When this yield is 2, the ratio is fairly low but when the yield is only 1 then the ratio is high. In contrast with these high values for many methylotrophs, the ratios for most typical heterotrophic growth substrates are very low.

In most methylotrophs a second condition for NAD(P)H limitation applies and this may result in such an extensive NAD(P)H limitation that ATP supply becomes relatively unimportant in determining growth yields. This condition is that oxidation of growth substrate to supply

Fig. 1.5 Metabolism of carbon substrate for provision of cell material, NADH and ATP

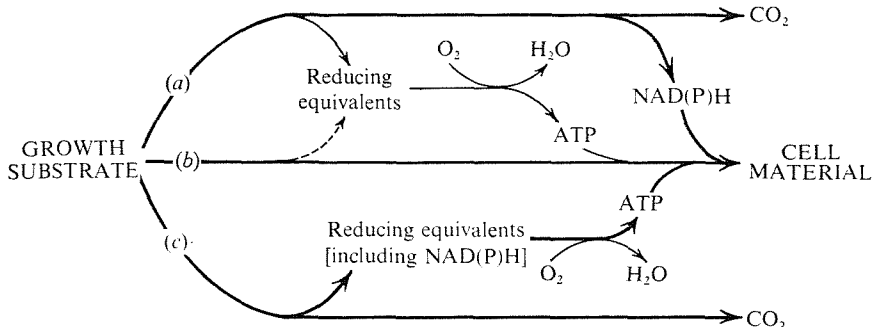


Fig. 1. The relationship between NAD(P)H production, oxygen consumption, ATP synthesis and production of cell material illustrating the conditions for NAD(P)H limitation of growth yield. The growth substrate (carbon source) is metabolized to produce: (*a*) NAD(P)H; (*b*) cell material; (*c*) ATP. The first condition for NAD(P)H limitation is that there should be a high NAD(P)H requirement for assimilation of growth substrate into cell material; a measure of this requirement is given by a/b. The second condition is that oxidation of substrate to supply carbon precursors (*b*) and NAD(P)H (*a*) for biosynthesis should also produce further additional reducing equivalents [other than NAD(P)H], whose oxidation by molecular oxygen may also be coupled to ATP generation. An indication of the overall extent of NAD(P)H limitation is given by $(a+b)/c$. In bacteria which are exclusively NAD(P)H-limited, oxidation of growth substrate exclusively for ATP production (*c*) is unnecessary.

carbon precursors and NAD(P)H for biosynthesis should also produce further additional reducing equivalents (other than NAD(P)H, whose oxidation via the electron transport chain to oxygen, may also be coupled to the production of ATP (summarized in Fig. 1.5). This ATP is not expressed in equations for the assimilation of the substrate in terms of extra substrate molecules because these molecules are already included as the source of NAD(P)H which is essential to give cell material at the appropriate level of reduction. If the ATP gained from the oxidation of these (non-NAD(P)H) reducing equivalents is sufficient for growth then the growth yield will be exclusively NAD(P)H-limited (rather than ATP-limited) and the direct relationships between P/O ratio, Y_{O_2}, Y_S and Y_{ATP} values will not apply. Should further ATP be required then the P/O ratio will determine how much more substrate will have to be oxidized to provide this. If this further ATP requirement is relatively small then the P/O ratio will have little effect on the growth yield and the bacteria will still be predominantly NAD(P)H-limited. If the ATP requirement for assimilation is very high then the supply of ATP, and hence the P/O ratio, will be relatively more important in determining growth yields. The enzymic basis for this unusual situation leading to the second condition for NAD(P)H-limitation in methylotrophs is that the final oxidation step, catalysed by formate dehydrogenase, is often the only source of NAD(P)H and the dehydrogenases for methanol (or methylamine) do not give rise to NAD(P)H. In typical heterotrophs, by contrast, the only alternative source of reducing power is usually reduced flavoprotein and the amount of this is small compared to the amount of NAD(P)H produced during the oxidation of most substrates.

The result of the above is that bacteria having the autotrophic RBP-pathway (such as Paracoccus denitrificans) and yeasts having the DHA pathway are the only methylotrophs whose growth yields are limited predominantly by ATP supply; serine pathway bacteria are predominantly NADH-limited and RMP pathway bacteria (such as Methylophilus methylotrophus) are usually limited by both ATP and carbon supply. (In the case of limitation by carbon supply, ATP and NADH are potentially in excess and the only way to increase yields would be by the provision of an additional more oxidized carbon source). In view of the constraints imposed by the substrate, assimilation and oxidation pathways of methylotrophs, it is of interest to compare their electron transport pathways, both between individual organisms and with non-methylotrophic organisms.

1.3 Electron Transport in Methylotrophs

Bacteria growing on methanol all have cytochromes of the b and c types and apparently typical oxidases, cytochromes a/a_3 or o (Tonge et al., 1974; Anthony, 1975b; Widdowson and Anthony, 1975; Cross and Anthony, 1978; Bamforth and Quayle, 1978; van Verseveld and Stouthamer, 1978; Keevil and Anthony, 1979a, 1979b; Netrusov and Anthony, 1979; and this thesis). Fig. 1.6 illustrates some of the electron transport chains which have been proposed for some methylotrophs, it is not yet known if there is a relationship between the types of electron transport system and the assimilation pathway. An important feature of methylotrophic respiratory chains is that methanol dehydrogenase is coupled to the electron transport chain at the level of cytochrome c (although not necessarily directly) and not at the level of cytochrome b (Widdowson and Anthony, 1975; Tonge et al., 1975; Higgins et al., 1976; Netrusov et al., 1977; Netrusov and Anthony, 1979). Drabikowska (1977) has suggested that in Methylomonas Pl 1 methanol dehydrogenase donates electrons to the electron transport chain at the level of cytochrome b, this conclusion was based on the data reproduced in Fig. 1.7. This data was obtained by following spectrophotometric changes in intact bacteria on the addition of substrates. The changes which occur are attributed to the reduction of cytochromes b and c (Fig. 1.7) although the traces shown could also be interpreted as mainly due to the reduction of cytochrome c. In trace (a) the initial absorption change following the addition of methanol may be due to the reduction of cytochrome c and not b (since cytochrome c will have a considerable contribution to the absorbance at this wavelength); on the addition of cyanide durther cytochrome c will be reduced, along with some cytochrome b due to endogenous reductant and further oxidation of formaldehyde; cytochrome b will also be reduced by equilibration of the redox components, cytochromes b and c, and the added substrate (E_{m7} of the methanol/formaldehyde couple is -182mV). It can also be seen in the difference spectra that the addition of methanol causes relatively little change in the extent of cytochrome b reduction while causing a large change in the reduction of cytochrome c. Drabikowska's conclusions are thus probably erroneous, there being no sound evidence that cytochrome b is ever involved in the first step of methanol oxidation.

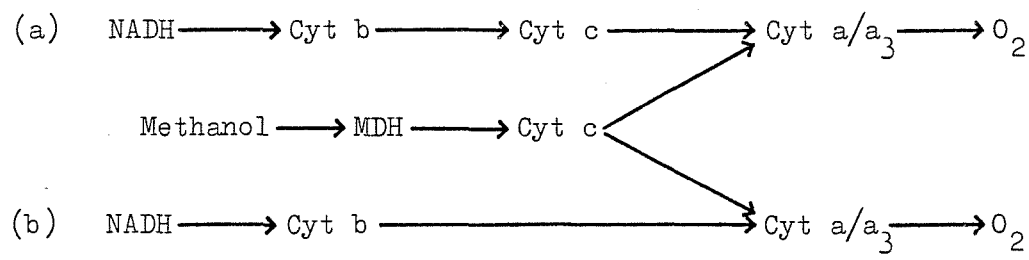
In the facultative methylotroph Pseudomonas AM1 this first step in methanol oxidation is coupled to the production of at least one

Fig. 1.6 Electron transport in methylotrophs

Pseudomonas AM1 (Keevil and Anthony, 1979b)

(a) Carbon-limited growth conditions.

(b) Carbon-excess growth conditions.



Paracoccus denitrificans (van Verseveld and Stouthamer, 1978)

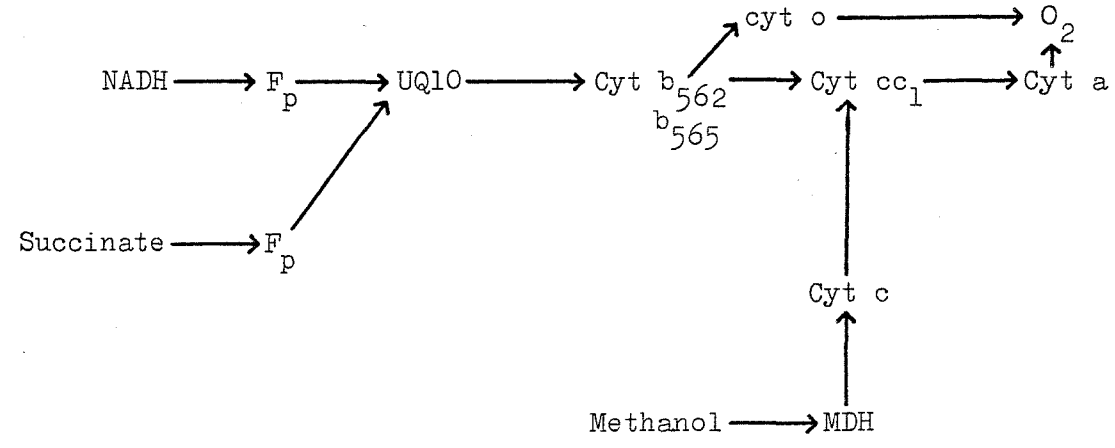


Fig. 1.6 (Cont'd.)

Methylosinus trichosporium (Higgins et al., 1977)

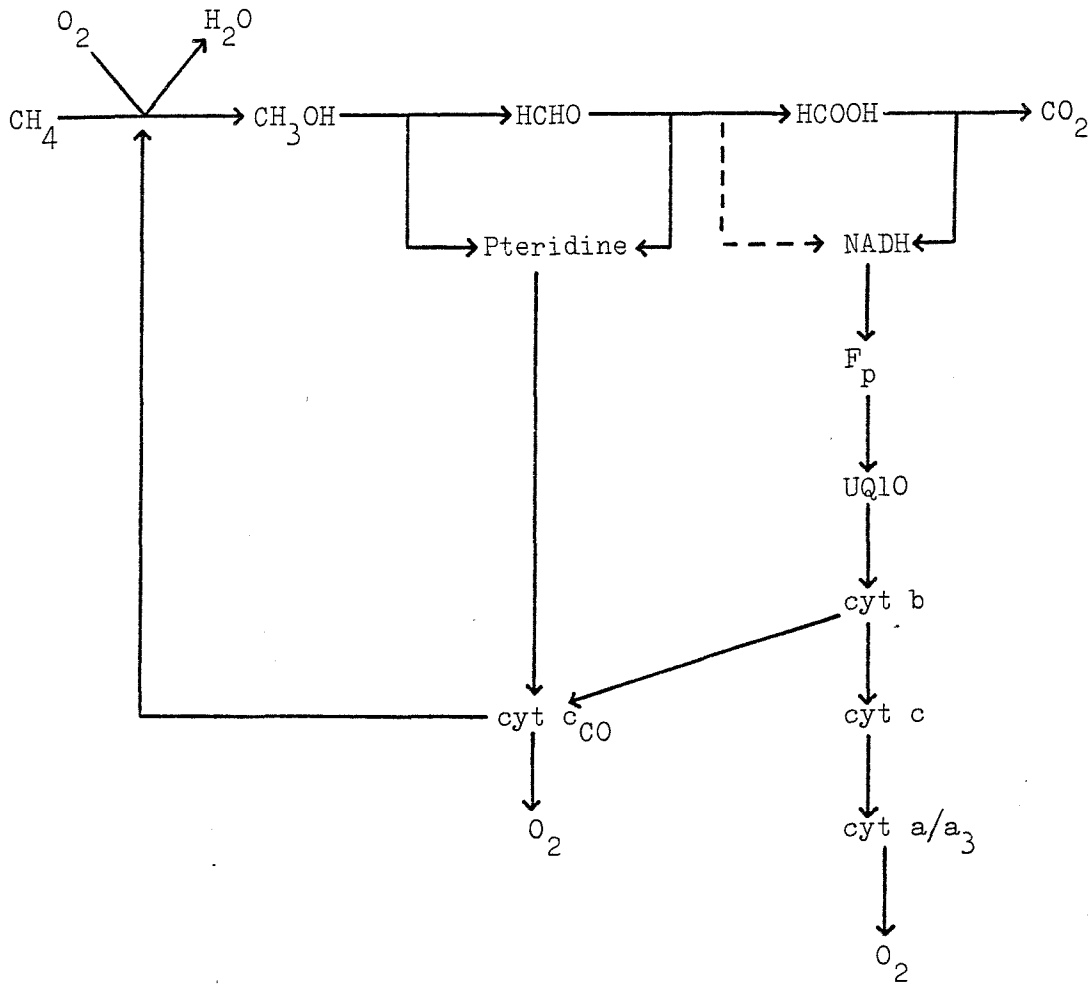


Fig. 1.7 Figures reproduced from Drabikowska (1977) "The Respiratory Chain of a Newly Isolated Methylomonas Pl 1"

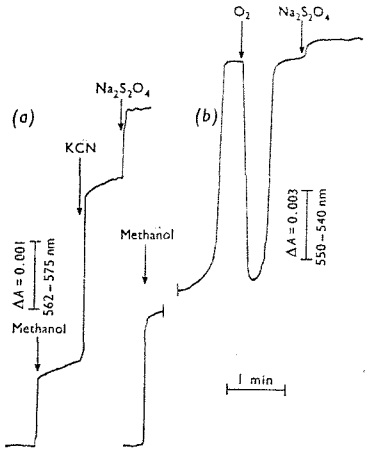


Fig. 7. *Redox changes of cytochromes b and c in the whole cells*

Cells were suspended in 10 mM-Hepes, pH 7.0. Where indicated, 5 mM-methanol and 1 mM-KCN were added to the aerobic cell suspension. (*a*) Redox changes of cytochrome *b* measured at $A_{562}-A_{575}$ (0.7 mg of protein/ml); (*b*) redox changes of cytochrome *c* at $A_{550}-A_{540}$ (0.43 mg of protein/ml). The complete reduction was achieved by the addition of a few crystals of dithionite. Optical path-length 1 cm.

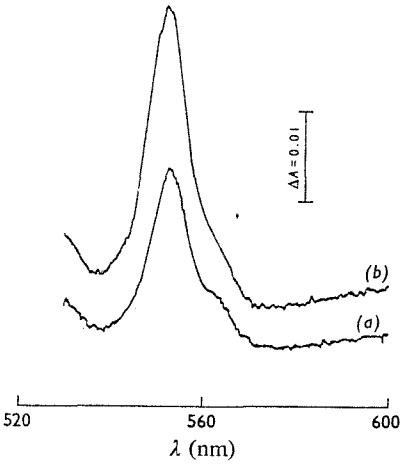


Fig. 8. *Room-temperature difference spectrum of the whole cells after thorough aeration* (10*h*)

Cell samples (0.9 mg of protein/ml) were suspended in 10 mM-Hepes buffer, pH 7.0. (*a*) Test sample was reduced by endogenous substrates after addition of 1 mM-KCN; (*b*) 5 min after the addition of KCN (1 mM), 5 mM-methanol was added.

molecule of ATP (Netrusov and Anthony, 1979). Proton translocation measurements in this organism initially indicated that cytochrome c may not be involved in the oxidation of other substrates such as NADH, thus perhaps limiting the P/O ratio to 2, but recent work has indicated that whether or not cytochrome c is involved in NADH oxidation may be determined by the growth conditions. It appears to be involved in proton translocation and ATP synthesis from NADH during carbon-limited growth but not during carbon excess growth (Keevil and Anthony, 1979b). It should be noted that the amount of NADH oxidation occurring during the growth of these serine pathway bacteria is small compared with that occurring during growth of RMP pathway bacteria such as Methylophilus methylotrophus (Anthony, 1978a).

The interpretation of cytochrome complements in methylotrophic bacteria is confused by the presence of more than one type of soluble cytochrome c which seem to be present in large quantities in methylotrophs and may bind carbon monoxide (O'Keeffe and Anthony, 1979; Cross and Anthony, 1978; van Verseveld and Stouthamer, 1978; Drabikovska, 1977; and this thesis) and some of these are described in the next section.

1.4 Cytochromes c in Methylotrophs

The properties of many cytochromes c from bacterial sources have been studied - including some cytochromes c from methylotrophs; Pseudomonas AM1 (Anthony, 1975b; O'Keeffe and Anthony, 1979); Methylosinus trichosporium OB3B (Tonge et al., 1977); Pseudomonas extorquens (Higgins et al., 1976b); and Paracoccus denitrificans (Scholes et al., 1971). Pseudomonas AM1 (a facultative serine pathway organism) contains two soluble cytochromes c; one of high molecular weight (20,000), a midpoint oxidation reduction potential (Em_7, 250mV) and α- absorbance maximum at 549nm in the reduced form; the other cytochrome was of lower molecular weight (10,000), a midpoint redox potential (Em_7, 285mV) and α- absorbance maximum at 550.5nm in the reduced form, both cytochromes bound carbon monoxide. The properties of these and other methylotrophic cytochromes c are given in Table 1.1. A mutant of Pseudomonas AM1 was obtained (Anthony, 1975b) which lacked cytochrome c. This mutant had the same growth properties as the wild type organism except it could not grow on methanol, ethanol or methylamine. This observation gave rise to the postulate that cytochrome c

Table 1.1 - Properties of some bacterial cytochromes c

Organism	Properties of soluble cytochrome(s) c				
	α abs max	MW	E_{m7}	% CO bound	Ref.
(a) Methylotrophs					
(i) Facultative methylotrophs					
Pseudomonas AM1	549	20,900	254	20%	1
	550.5	11,000	294	33%	
Hyphomicrobium X	551	nk	nk	61%	2,9
Pseudomonas extorquens	551	13,000	295	60%	3,9
Paracoccus denitrificans*	550	14,100	250	nk	4
(ii) Obligate methylotrophs					
Methylomonas methicana	550	18,000	nk	+	5
Methylomonas albus**	553	nk	nk	+	6
Methylomonas Pl 1	552.5	nk	nk	nk	7
	549	nk	nk	nk	
Methylosinus trichosporium	551	12,500	310	70%	8
Methylophilus methylotrophus	551.25	8,500	373	7%	10
	550.75	16,800	336	24%	
	549.75	21,000	310	60%	
(b) Non-methylotrophs					
Mammallian c	550	12,000	255	-	11
Rhodospirillum rubrum c_2	550	12,500	320	-	11
cc'	550	29,800	-8	+	11
Rhodospirillum molischianum	550	13,400	288	nk	11
c_2	550	10,200	381	nk	11
Pseudomonas aeruginosa	551	8,100	286	nk	11
Azotobacter vinelandii c_4	551	25,000	300	-	
c_5	555	25,000	320	10%	11
Beneckea natriegens	552	nk	380	80%	12

/Cont'd...

Table 1.1 (Cont'd.)

References :

1. O'Keeffe and Anthony, 1979
2. Large et al., 1979
3. Higgins et al., 1976b
4. John and Whatley, 1977
5. Patel et al., 1979
6. Davey and Mitton, 1973
7. Drabikowska, 1977
8. Tonge et al., 1977
9. Tonge et al., 1974
10. This thesis
11. Dickerson and Timkovitch, 1975
12. Weston and Knowles, 1973

* An additional, carbon monoxide binding, cytochrome c is induced during growth on methanol.

** The carbon monoxide binding cytochrome was described as cytochrome o by these workers but it has been suggested that it is a c type cytochrome (Tonge et al., 1974).

was only involved in the oxidation of methanol. Results from Methylosinus trichosporium (Tonge et al., 1975) and Pseudomonas extorquens (Higgins et al., 1976b) support the view that cytochrome c is essential for the oxidation of methanol. As described in the previous section O'Keeffe and Anthony (1978) proposed a scheme whereby cytochrome c was only essential for respiration and respiration-driven proton translocation from methanol and there was no conclusive evidence that cytochrome c mediated between cytochromes b and a/a_3 under the conditions in which they grew the organism. Later work suggests, however, that cytochrome c is involved in NADH oxidation in carbon limited conditions (Keevil and Anthony, 1979b). It may be that where more than one type of cytochrome c is present in these organisms, one may be involved in methanol oxidation while the other may only be involved in the oxidation of NADH.

Methylotrophic bacteria have several points of similarity with the photosynthetic bacteria;. the ribulose phosphate pathways for carbon assimilation, the possession of carotenoid pigments by many methylotrophs, induction of bacteriochlorophyll by light in Pseudomonas AM1 and Protaminobacter ruber (Sato, 1978). Furthermore Sahm et al., (1976) have recently reported the first methanol-utilizing photosynthetic bacterium; Rhodopseudomonas acidophila. For this reason the properties of the cytochromes c from some photosynthetic organisms have been included in Table 1.1.

A common feature of the cytochromes c of methylotrophs is the ability to combine with carbon monoxide. Cytochromes c able to react with carbon monoxide are not uncommon however (Lemberg and Barrett, 1973), and even when these cytochromes are autoxidizable there are very few known to function by the activation of oxygen, i.e. as an oxygenase or terminal oxidase. The formation of a carbon monoxide complex is not sufficient in itself to demonstrate that cytochromes ability to function as an oxidase without also demonstrating the relief of respiratory inhibition by light corresponding to the wavelength of the absorption of the cytochrome-CO complex. This has not been shown for any cytochrome c from methylotrophs, although it has been shown for the cytochrome c_{CO} of the marine bacterium Beneckea natriegens (Weston and Knowles, 1974). The soluble ferrocytochrome c found in methylotrophs combines slowly with CO; it is not rapidly oxidized by molecular oxygen, and is present

in bacteria grown on substrates other than C_1 compounds and in bacteria grown with nitrate in the absence of oxygen. These observations do not support the speculation (Tonge et al., 1974; Ferenci, 1974) that the soluble cytochrome c of Pseudomonas AM1 and of other methylotrophs has an oxidase function in methanol oxidation. Reduced cytochrome c_{CO} has also been suggested as the electron donor in the methane mono-oxygenase system of Methylosinus trichosporium OB3B (Tonge et al., 1975; 1977) but this is contrary to the findings of Colby and Dalton (1978) and Stirling and Dalton (1979) in Methylosinus capsulatus and Methylosinus trichosporium OB3B. These workers showed that cytochrome c_{CO} was not required, and that neither methanol nor ascorbate would act as donor, NAD(P)H being the only active electron donor. The electron transport system proposed for Methylosinus trichosporium shown in Fig. 1.6 should be reconsidered in the light of these observations.

1.5 Conclusions and Aims

Methylophilus methylotrophus (Pseudomonas methylotropha; Pseudomonas AS1) is an obligate methylotroph which uses the ribulose monophosphate pathway (Strom et al., 1974) for the assimilation of its sole carbon and energy source, methanol. The aim of this work is an exploration of the relationship between electron transport, respiration-driven proton translocation, ATP synthesis and growth yields in methylotrophs and how these characteristics may be affected by alterations in the growth conditions in continuous culture; this in turn has general relevance to oxidative energy transduction in bacteria and mitochondria.

Previous work on methylotrophs in this field has concentrated on either methane-utilizing organisms, where the evaluation of energetics is more complex due to the initial methane to methanol oxidation step, or on facultative methylotrophs particularly Pseudomonas AM1 which is a serine pathway organism, and the less typical methylotroph Paracoccus denitrificans, a ribulous biphosphate pathway organism. Methylophilus methylotrophus differs from these previously studied organisms in being an obligate methylotroph and a ribulose monophosphate pathway bacterium. It is therefore of interest to compare the electron transport of Methylophilus methylotrophus with other methylotrophs, in relation to their ATP and NAD(P)H requirement, which will relate to their respective yields, since it has already been shown that growth yields for bacteria

growing on methanol are not an accurate guide to the number of sites of ATP synthesis (Anthony, 1978). This work is also of relevance to industry as methylotrophic bacteria (growing on methane and methanol) are being widely considered (and used) as a source of single cell protein and cell products (enzymes and storage compounds) and extracellular products (amino acids and polysaccharides); in the former case high cell yields are required but in the latter low cell yields are required; for this reason it is economically important to know the factors which affect growth yields with respect to carbon and oxygen consumption. Methylophilus methylotrophus was chosen by Imperial Chemical Industries Ltd., for the production of single cell protein in view of its high efficiency of methanol assimilation, high protein content, high growth rate, high nutritional value and absence of toxicity. The ICI process involves the continuous culture of Methylophilus methylotrophus in a 4,000 litre pressure cycle fermenter under methanol limitation at a dilution rate of 0.2 hr at 30g dry wt/l, at 40°C. A commercial plant of 150,000 litre volume is due to be commissioned in 1980.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

All chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, except those listed below :

Aldrich Chemical Co. Ltd., Gillingham, Dorset.

- Anthraquinone 2-sulphonic acid, sodium salt
- Methyl viologen
- 2,3,5,6-Tetramethyl-p-phenylenediamine
- Trimethylhydroquinone

Hopkins and Williams, Chadwell Heath, Essex.

- Antifoam A emulsion
- Repelcote

Koch-Light Laboratories Ltd., Colnbrook, Bucks.

- Acrylamide
- 2-Hydroxy 1,4 napthoquinone

L.K.B. Instruments Ltd., South Croydon, Surrey.

- Carrier Ampholytes pH 3.5 - 5.5
- Carrier Ampholytes pH 4.0 - 6.0

Oxoid Ltd., London.

- Ionagar No. 2

Pharmacia Fine Chemicals, Hounslow, Middlesex.

- Sephadex G-25
- Sephadex G-50
- Sephadex G-75
- Sephadex G-150

Sigma London Chemical Company Ltd., Kingston-upon-Thames, Surrey.

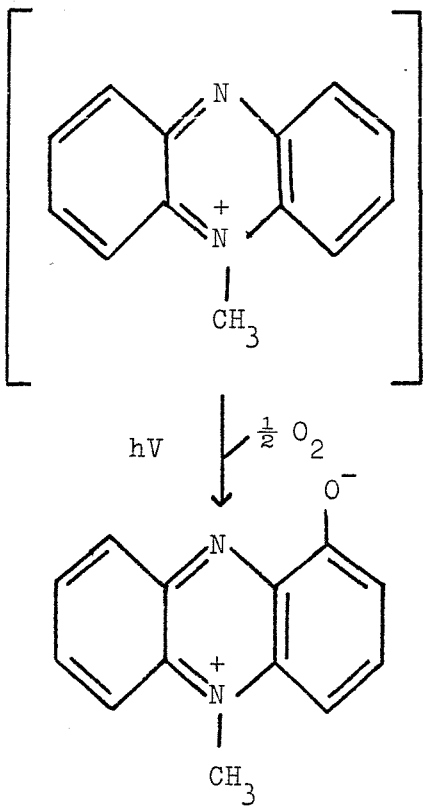
- Antimycin A
- 2(N-Cyclohexylamino)ethane sulphonic acid (CHES)

Cyclohexylaminopropane sulphonic acid (CAPS)
Hydroquinone
N-2-Hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES)
2-Heptyl-4-hydroxyquinoline-N-oxide (HQNO)
Menadione
Morpholinopropane sulphonic acid (MOPS)
1,2 Napthoquinone-4-sulphonic acid, sodium salt
Nicotinamide adenine dinucleotide, reduced form (NADH)
Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)
Phenazine methosulphate (PMS)
Phenazine ethosulphate (PES)
Proflavin hemisulphate
Rotenone
N,N,N',N'-Tetramethyl-p-phenylenediamine (TMPD)
Tris(hydroxymethyl)aminomethane (Tris)

Whatman Ltd., Maidstone, Kent.

Carboxymethyl cellulose CM52
Diethlaminoethyl cellulose DE52

Pyocyanine was synthesized by the photo-oxidation method of McIlwain (1937) as follows; 2.0g of PMS was dissolved in 200ml distilled water and the pH adjusted to pH 8.0 with NaOH. The solution was exposed to light from a 60W lamp overnight during which time the colour of the solution changed from yellow to purple and the pH decreased. The pH was adjusted back to pH 8.0 and the colour changed from purple to blue (characteristic of pyocyanine). The pyocyanine was extracted into chloroform and re-extracted into 0.1M HCl, the pH was adjusted to alkaline again, (as judged by the colour change), followed by re-extraction into chloroform. This process was repeated 3 times. The final chloroform extraction was dried on a rotary evaporator and the product weighed. It was then dissolved in 0.1M HCl to a concentration of 10mM and stored at $-20^{\circ}C$ as the acid salt.



Phenazine methosulphate

$CH_3\ SO_4^-$

Pyocyanine

2.2 Bacterial Strain

Methylophilus methylotrophus (NCIB 10515) was a gift from Dr. I. Taylor (I.C.I. Ltd., Agricultural Division, Billingham, Teeside).

2.3 Media and Maintenance of Cultures

The following stock solutions were used for batch and continuous culture media :-

(a) Trace elements solution : 2 ml per litre of batch medium, 10 m. per litre of continuous culture medium.

Stock concentration		Final concentration	
		Batch	Continuous
$CaCl_2.2H_2O$	1.06 g/l	2.12 mg/l	10.6 mg/l
$MnSO_4.5H_2O$	40 mg/l	0.08 mg/l	0.4 mg/l
$CuSO_4.5H_2O$	8 mg/l	0.016 mg/l	0.08 mg/l
$ZnSO_4.7H_2O$	40 mg/l	0.08 mg/l	0.4 mg/l
$FeSO_4.7H_2O$	800 mg/l	1.6 mg/l	8.0 mg/l
$NaMoO_4.2H_2O$	8 mg/l	0.016 mg/l	0.08 mg/l
$CoCl_2.6H_2O$	8 mg/l	0.016 mg/l	0.08 mg/l
H_3BO_4	6 mg/l	0.012 mg/l	0.06 mg/l

0.3 ml 98% H_2SO_4 per litre was added to prevent precipitation.

(b) 36% $(NH_4)_2SO_4$: 5 ml per litre of batch medium (final concentration 1.8 g/l). 10 ml per litre continuous culture medium final concentration 3.6 g/l).

(c) 40% $MgSO_4.7H_2O$: 0.5 ml per litre batch medium (final concentration 0.2 g/l). 1 ml per litre continuous culture medium (final concentration 0.4 g/l).

(d) 1.046M K/Na phosphate pH 6.8 : (K_2HPO_4 5 g/l, NaH_2PO_4 78 g/l). 20 ml/l medium 21mM) batch cultures only. Continuous culture medium used 0.75 ml/l of 13.3M orthophosphoric acid to give a final phosphate concentration of 10mM. The pH of the medium was adjusted in the culture vessel to give a pH of 6.8 by the automatic addition of 0.5M KOH/0.5M NaOH. Solutions were added to most of the water and made up to the correct volume immediately before autoclaving. The pH was also checked at this stage in the case of batch cultures.

(e) Carbon source : Methanol was added as carbon source at a concentration of 1% v/v after autoclaving. Methanol was autoclaved separately in tightly stoppered bottles.

Solid media

Solid media were prepared by the addition of Ionagar No. 2 1% w/v to the salts medium. The mixture was autoclaved, cooled to 45°C before adding the carbon source and pouring plates.

Stock cultures

Stock cultures were maintained in 30% glycerol at -15°C. They were prepared by the addition of 2.5 ml of a log phase culture to a bijou bottle containing 1.5 ml of sterile 80% glycerol. Stock cultures were subcultured monthly.

2.4 Sterilization

Heating : Glassware was sterilized by heating to 160°C for 2 hours.

Autoclaving : Continuous culture media and apparatus was autoclaved at 121°C for 2 hours, all other solutions were autoclaved at 121°C for 20 minutes.

2.5 Purity

Culture purity was checked by streaking out cultures on nutrient agar, nutrient agar plus methanol and minimal medium plus methanol. Methylophilus methylotrophus forms only pinhead colonies on nutrient agar alone. Samples were also checked for purity by microscopy after Gram staining.

2.6 Growth and Harvesting of Cultures

1 litre cultures were grown in 2 litre shake flasks at 37°C in minimal medium (Section 2.3) supplemented with 1% methanol. Cells were harvested in the late logarithmic phase of growth, except where stated, by centrifugation at 10,000g for 10 min. at 4°C. Cells used for proton translocation measurements were washed twice with 140mM KCl on 20mM glycyl-glycine pH 7.0, and resuspended in the same to a density of about 70 mg/ml and stored at 0-4°C. For other experiments cells were washed twice with 25mM MOPS buffer pH 6.8, resuspended in buffer and stored at 0-4°C. Cells were always used within 6 hr. of harvesting.

Growth of cells in continuous culture is described in Section 2.7.

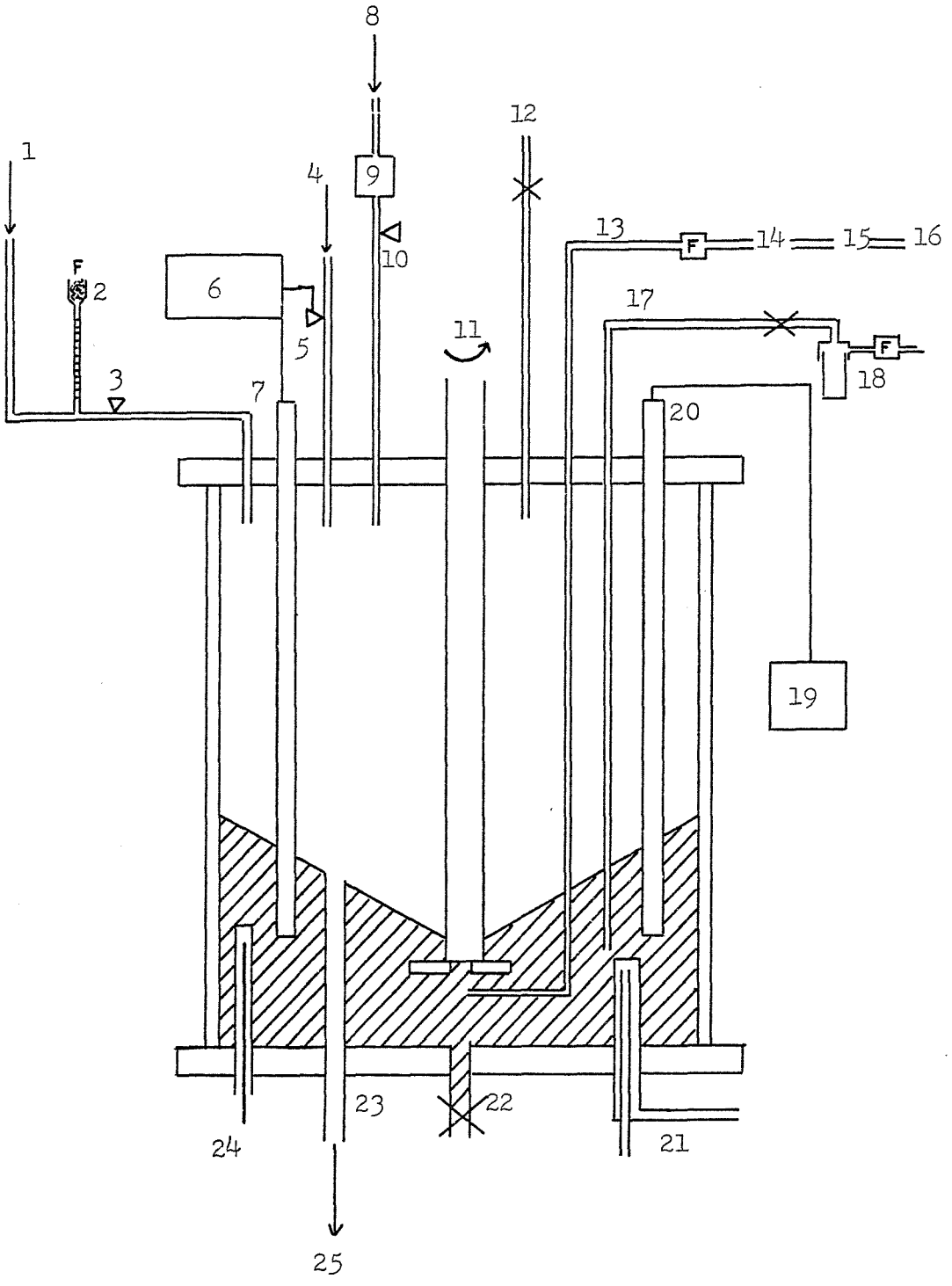
2.7 Growth of Bacteria in Continuous Culture

Chemostat cultures were set up in a 5 litre LHE fermenter (L.H. Engineering Company Ltd., Bells Hill, Stoke Poges, Bucks.); the apparatus is shown in Fig. 2.1. Internal surfaces of the vessel were treated with Repelcote before autoclaving to discourage wall growth. The medium used is described in Section 2.3 and was supplied to the vessel by means of a Watson-Marlow peristaltic pump (Watson-Marlow Ltd., Falmouth, Cornwall.) and the pH of the culture maintained at 6.8 by the automatic addition of 0.5M KOH/0.5M NaOH controlled by a pH meter/controller connected to a peristaltic pump. Antifoam was added as a 5% solution of Dow Corning Antifoam A emulsion, the addition sequence being controlled by a Crouzet doser/timer connected to a peristaltic pump. The temperature was maintained at 40°C by a Churchill thermostated water circulator. Air was supplied at volumes of 500 ml to 5l/min by air pump (Charles Austin Pumps Ltd.) and the culture stirred at about 1000 rpm by a $\frac{1}{4}$ hp. motor. Air and culture were removed through a drop weir and the gas disengaged at the receiver. The oxygen

Fig. 2.1 - Continuous Culture Apparatus

1. Medium reservoir.
2. Burette for medium flow measurement.
3. Peristaltic pump (Watson-Marlow).
4. Alkali reservoir.
5. Peristaltic pump (Watson-Marlow).
6. pH meter/controller.
7. pH electrode (LHE).
8. Antifoam reservoir.
9. Antifoam doser/timer (Crozet).
10. Peristaltic pump (Watson-Marlow).
11. Stirrer.
12. Inoculum line.
13. Air inlet.
14. G.A.P. gas flow meter.
15. Air flow adjustment.
16. Gas flow stabilizer and humidifier.
17. Sample line.
18. Sample vial.
19. Dissolved oxygen tension meter.
20. Lead/silver oxygen electrode (LHE).
21. Hot finger from thermostated water circulator (Churchill).
22. Drain for large samples.
23. Drop weir.
24. Thermometer.
25. Receiver.

F. Sterile filter.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tension was monitored with a lead/silver oxygen electrode (LHE), which was calibrated before inoculation. Dilution rates were calculated from the culture volume and the combined flow rates of medium, antifoam and alkali; these measurements were made daily. Gas flow was determined with a bubble meter. Limiting substrates were checked by adding a higher concentration of the limiting substrate and following the subsequent increase in dry weight. Culture purity and pH were checked daily by removal of samples through the sample line. The concentration of methanol in the flow medium was always 1% (v/v). The ammonium sulphate concentration in the flow medium was normally 3.6 g/l; nitrogen-limitation was achieved by reducing the concentration to 1.0 g/l. Oxygen was normally supplied as air at a flow rate of 6 l/min; oxygen-limitation was achieved by reducing the flow rate to 1.5 l/min.

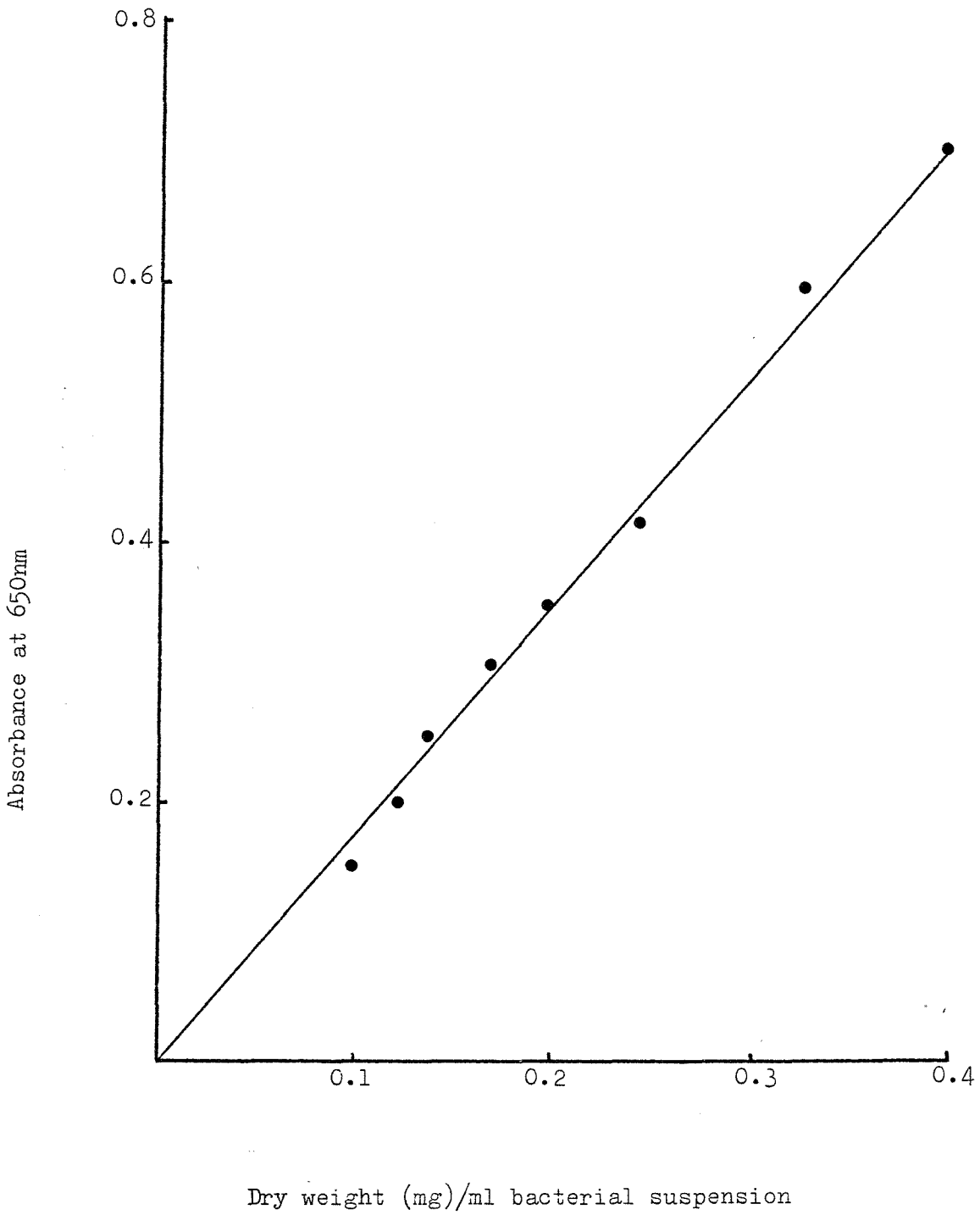
2.8 Estimation of Dry Weights

Dry weights of bacteria in suspensions were estimated from a calibrated curve of OD_{650} (measured in a Unicam SP 600 spectrophotometer), against dry weight for approximate measurements. Direct dry weight measurements were made by centrifugation of cells in samples, washing twice with distilled water and drying to constant weight at 105°C. The latter method was used to construct the dry weight calibration curve in Fig. 2.2.

2.9 Respiration-driven Proton Translocation Measurements

The pulse oxidant method of Mitchell and Moyle (1967) was used. The reaction vessels were of 6 ml volume maintained at 30°C by water jackets. The reaction mixture consisted of 140mM KCl, 40mM potassium thiocyanate, 7.5mM substrate and about 6 mg dry weight cells ml^{-1} in a total volume of 4 ml. The mixture was stirred magnetically and the pH changes measured with a Russell CMAT 7/2 microelectrode connected to a Pye Unicam 290 pH meter and recorded on a Servoscribe RE 541.2 potentiometric recorder with a full scale deflection corresponding to a 0.1 pH unit change. The suspension was kept anaerobic by means of a perspex lid through which a syringe needle passed, carrying a stream of oxygen-free nitrogen over the surface of the suspension. The system was allowed to equilibrate for 30 min. after which oxygen was added as 5.25µl volumes of air-saturated, 140mM, KCl at 30°C; at least 4 different

Fig. 2.2 - Dry weight calibration curve (Section 2.8)



volumes were used for each determination. It was assumed that 10µl of air-saturated KCl contains 4.45ng atoms of oxygen at 30°C. The resulting acidification of the external medium was standardized with 5-25µl volumes of anaerobic 2mM HCl. The changes in pH on addition of acid or oxygen were calculated as described by Mitchell and Moyle (1965) and graphs were plotted to relate the pH changes to the amount of oxygen added. The ratio of protons translocated from the bacteria to the external medium per atom of oxygen consumed could then be calculated.

Bacterial respiration rates, of the samples used for the measurement of proton translocation, were also measured in an oxygen electrode as described in Section 2.13, but using the same reaction mixture used for the respiration-driven proton translocation measurements.

2.10 Preparation of Membrane Fractions

Membrane preparations were made from approximately 250 ml samples of bacteria taken directly from the chemostat pot. The bacteria were harvested by centrifugation and washed twice with ice-cold 25mM MOPS buffer (pH 7.0), containing 5mM $MgCl_2$ and 5mM methanol and resuspended in the same buffer to a density of about 30 mg dry wt/ml. The bacterial suspension was passed twice through a French pressure cell press (Aminco, Silver Spring, MD 20910 USA) at 20,000 lb/in^2 (135 MPa) and the resulting extract centrifuged at 6000g for 10 min to remove whole bacteria and cell debris. The cell free extract was centrifuged at 350,000g for 1 hour to separate the particulate and soluble fractions. The particulate fractions were washed twice in buffer and stored in 0.7 ml lots in liquid nitrogen. High speed supernatants were also retained for analysis.

2.11 Protein Estimations

Protein concentrations of samples were estimated by the method of Lowry *et al.*, (1951); procedures were those of DeMoss and Bard (1957). Crystalline bovine serum albumen (fraction V) was used as standard.

2.12 Cytochrome Estimations

Cytochrome spectra were recorded on a Cary 118C dual beam spectrophotometer (Varian Associates Ltd., Walton-on-Thames, Surrey) at

ambient temperature or at 77°K with a 2mm light-path low temperature attachment. Reduced-minus-oxidized difference spectra were obtained by recording the spectra of samples, (whole cell suspensions, membrane and soluble cell fractions and washings), reduced with sodium dithionite (a few crystals of solid) with the reference sample oxidized with approximately 0.05 ml of a 0.03% solution of hydrogen peroxide or a small amount of solid potassium ferricyanide. In some cases a few microlitres of a saturated solution of hydroquinone was used to reduce preparations instead of sodium dithionite. Aeration of bacterial suspensions was achieved by passing air through cuvettes or by vigorous shaking immediately before recording the spectrum against an untreated or reduced sample. Spectra were recorded at the temperature of liquid nitrogen (77°K) by plunging samples into a Dewar flask containing liquid nitrogen and running the spectrum while maintaining the base of the aluminium cell holder in a reservoir of the liquid gas. For the measurement of carbon monoxide-binding pigments, suspensions were reduced with sodium dithionite and, after reduction, CO was passed through the test cuvette and the (reduced plus CO)-minus-reduced spectrum read. Baselines were always checked before recording spectra by recording the spectrum with untreated samples in both cuvettes. The proportion of cytochrome c released into the medium during growth was calculated by measuring the quantity of cytochrome c (by means of the reduced-minus-oxidized difference spectra) in whole culture, in whole bacteria obtained by centrifugation and in the supernatant after removal of whole bacteria by centrifugation. Cytochrome contents were estimated using the molar absorption coefficients of Chance (1957); cytochrome a/a_3 601-635nm = 16 litre/mmol/cm; cytochrome b 556-575nm = 22 litre/mmol/cm; cytochrome c 549-535 = 19 litre/mmol/cm. Binding of carbon monoxide to cytochrome c was estimated using a molar absorption coefficient of 55 litre/mmol/cm (peak to trough).

2.13 The Effect of Inhibitors on Respiration

Respiratory activities of whole bacteria and cell fractions were measured in a Clark type oxygen electrode (Rank Bros., Bottisham, Cambs.,) in a 2 ml reaction volume in the presence of 25mM MOPS (pH 7.0) with substrates at the following concentrations; methanol, 7.5mM; NADH, 1mM; NADPH, 1mM; ascorbate/TMPD, 2mM and 0.2mM respectively. Ascorbate and TMPD were mixed at least 10 minutes before use; this was

found to give the lowest non-enzymic rate. The following inhibitors were used; potassium cyanide (in 0.5mM Tris-HCl, pH 8.0); sodium azide (in 5mM Tris-HCl, pH 8.0); Antimycin A (in DMSO); rotenone (in DMSO); HQNO (in DMSO). Inhibitors were incubated with samples until a new linear rate was obtained or for 10 minutes if no inhibition was observed.

Initial oxidation rates (before the addition of inhibitors) were always greater than 30µl O_2 per hour (22.3 nmol O_2/min) to ensure that rates in the presence of inhibitors were large enough to measure accurately.

2.14 Measurement of Midpoint Oxidation-reduction Potentials

The procedure used for the measurement of oxidation-reduction midpoint potentials of the cytochromes in soluble or membrane preparations were essentially those of Dutton (1971) using either the purpose built dual wavelength spectrophotometer of Dr. O.T.G. Jones at Bristol University, a purpose built dual wavelength spectrophotometer (Applied Photophysics Ltd., Albemarle St., London.), or a Cary 118C dual beam spectrophotometer. The reaction vessel contained 5.5 ml of suspension in 25mM MOPS buffer (unless stated otherwise) pH 7.0, and 100mM KCl which was stirred by an Electrothermal stirrer (Electrothermal Engineering Ltd., Neville Rd., London.) passed through the top of the vessel. The vessel was fitted with side arms sealed with septa through which additions were made with microlitre syringes. There was also a gas inlet and exit through which oxygen-free nitrogen was passed to maintain the contents under an atmosphere which was essentially oxygen-free. To remove traces of oxygen which are present in commercial white-spot nitrogen the gas was first passed through a solution containing proflavin hemisulphate (224 mg/l), methyl viologen (270 mg/l) and EDTA (7.5 g/l) in 250mM Na/K phosphate buffer (pH 6.5), which was activated by light during use (Sweetzer, 1967). The potential in the cell was measured with a platinum electrode sealed into the vessel with the circuit completed by a salt bridge to a saturated calomel electrode (Radiometer, Copenhagen). The difference in potential between the platinum and reference electrodies was measured with a digital voltmeter and a correction made to give the potential with respect to the standard hydrogen half cell (an addition of 247mV). Preparations were reduced with ascorbic acid or NADH and oxidized with potassium ferricyanide.

For membranes the following mediators were normally used; hydroquinone, 25μm (E_{m7} 280mV); 2,3,5,6 tetramethyl phenylene diamine, 50μM (E_{m7} 220mV); phenazine methosulphate, 25μM (E_{m7} 80mV); phenazine ethosulphate, 25μM (E_{m7} 55mV); pyocyanine, 6μM (E_{m7} -34mV); 2 hydroxy 1,4, napthoquinone, 20μM (E_{m7} -145mV). Soluble preparations were titrated in the presence of 2,3,5,6, tetramethylphenylenediamine, hydroquinone and 50μM potassium ferricyanide.

The apparatus was used to make simultaneous recordings of absorption and electrode potential which were then used to construct graphs to make a preliminary identification of the redox components present. For each component :

$$\log \frac{(\text{oxidized cytochrome})}{(\text{reduced cytochrome})}$$

was plotted against electrode potential using the programme given in Section 2.15. Having obtained an approximate midpoint redox potential by this method, the potential range corresponding to each component was then more accurately selected and the procedure repeated to obtain more accurate midpoint redox potentials for each component. The accuracy of the system was checked by using it to determine the midpoint redox potential of horse heart cytochrome c, which was found to be within 5mV of the published value of +255mV (Henderson and Rawlinson, 1956).

2.15 Programme for the Calculation of Midpoint Potentials

The equation relating the tendency of a redox couple to accept or donate electron(s), (its electrical potential), is the Peters-Nernst equation :-

$$E_h = E_o + \frac{R\,T}{n\,F} \log \frac{(Ox)}{(Red)}$$

where E_h is the electrical potential exhibited by the half cell relative to the standard hydrogen half cell; E_o is the standard half reduction potential (pH 0) characteristic of the redox couple. Substituting values for R, T and F at 25°C this simplifies to :-

$$E_h = E_o + \frac{0.059}{n} \log \frac{(Ox)}{(Red)}$$

Under non-standard conditions of pH this can be written :-

$$E_m = E_h - \frac{0.059}{n} \log \frac{(Ox)}{(Red)}$$

where E_m is the half reduction potential characteristic of the redox couple at a given pH; for example E_{m7} is the half reduction potential at pH 7.0.

Since the amount of oxidized or reduced cytochrome is related directly to its absorbance, and knowing (or being able to estimate) the total absorbance change between fully reduced and fully oxidized cytochrome, the midpoint potential can be calculated if the absorbance is known at various potentials :-

$$E_m = E_h - \frac{0.059}{n} \log \frac{(\text{Absorbance at 100\% Red} - \text{Absorbance at } E_h)}{(\text{Absorbance at } E_h - \text{Absorbance at 100\% Ox})}$$

This formula was converted to a programme for a Texas Ti59 calculator as follows :-

```
LRN   2nd Lbl A, STO 01, R/S,
      2nd Lbl 2nd A', STO 02, R/S,
      2nd Lbl B, STO 03, R/S,
      2nd Lbl C, STO 04, R/S,
      2nd Lbl D, STO 05, R/S,
      2nd Lbl E, Rcl 01, 2nd Prt,
      Rcl 02, 2nd Prt,
      Rcl 03, 2nd Prt,
      Rcl 04, 2nd Prt,
      Rcl 01, -, Rcl 03, =, STO 11,
      Rcl 04, -, Rcl 01, =,
      ÷, Rcl 11, =,
      2nd log, 2nd Prt, x, Rcl 05, =, STO 13,
      Rcl 02, -, Rcl 13, =, 2nd Prt,
      2nd Adv,
      R/S,

LRN
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To operate the programme the data were entered as follows :-

Enter A; the absorption at the selected point.
Enter A'; the potential at that point in volts.

Enter B; absorption at 100% oxidized.

Enter C; absorption at 100% reduced.

Enter D; 0.059 volts (for 1 electron transfer).

To run programme enter E.

The printout gives A, A', B,C and the value of log $\frac{(Ox)}{(Red)}$ at the selected point with the midpoint potential of the absorbing components.

2.16 Purification of the Soluble Cytochromes c of Methylophilus methylotrophus

500g (wet weight) of frozen continuous culture grown bacteria (grown under conditions of methanol and oxygen limitation) were taken and homogenized in 500 ml of 20mM phosphate buffer, pH 7.0. 50mg DNAase was added and the cells were passed twice through a French pressure cell at 20,000 lb/in^2 (135MPa) and the resulting deep red solution was centrifuged at 23,000g for 1 hour to remove whole bacteria and cell debris. The resulting cell free extract was acidified with 1M HCl to pH 4.2 and the precipitated protein and nucleic acid removed by centrifugation. The pH was again adjusted to pH 4.2 and the centrifugation repeated. The deep red supernatant was adjusted to pH 8.0 with 1M NaOH and concentrated in a Chemlab 400 ml concentrator (Chemlab Instruments Ltd., Upminster Rd., Hornchurch, Essex), with a G-05-T membrane (5,000MW cut-off) under an atmosphere of nitrogen to a volume of 300 ml. This was dialysed in 3 lots, each against 10 l of 10mM Tris-HCl (pH 8.0) in a 100 ml beaker dialyser (Bio-Rad Laboratories Ltd.) and applied to a column(4 x 23cm)of DEAE cellulose (Whatman DE 52) equilibrated in the same buffer. Some of the cytochrome came straight through the column (designated cytochrome C_H) and two other cytochrome bands were left bound to the column. The unbound cytochrome was pooled, concentrated and dialysed against 5 l of 5mM sodium acetate (pH 5.6). The lower band on the column was eluted with 50mM Tris (pH 8.0) in 80 ml and was designated cytochrome C_K. The upper band on the column was eluted in 100mM NaCl/100mM Tris (pH 8.0) and was designated cytochrome C_L.

The cytochrome C_H was applied to a Sephadex G-150 upward flow column (90 x 2cm) and two coloured fractions were obtained; the first was found to be methanol dehydrogenase and the second was the cytochrome. The cytochrome C_H was applied to a 8.5 x 3cm column of CM cellulose

(Whatman CM52) in 5mM sodium acetate (pH 5.6) and it bound as a dense band at the top of the column. The cytochrome was eluted with 100mM buffer and a number of coloured bands were seen, the major band (more than 90%) was in the reduced form and eluted first and was used for further purification. The minor band appeared to be the oxidized form of the same cytochrome and was discarded. The cytochrome did not bind to hydroyapatite in 1mM phosphate buffer (pH 7.0) and was re-applied to a CM cellulose column (8 x 3cm) in 5mM acetate buffer. The cytochrome was eluted with a linear gradient of 5-120mM sodium acetate (pH 5.6) containing 5mM ascorbic acid; the cytochrome eluted in about 60mM buffer. The UV absorption of the eluate was monitored with a Uvicord recorder (LKB) and the cytochrome peak corresponded exactly with the UV absorbance. The cytochrome was dialysed against 10 l of distilled water in a beaker dialyser and was stored frozen at $-20^{\circ}C$. the cytochrome appeared as one band on SDS polyacrylamide gels after electrophoresis.

Cytochrome c_K was concentrated to 25 ml in a concentration cell and applied to an upward flow Sephadex G-150 column (90 x 2cm) in 100mM Tris-HCl (pH 8.0). The purest fractions were pooled and applied to a DEAE column (10 x 3cm) in 5mM Tris-HCl (pH 8.0) and eluted with a linear gradient of 5-100mM buffer. The cytochrome eluted in 40mM buffer and was dialysed against 6l of distilled water in a beaker dialyser and stored frozen at $-20^{\circ}C$. The cytochrome appeared as one band on SDS polyacrylamide gels after electrophoresis.

Cytochrome c_L was concentrated to 30 ml in a concentration cell and was applied to an upward flow Sephadex G-150 column (90 x 2cm) in 100mM Tris-HCl (pH 8.0). It eluted before a yellow fraction of unknown composition. The cytochrome fraction was applied to a DEAE column (9 x 3cm) in 10mM Tris-HCl (pH 8.0) and eluted with a linear gradient of 10mM Tris-HCl to 125mM NaCl/125mM Tris-HCl (pH 8.0). The cytochrome eluted in approximately 90mM NaCl/90mM Tris. The cytochrome was concentrated to 15 ml in a concentration cell and applied to a Sephadex G-75 superfine column (100 x 2cm) in 100mM Tris-HCl (pH 8.0). Two overlapping cytochromes were eluted, fractions were pooled from the earliest part of the first band; the mixed part of the bands and the latest part of the second band. The early and late fractions both gave single bands on polyacrylamide gels after electrophoresis but of

differing molecular weight; the mixed fraction had both bands. The mixed fraction was subjected to isoelectric focussing as described in Section 2.19.

A summary of the purification procedure is given in Fig. 2.3.

2.17 Estimation of Molecular Weights of Cytochrome c

Molecular weights were determined by SDS polyacrylamide electrophoresis based on the method of Swank and Munkres (1971). Stock solutions were made up as shown in Table 2.1. The stock solutions were mixed in the proportions indicated and deaerated for 5 minutes under vacuum on a water pump. Ammonium persulphate was then added to initiate polymerization. The gel solution was cast in Perspex tubes (6 x 120mm), which had been previously sealed with Parafilm, to a depth of 110mm. Immediately 0.2 ml of distilled water was layered over the top of the gel solution to ensure a level meniscus; when polymerization was complete (after about 20 minutes) the layer of water was removed and the gels were placed in a Shandon disc electrophoresis apparatus. The upper and lower reservoirs were filled with buffer and the sample was applied to the gel with a microlitre syringe.

Samples for electrophoresis were prepared by the addition of an equal volume of 5% SDS (w/v) containing 10% (w/v) 2-merceptoethanol and 50mM sodium carbonate to the protein solution followed by heating for 5 minutes at 100°C; the protein concentration was generally of the order of 2 mg/ml. A drop of bromophenol blue solution was added as tracking dye together with a few crystals of sucrose. The samples (25-100µl) were applied to the gels through the upper reservoir and a constant current of 5mA per gel was applied for electrophoresis. When the dye front was about 5mm from the end of the gel (after about 4.5 hours) the gel was removed from the Perspex tube, the position of the tracking dye marked by the insertion of a piece of copper wire through the gel and the gel was stained as described in Section 2.18.

The standard proteins used for molecular weight determination were insulin (M.W.5,700), horse heart cytochrome c (11,700), lysozyme (14,100), myoglobin (17,200) trypsin (23,000) and ovalbumen (43,000). A standard curve was then plotted of Rf. against log molecular weight. The standards and cytochrome samples were run on separate gels and electro-

Fig. 2.3 - Summary diagram of the purification of the soluble cytochromes c from Methylophilus methylotrophus

The method used to purify the cytochromes c is described in Section 2.16.

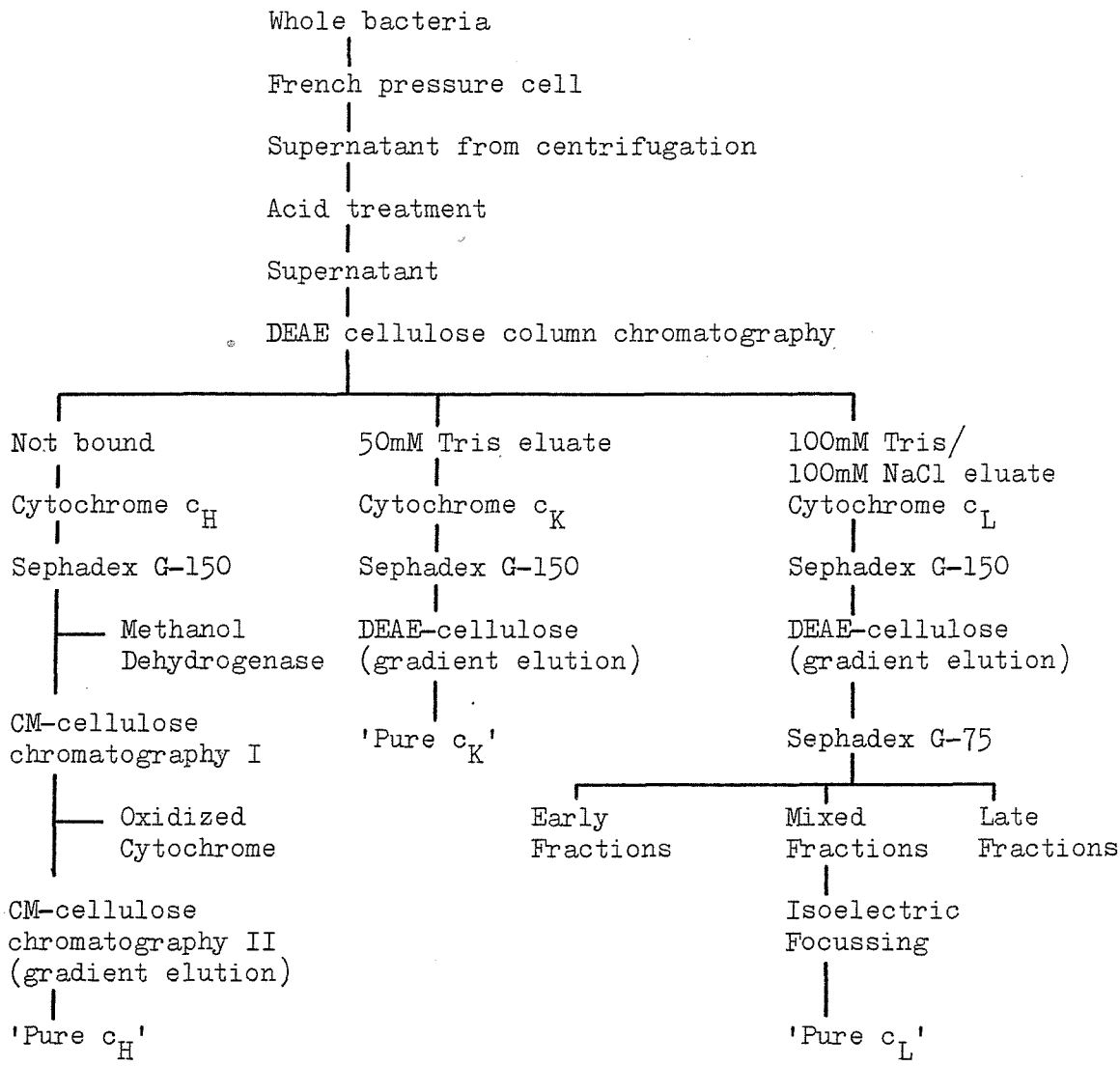


Table 2.1 - Solutions for polyacrylamide gel electrophoresis

a. Solubilizing solution

5% w/v sodium dodecyl sulphate
50mM sodium carbonate
10% v/v 2-mercaptoethanol

b. Gel solutions

Solution A : 38.7g acrylamide, 1.33g bis acrylamide — made up to 100 ml with water

Solution B : 0.6 ml TEMED, 0.8g sodium dodecyl sulphate, 4.48 ml phosphoric acid — made up to 100 ml with water and adjusted to pH 5.0 with solid Tris

c. Buffer

0.1% sodium dodecyl sulphate in 0.1M phosphoric acid, adjusted to pH 6.8 with solid Tris.

For 100 ml of gel 32.3 ml of A was mixed with 12.5 ml of B and 36.04g of urea was added to give a final concentration of 6M. The volume was made up to 100 ml with distilled water and the pH adjusted to 6.8 with solid Tris. After degassing 60mg of ammonium persulphate was added to initiate polymerization.

phoresed at the same time, 2 determinations were made. The results of one of the experiments are shown in Fig. 2.4.

2.18 Staining for Protein in Polyacrylamide Gels

Gels were stained for 2 hours at room temperature in 0.2% Coomassie Brilliant Blue dissolved in water: glacial acetic acid: methanol (65:10:25 v/v/v). The gels were destained in water: glacial acetic acid: methanol (65:10:25 v/v/v) until the background was clear, before scanning at 550nm in a Joyce Loebl Chromoscan 200.

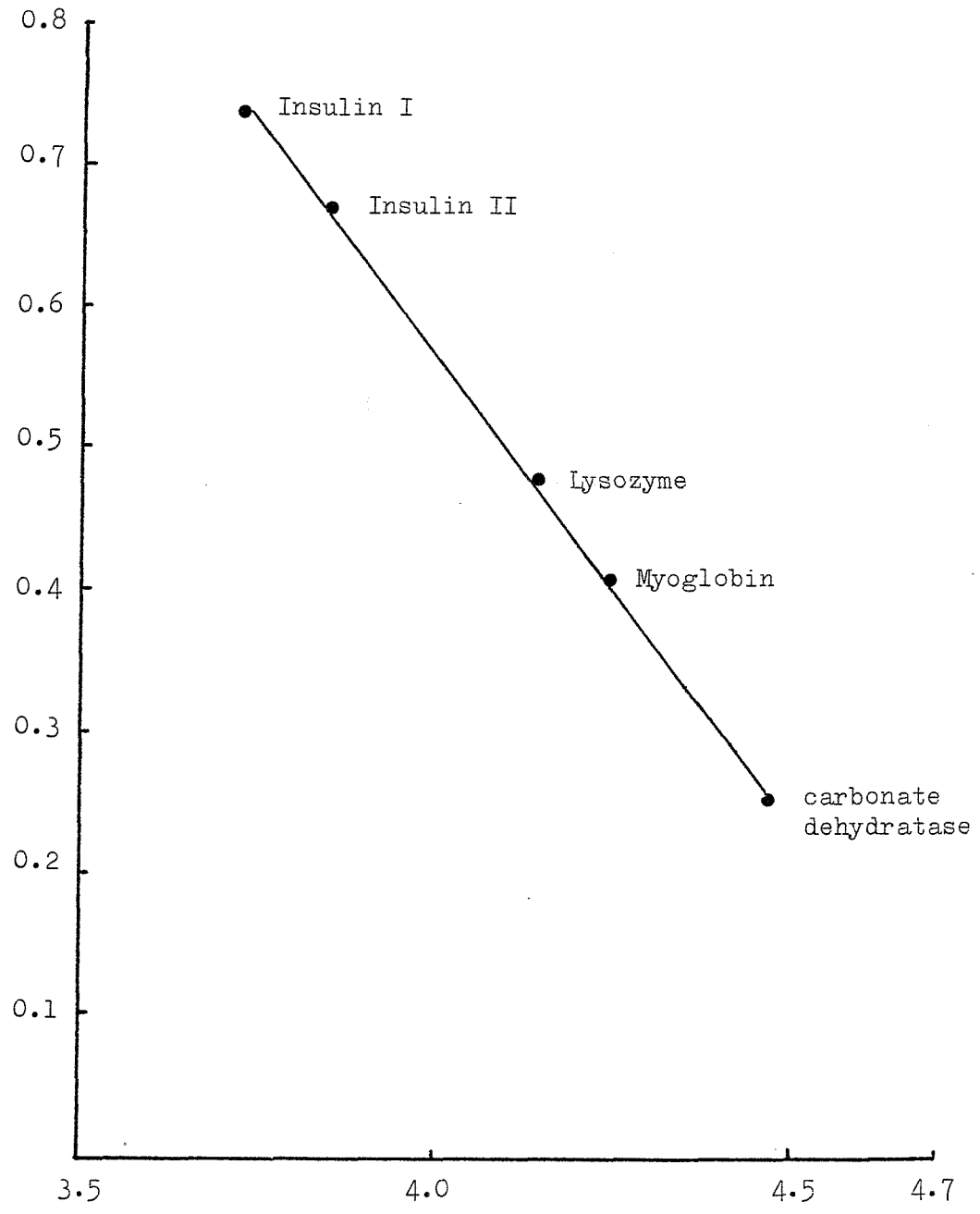
2.19 Preparative Isoelectric Focussing

40g of Sephadex G-75 Superfine was placed in 1 litre of distilled water for 10 minutes to swell. The gel was then filtered in a large Buchner funnel under slight suction with a 15cm Whatman No. 54 filter (25µm porosity). The gel bed was washed with a further 5l of distilled water over a period of 3 hours. The gel was then dehydrated with repeated 100-150 ml portions of absolute ethanol, and the residual white powder was dried overnight in a vacuum dessicator.

Before isoelectric focussing it was necessary to determine the evaporation limit of the batch of Sephadex by the following method. A gel bed was prepared from 5g of the washed Sephadex in 100 ml of distilled water by adding the gel slowly to the water in a 200 ml beaker and weighing. A LKB 2117-501 electrofocussing tray was then taken (part of the LKB 2117 Multiphor system; LKB Instruments Ltd., South Croydon, Surrey) and six 10.5cm electrode strips (which had been presoaked in distilled water) were placed 3 deep at each end of the tray, which was then weighed. The gel was then homogenised by gentle stirring and immediately poured into the tray; the beaker and the small remainder of the suspension were then weighed. A small hairdryer was then mounted 70cm above the tray to evaporate the water from the suspension. The initial slurry weight was calculated from the weighings of the beaker before and after pouring of the slurry and water was evaporated until small cracks appeared in the gel bed. The water loss was determined by weighing the tray and calculated as a percentage of the initial weight of the slurry on the tray; 75% of this figure was then taken as the evaporation limit for the batch of Sephadex.

Fig. 2.4 - Standard curve of molecular weights determined by SDS gel electrophoresis

The method is described in Section 2.17.



The sample (80 ml of cytochrome c_L) was pretreated by dialysis against 3l of 1% glycine in a beaker dialyser for 1 hour. 2.5 ml of Ampholine carrier ampholytes (LKB) (pH 3.5-5.5)and 2.5 ml Ampholine carrier ampholytes (pH 4.0-6.0)were added to the cytochrome in a 200 ml beaker and the volume made up to 100 ml with distilled water. 5g of the washed Sephadex G-75 superfine were stirred in and the contents weighed. Six 10.5cm electrode strips were soaked in the ampholine solution and placed 3 deep at each end of the tray. The cytochrome slurry was then poured in and dried to the evaporation limit as described above. The tray was then transferred to the cooling plate of the Multiphor unit with a film of 1% SDS between the tray and plate. An electrode strip soaked in 1M phosphoric acid was placed at the anodic side and another strip soaked in 1M NaOH at the cathodic side, each on top of the strips already on the tray. The protruding parts of the strips were cut off to fit the tray exactly. The lid of the apparatus was then placed over the tray and connections were made to a LKB 2130 power supply. The current was set to 15mA maximum, the voltage to 1500V maximum, the apparatus set to limit the power to 8W and the gel run for 16 hours. The plate was maintained at 10°C by a Grant LB 50 refrigeration unit (Grant Instruments, Cambridge).

After focussing, a 30 compartment fractionating grid was placed over the tray (LKB 2117) and the cytochrome bands were scraped off into 5 ml plastic columns (LKB) and the cytochrome eluted. Alternate bands were also removed from the grid along the length of the gel to determine the pH gradient. Spectra of the cytochrome bands were recorded and samples were run on SDS gels.

2.20 Analytical Isoelectric Focussing

Isoelectric focussing of purified cytochromes was performed on LKB Ampholine polyacrylamide gel plates (pH 3.5-9.5) (LKB 1804-101); half a plate was used. A LKB 2117 Multiphor cooling plate was used as the support and the template maintained at 10°C by a Grant LB 50 refrigeration unit. White spirit was used as insulating fluid between cooling plate and template, and between template and PAG plate. The cathode electrode strip (soaked in 1M NaOH) and anode electrode strip (soaked in 1M phosphoric acid) were placed on the plate and the samples were applied as approximately 15µl of sample on a sample

application paper (5 x 10mm). The samples were electrofocussed across the width of the plate. After 45 minutes the sample application pieces were removed and the experiment continued; total running time was 1.5 hours. The pH gradient across the gel was measured with pH indicator paper, and after measurement the zones were sharpened for 10 minutes. The power supply was from a LKB 2130 power pack using 15W. The voltage was limited to 1500V and the current to 50mA.

After focussing, the electrode strips were removed and the gel fixed for 1 hour in fixing solution (57.5g trichloroacetic acid and 17.25g sulphosalicyclic acid in 500 ml distilled water). After fixing, the plate was washed for 5 minutes in destain (ethanol: acetic acid: water, 25/8/67 v/v/v) and stained for 10 minutes at 60°C in Kanacid blue R (0.46g in 400 ml destain). The plate was then destained with several changes of destain for 24 hours. After this time the plate was placed in preserving solution (40 ml glycerol in 400 ml destain) and dried. A plastic protection sheet was then placed over the gel surface.

2.21 Methanol Dehydrogenase Assay

Methanol dehydrogenase was assayed according to the method of Anthony and Zatman (1964) in an oxygen electrode. The reaction mixture contained 100mM Tris-HCl (pH 9.0); 150mM ammonium sulphate; 0.05% PMS; 7.5mM methanol. The reaction was started by the addition of cell extract. The reaction volume was 2 ml and was performed at 30°C.

CHAPTER 3

MEASUREMENT OF RESPIRATION-DRIVEN PROTON TRANSLOCATION IN METHYLOPHILUS METHYLOTROPHUS

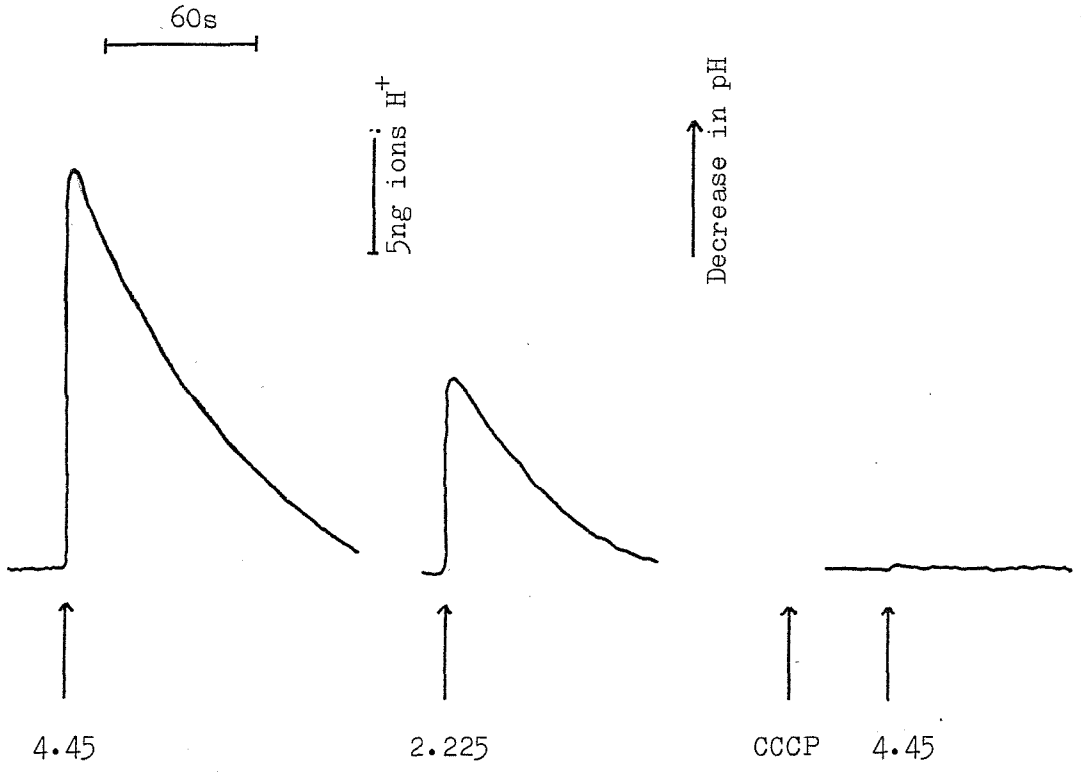
3.1 Introduction

It has been established that the arrangement and properties of the components of the electron transport chain of bacteria determines the stoicheiometry of respiration-driven proton translocation (Haddock and Jones, 1977; Jones, 1977). Bacteria with cytochromes b, c and a/a_3 usually have 3 proton translocating segments, whereas bacteria lacking cytochrome c usually have only 2 segments. Previously published work on obligate methylotrophs indicates a maximum of one site of ATP synthesis for all substrates (Tonge et al., 1977) although more sites have been found in facultative organisms such as Pseudomonas AM1 (O'Keeffe and Anthony, 1978; Keevil and Anthony, 1979). Proton translocation measurements are often used as a guide to the amount of ATP likely to be available for growth and hence may relate to the efficiency of conversion of carbon source to cell materials. It is of interest to measure proton translocation in Methylophilus methylotrophus in view of its high growth yield, and this chapter is concerned with the measurement of respiration-driven proton translocation in this organism.

3.2 Measurement of Respiration-Driven Proton Translocation in cells from batch cultures

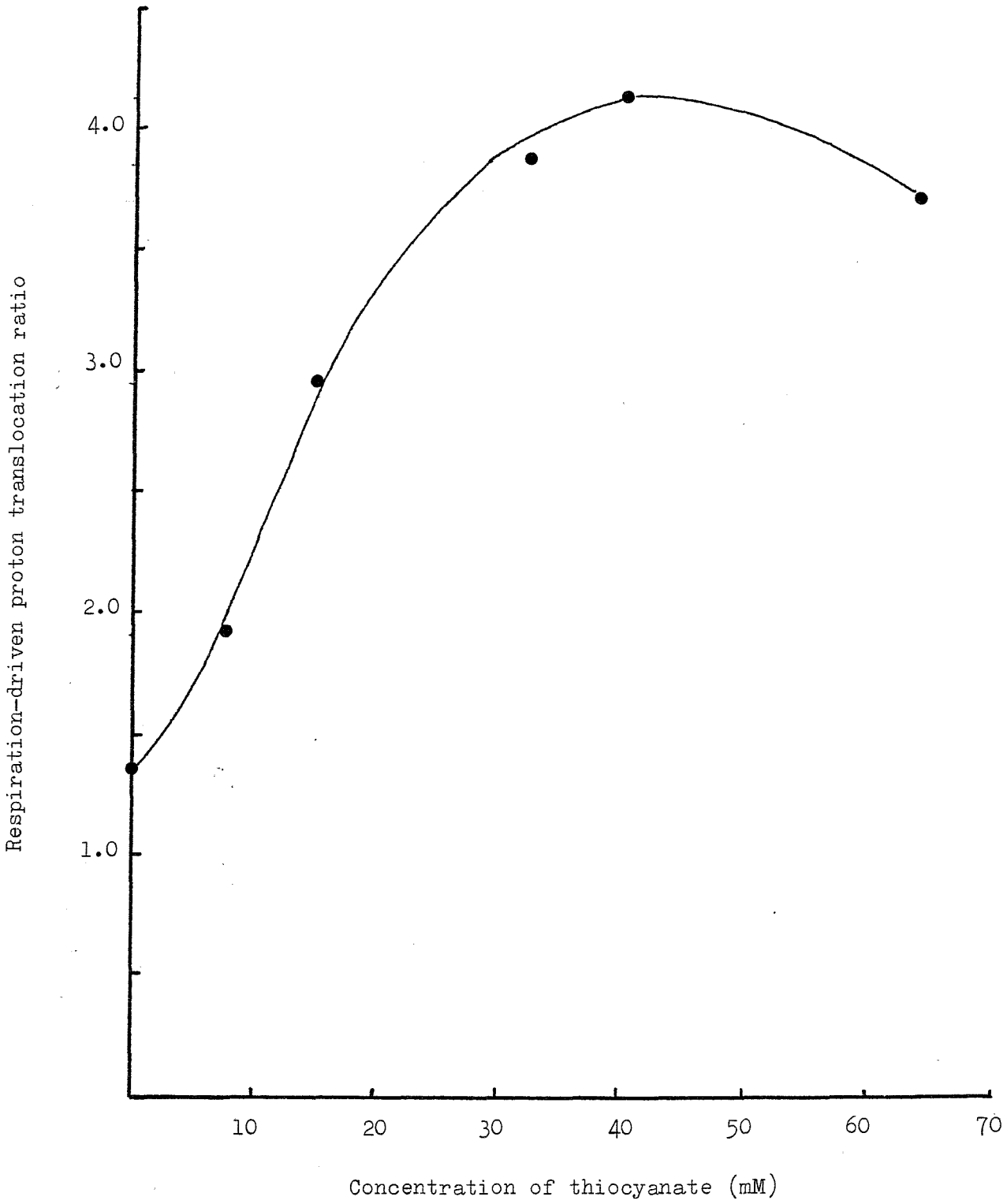
Respiration-driven proton translocation was measured in batch-grown bacteria as described in Section 2.9. Fig. 3.1 shows some typical traces obtained after the injection of oxygen (as air-saturated 140mM KCl) into anaerobic suspensions of Methylophilus methylotrophus. The decay of the pH change showed first order kinetics with a $t_{\frac{1}{2}}$ of about 40 sec. The addition of the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (50nmol) abolished the response to further additions of oxygen. The addition of carbonate dehydratase (40µg/ml) had no effect on the respiration-driven proton translocation ratio measured in the presence or absence of substrate. Fig. 3.2 shows the effect of various potassium thiocyanate concentrations on the measured respiration-driven proton translocation ratio; 40mM thiocyanate was

Fig. 3.1 - pH changes resulting from addition of O_2 to anaerobic suspensions of Methylophilus methylotrophus



The apparatus and methods are described in Sections 2.9 and 3.2. The amount of O_2 (ng-atoms) added in each pulse of air-saturated KCl is indicated; addition of anaerobic KCl elicited no change in pH. The addition of the uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 50 nmol) is indicated by an arrow.

Fig. 3.2 - The effect of the potassium thiocyanate concentration on the respiration-driven proton translocation ratio of Methylophilus methylotrophus



The effect of the potassium thiocyanate concentration on the respiration-driven proton translocation ratio for the endogenous substrate was determined as described in Sections 3.2 and 2.9.

used for all subsequent experiments. A concentration of 66mM potassium thiocyanate was used by O'Keeffe and Anthony (1978), in their work using the facultative methylotroph Pseudomonas AM1, as this concentration was found to give the best results. It was found that the storage of bacteria in 140mM KCl alone caused a rapid decrease in the ratios measured and in later experiments bacteria were kept in 20mM glycylglycine pH 7.0 (final concentration 1.5mM in the reaction mixture). Respiration rates were measured in the presence of potassium thiocyanate and were typically 110$\mu l O_2$/h/mg dry weight for methanol and formaldehyde, and 10$\mu l O_2$/h/mg dry weight for the endogenous substrate (probably NADH); the same values were found in the absence of potassium thiocyanate, this contrasts with the greatly reduced methanol oxidation rate in the presence of potassium thiocyanate found by O'Keeffe and Anthony (1978), in the facultative methylotroph Pseudomonas AM1.

Unfortunately the respiration-driven proton translocation ratios were very variable, but in general the values for endogenous substrate were between 3.5 and 6.0; for methanol they were between 2.0 and 4.0; and for formaldehyde they were about 4. It was noted however that the ratios for endogenous substrate tended to fall into two groups; 43% (of 42 determinations) were between 3.0 and 4.0 and 36% were between 5.0 and 6.0 protons per oxygen atom, and this may reflect a variation in the number of proton translocating segments. It was also found that respiration-driven proton translocation ratios tended to increase during growth and that this was concomitant with an 8 fold increase in the amount of cytochrome a/a_3 content during growth. This suggested that the electron transport system and the associated respiration-driven proton translocation might be different during growth phase in batch culture. Because this is difficult to control, the effect of growth conditions on cytochrome content and proton translocation ratios were investigated by use of continuous culture.

3.3 Measurement of Respiration-Driven Proton Translocation ratios in Bacteria grown in Continuous Culture

Bacteria were grown in continuous culture as described in Section 2.7; bacteria from methanol-limited continuous cultures were found to give proton translocation ratios too variable to be of use; simultaneous measurement of two identical samples frequently gave

ratios of more than 2 units apart. The cytochrome content of the bacteria was found to differ however, and this is described in the next chapter.

3.4 Summary

Respiration-driven proton translocation has been shown to occur in Methylophilus methylotrophus and although no firm conclusions can be made as to the exact number of proton translocating segments for any substrate, it does appear that there is more than one site of respiration-driven proton translocation by contrast with the single site proposed for the obligate methylotroph Methylosinus trichosporium by Tonge et al., (1977a).

CHAPTER 4

THE CYTOCHROMES OF METHYLOPHILUS METHYLOTROPHUS

4.1 Introduction

From the earliest research into cytochromes the spectrophotometer has proved the most useful tool. The alpha, beta and Soret absorption bands are characteristic of the cytochromes and the first step in the study of the electron transport system of any new organism is to use this fact to determine which cytochromes are present and in what quantity. Spectra may be recorded at room or liquid nitrogen temperatures, of whole bacteria, extracts or membrane preparations in the presence or absence of ligands such as carbon monoxide. Visible and UV spectra of molecules are associated with transitions between electronic energy levels, usually from a ground state to an excited state. The excitation of electrons is accompanied by changes in their vibrational and rotational quantum numbers so that what would otherwise become an absorption line becomes a broad peak containing vibrational and rotational fine structure. Due to interactions with solvent molecules this is usually blurred out and a smooth curve is obtained. The effect of lowering the temperature of a sample is to reduce the vibrational and rotational components, and the solvent interaction. This results in a sharpening of the absorption bands and sometimes a resolution of overlapping bands. For this reason spectra of samples containing cytochromes are often recorded at the temperature of liquid nitrogen ($77^{\circ}K$). Care must be taken when interpreting spectra because the alpha absorption bands of some cytochromes (usually cytochrome c) split at low temperature and this has led to the absorption of a single cytochrome erroneously being attributed to two cytochromes; and the converse, two separate cytochromes absorption bands being attributed to a single cytochrome with a split alpha peak.

Cytochromes of the a-type have characteristic spectra in the reduced state, with alpha and Soret bands at 600-605nm and 440-445nm respectively. The alpha and Soret bands in reduced-minus oxidized difference spectra are in the same spectral region (Bartch, 1968); Kamen and Horio, 1970; Horio and Kamen, 1970). Cytochromes of the b-type have characteristic absorption spectra in the reduced state, with

alpha, beta and Soret peaks at 556-565nm, 525-535nm and 430nm, the peaks in reduced-minus-oxidized difference spectra being in the same spectral regions. Cytochromes of the c type have characterstic absorption spectra in the reduced state, with alpha, beta and Soret bands at 550-552nm, 520-523nm and 416-418nm respectively; the peaks in reduced minus oxidized difference spectra are in the same spectral range.

The measurement of oxidation-reduction potentials is a useful technique for further characterisation of the cytochromes and is also helpful in establishing the order of the components. Differences of a few tens of millivolts between the midpoint redox potentials of two components is not enough to establish their relative order however, since relative concentrations could reverse the actual order in which they may function.

The experiments described in this chapter were designed to identify the cytochromes of Methylophilus methylotrophus and to characterize them with respect to their midpoint redox potentials.

Results and Discussion

4.2 Difference Spectra measured at Room Temperature

Reduced-minus-oxidized difference spectra were recorded of whole Methylophilus methylotrophus cells which had been grown in batch and continuous culture under conditions of methanol-, oxygen- or nitrogen-limitation (Sections 2.6; 2.7). A membrane-bound cytochrome of the a-type (absorption maximum at 602nm in reduced-minus-oxidized difference spectra; Fig. 4.1) has been found in bacteria grown in batch cultures, (the quantity increasing approximately 8-fold during growth) and also in methanol-limited continuous culture. This cytochrome was probably a mixture of cytochromes a and a_3, since carbon monoxide binding was observed (Fig. 4.3). The Soret band of cytochrome a/a_3 could not be seen in whole bacteria or bacterial membranes at room temperature because of the large quantity of cytochrome c also present in the preparations. Cytochrome a/a_3 could not be detected in bacteria (or bacterial membranes) grown under conditions of oxygen- or nitrogen-limitation (when methanol was in excess). Since the cytochrome was absent under both limitations, but was present when methanol was the limiting substrate, it was apparent that it was the conditions of

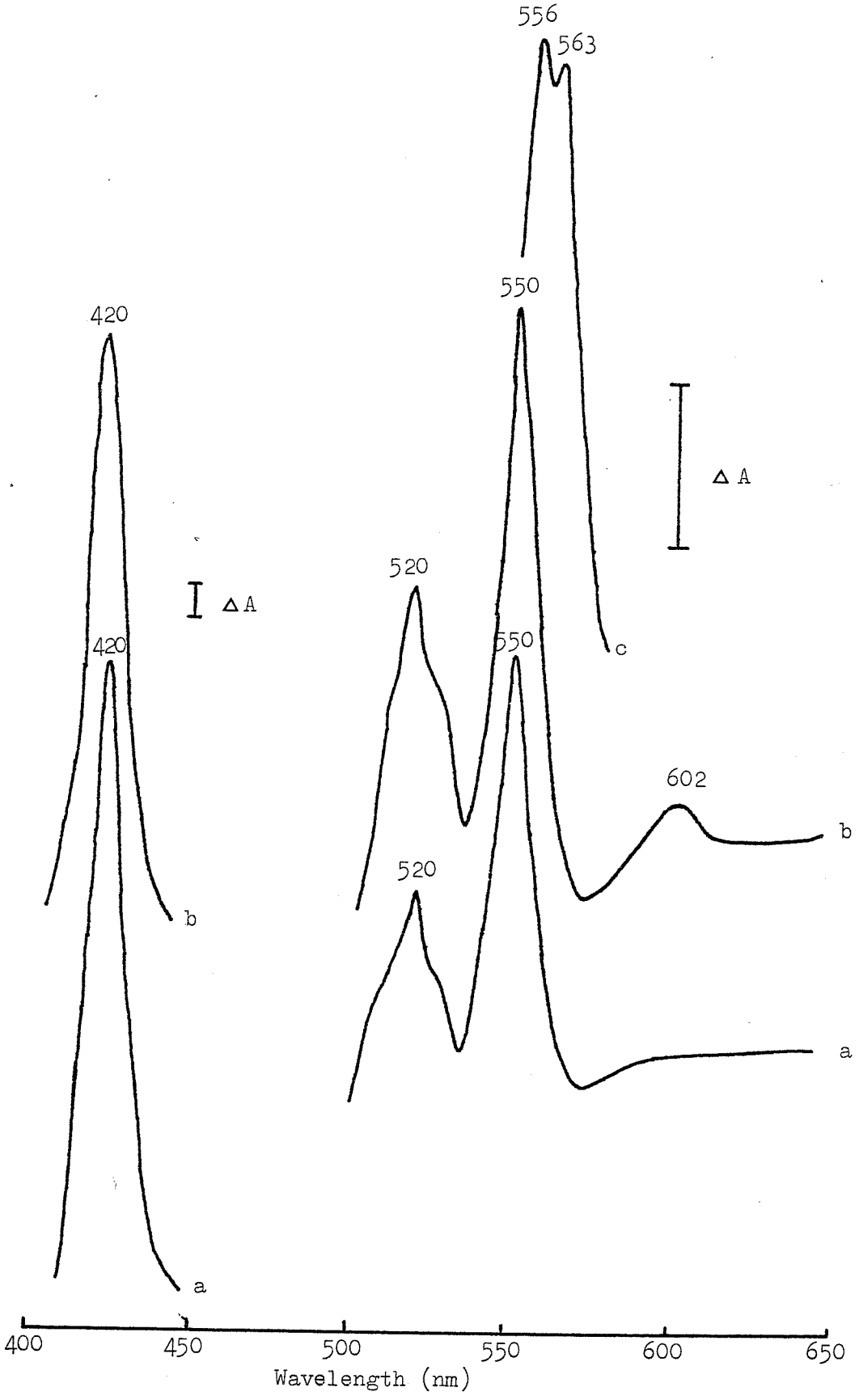
Fig. 4.1 - Reduced-minus-oxidized difference spectra of whole Methylophilus methylotrophus cells

Details of the methods used to grow bacteria and for recording spectra are given in Sections 2.6; 2.7 and 2.12.

a. (Dithionite reduced)-minus-(H_2O_2 oxidized) difference spectrum of whole Methylophilus methylotrophus cells grown in continuous culture under conditions of nitrogen-limitation (6.7mg dry weight bacteria/ml). Absorbance marker: 0.02 (10mm light path).

b. (Dithionite reduced)-minus-(H_2O_2 oxidized) difference spectrum of whole Methylophilus methylotrophus cells grown in continuous culture under conditions of methanol-limitation (8.0mg dry weight bacteria/ml). Absorbance marker: 0.02 (10mm light path).

c. Difference spectrum obtained after aeration of a suspension of whole Methylophilus methylotrophus cells; reduction of the sample with dithionite and allowing endogenous reduction to occur in the reference cuvette. A similar result was obtained from (dithionite reduced)-minus-(aerobic plus either methanol or ascorbate/TMPD) difference spectra. 47mg dry weight bacteria/ml, absorbance marker: 0.005 (2mm light path).


556
563
550
420
ΔA
520
550
602
420
ΔA
520
c
b
b
a
a
400
450
500
550
600
650
Wavelength (nm)

methanol-excess or methanol-limitation which were determining the absence or presence of the cytochrome rather than the oxygen tension or nitrogen supply as such.

Two species of cytochrome b were identified in reduced-minus-oxidized difference spectra (Fig. 4.1). Cytochrome c usually obscures the absorption bands of the cytochromes b. In these experiments the demonstration of the cytochromes b depended on the fact that, after aeration, cytochrome c becomes reduced before the cytochromes b by endogenous reductants and so the total quantity of the b-type cytochromes could not be estimated from these spectra. It was estimated that the total amount of b-type cytochromes was not markedly different in bacteria grown under different conditions.

Cytochrome(s) c having absorption maxima in reduced-minus-oxidized difference spectra at 550, 520 and 420nm was always produced by Methylophilus methylotrophus in high quantities and an unusual feature was that 35% of the total was released into the growth medium in both batch and continuous culture (Fig. 4.2; Table 4.1). This amount was far too high to be attributed to cell lysis. Washing of the cells did not remove significantly more cytochrome nor did it affect the rate of methanol oxidation. The cytochrome released into the growth medium was fully reduced when kept in the dark, but on exposure to light the cytochrome was reversibly photo-oxidized. This effect was lost on concentration (7x) using a 10,000MW cut-off filter and restored on addition of the ultrafiltrate, indicating that the molecular weight of at least one of the components involved in the photo-oxidation was less than 10,000 (the cytochrome was retained by the filter). The 'spent' growth medium and ultrafiltrate showed a freen fluorescence in UV light and it is possible that the photo-oxidation was catalysed by a molecule such as a flavin which may have been released into the growth medium.

Disruption of the bacteria by passage through a French pressure cell released further quantities of cytochrome(s) c, but even after washing of particulate fractions with strong salt solutions (500mM-KCl) and further sonication to disrupt vesicles, half the cytochrome c remained bound to the membranes (Fig. 4.2; Table 4.1). The cytochrome remaining bound to the membrane did not appear to be a different type on the basis of redox potential (Sections 4.5 and 5.5) or carbon monoxide binding (Section 4.3).

Fig. 4.2 - Difference spectra of the growth medium and broken-cell preparations of Methlophilus methylotrophus

The methods used for recording spectra are described in Section 2.12. All spectra were recorded as (dithionite reduced)-minus-(H_2O_2 oxidized) difference spectra; light path 10mm.

a. Growth medium after removal of whole bacteria by centrifugation. Absorbance marker: 0.005.

b. Membranes from methanol-limited bacteria (1.42mg protein/ml). Absorbance marker: 0.01.

c. Soluble cell fraction after the removal of whole bacteria and cell debris (5mg protein/ml). Absorbance marker: 0.02.

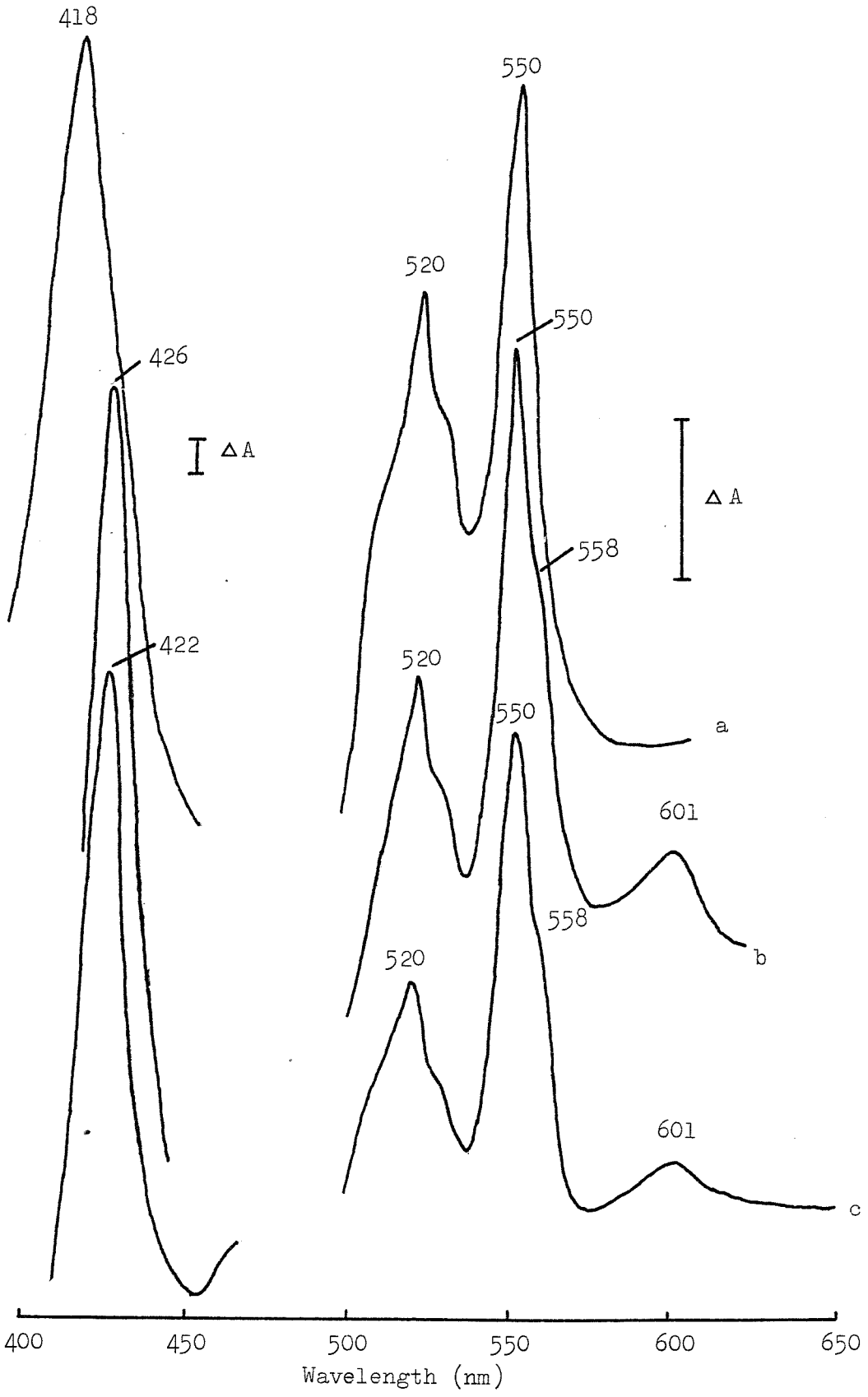

418
426
422
ΔA
550
520
550
558
ΔA
520
550
601
558
520
601
a
b
c
400
450
500
550
600
650
Wavelength (nm)

Table 4.1 - The cytochromes produced by Methylophilus methylotrophus

The methods used to grow bacteria, to estimate the quantities of the cytochromes and for recording difference spectra are described in Sections 2.6, 2.7 and 2.12.

Cytochrome	Growth conditions	Absorption maxima in difference spectra (nm)		Quantity		% of cytochrome binding CO
		22°C	77°K	i. pmol/mg dry wt.bacteria	ii. nmol/mg membrane prot.	
Cytochrome a/a_3	Batch	602	600.5, 440	nd-160		n.k.
	Methanol-limited	602	600.5, 440	160	0.2	n.k.
	Methanol-excess	nd	-	-	-	-
Cytochrome b	All conditions	563, 555, 428	563, 558*, 554.5	220	0.55	n.k.
Cytochrome c	All conditions					
	i. whole bacteria	550, 520, 420	548.5/545.5** (split)	835	-	30%
	ii. membranes			400	1.22	31%
	iii. soluble preparations			432	-	33%
	iv. growth medium			480	-	35%

n.d. = not detected.

n.k. = not known.

* This b_{558} peak was not present in methanol-limited preparations.

** Cytochrome c did not show splitting on membranes from methanol-limited bacteria.

4.3 Reaction of the Cytochromes of Methylophilus methylotrophus with Carbon Monoxide

Carbon monoxide-binding cytochromes of three types were detected in whole bacteria and cell fractions (Fig. 4.3). The presence of a c-type cytochrome able to bind carbon monoxide was indicated by the trough at 550nm and the peaks at 535 and 410nm. This binding was exhibited to the same extent (about 30%; Table 4.1) in whole bacteria, growth medium and both soluble and particulate cell fractions. In bacteria grown under conditions of methanol-excess, a b-type cytochrome capable of binding carbon monoxide was indicated by the trough at 429nm and the shoulder at 556nm (Fig. 4.3b); a small amount of this carbon monoxide-binding cytochrome b was also indicated in spectra of methanol-limited bacteria (Fig. 4.3a). Cytochromes of the b-type which react with carbon monoxide are often assumed to have an oxidase function and are thus called cytochrome o (Castor and Chance, 1955). Although the demonstration of carbon monoxide-binding alone is not sufficient to justify the conclusion that this carbon monoxide-binding cytochrome b is an oxidase, the results given elsewhere in this thesis support this conclusion and it is therefore referred to throughout the rest of this work as cytochrome o.

Cytochrome a_3 was indicated by the trough at 440nm in spectra of methanol-limited bacteria (Fig. 4.3). Under growth conditions where cytochrome a/a_3 was not present, no carbon monoxide binding a-type cytochrome was detected. The quantity of the cytochromes and their spectral characteristics are given in Table 4.1. Exposure to carbon monoxide for 15 seconds was sufficient to cause maximal binding to cytochrome a_3. Approximately 30 seconds exposure was necessary for the maximal formation of the cytochrome o-CO complex depending on the preparation. Cytochrome c required at least 10 minutes incubation with carbon monoxide for full combination.

4.4 Difference Spectra measured at 77°K

Reduced-minus-oxidized difference spectra of bacterial membranes at 77°K (Fig. 4.4) revealed the presence of 3 b-type cytochromes with α-absorption maxima at 563, 558 and 554.5nm. In membranes prepared from bacteria grown under conditions of methanol excess, cytochrome a/a_3 was absent, but there appeared to be more of the 558nm

Fig. 4.3 - The reaction of carbon monoxide with the cytochromes of *Methylophilus methylotrophus*

Spectra were recorded as described in Section 2.12.

a. Methanol-limited whole bacteria (2.76mg dry weight bacteria/ml). (Dithionite-reduced plus CO)-minus-(dithionite-reduced) difference spectrum; CO was passed through the suspension for 15 sec. Absorbance marker: 0.005.

b. Methanol-excess whole bacteria (oxygen-limited) (5mg dry weight bacteria/ml). (Dithionite-reduced plus CO)-minus-(dithionite-reduced) difference spectrum; CO was passed through the suspension for 30 sec. Absorbance marker: 0.01.

c. Soluble cell fraction (7mg protein/ml). (Dithionite-reduced plus CO)-minus-(dithionite-reduced) difference spectrum; CO was passed through the preparation for 5 min followed by a 10 min incubation in the dark. Absorbance marker: 0.01.

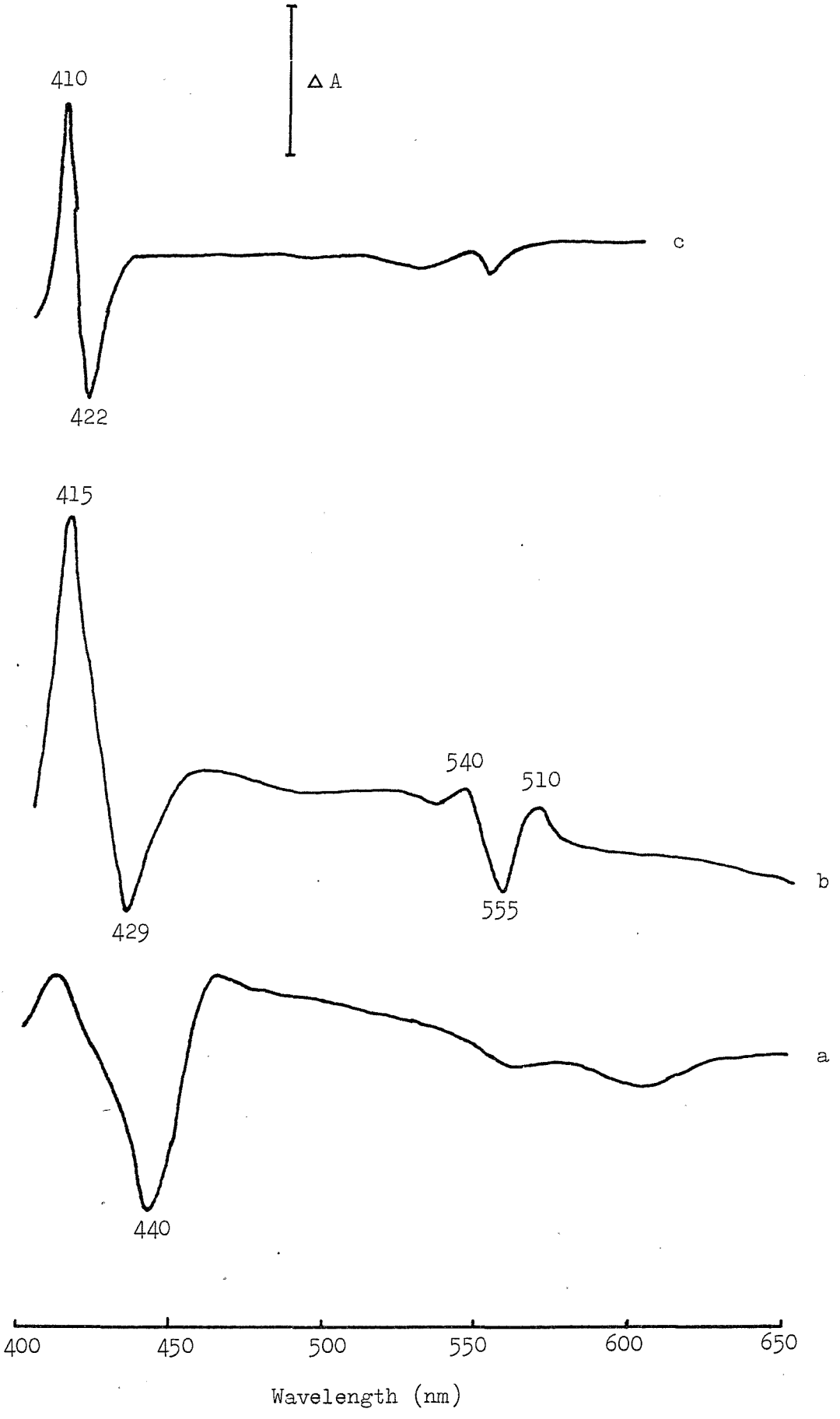

410
ΔA
c
422
415
540
510
b
429
555
a
440
400
450
500
550
600
650
Wavelength (nm)

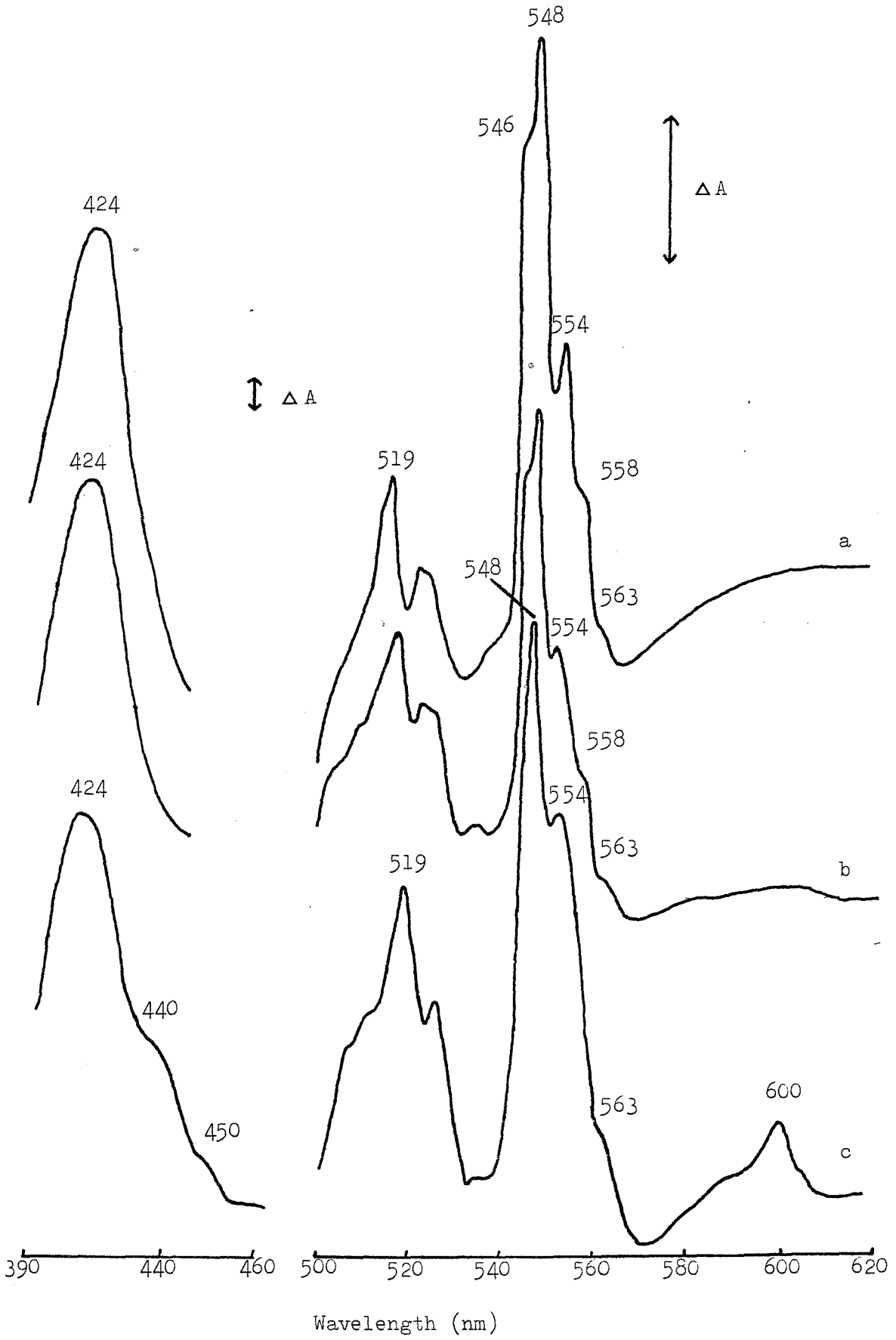
Fig. 4.4 - Reduced-minus-oxidized difference spectra of Methylophilus methylotrophus membrane preparations at 77°K

Details of the methods used for the growth of bacteria and for recording spectra are given in Sections 2.6; 2.7 and 2.12. All spectra were recorded as (dithionite-reduced)-minus-(H_2O_2-oxidized) difference spectra; the light path was 2mm. Identical spectra were obtained when NADH was used to replace dithionite as the reductant.

a. Membranes from methanol-excess bacteria (oxygen-limited); 15.8mg protein/ml. Absorbance marker: 0.05.

b. Membranes from methanol-excess bacteria (nitrogen-limited); 10.1mg protein/ml. Absorbance marker: 0.02.

c. Membranes from methanol-limited bacteria; 12mg protein/ml. Absorbance marker: 0.03.


548
546
ΔA
424
554
ΔA
519
424
558
a
548
563
554
424
558
554
563
519
b
440
450
600
563
c
390
440
460
500
520
540
560
580
600
620
Wavelength (nm)

cytochrome. It may be that this cytochrome was the cytochrome o because the quantity of the cytochrome o appeared to be higher in these growth conditions as judged on the basis of respiratory activity (Section 6.3).

Because spectra were identical with NADH or dithionite as reductant it was concluded that there was an electron transport pathway between NADH and all the cytochromes demonstrated in these spectra.

Spectra of (hydroquinone-reduced)-minus-(ferricyanide-oxidized) membrane preparations showed reduction of all the cytochrome components with the exception of cytochrome b_{563} indicating that this cytochrome had the lowest redox potential.

The soluble cell fraction of both methanol-limited and methanol-excess bacteria (oxygen- or nitrogen-limited) exhibited a split α cytochrome c peak at low temperature (Fig. 4.5). It is shown later that there are 3 soluble cytochromes c in cell extracts and that the 2 major components both show α-band splitting at 77°K (Section 5.4). The α-peak of cytochrome c was also split on membranes of methanol-excess (oxygen- or nitrogen-limited) bacteria giving a peak at 548.5nm and a shoulder at 545.5nm; these are similar to the split peaks of the pure cytochromes c (Section 5.4) indicating the α-peaks of some of the cytochromes c also split at 77°K when the cytochromes are bound to the membrane. On membranes of methanol-limited bacteria no splitting of the α-peak was observed (Fig. 4.4). There is insufficient evidence to distinguish between the possibility that the cytochromes are arranged on the membrane in such a way that splitting no longer occurs; and the possibility that the small amount of a third different cytochrome c (which does not show splitting) present on these membranes (Section 4.5) might obscure any splitting of the α-band of the other cytochromes that may occur.

Membranes of methanol-limited bacteria always had a component detected at 77°K in the (dithionite-reduced)-minus-(H_2O_2 -oxidized) difference spectrum at 450nm which was absent from membranes of methanol-excess bacteria. The identity of this component is unknown.

4.5 Redox Potentiometry of the Membrane-bound Cytochromes

Oxidation reduction midpoint potentials of the membrane-bound cytochromes were determined as described in Section 2.14 in preparations

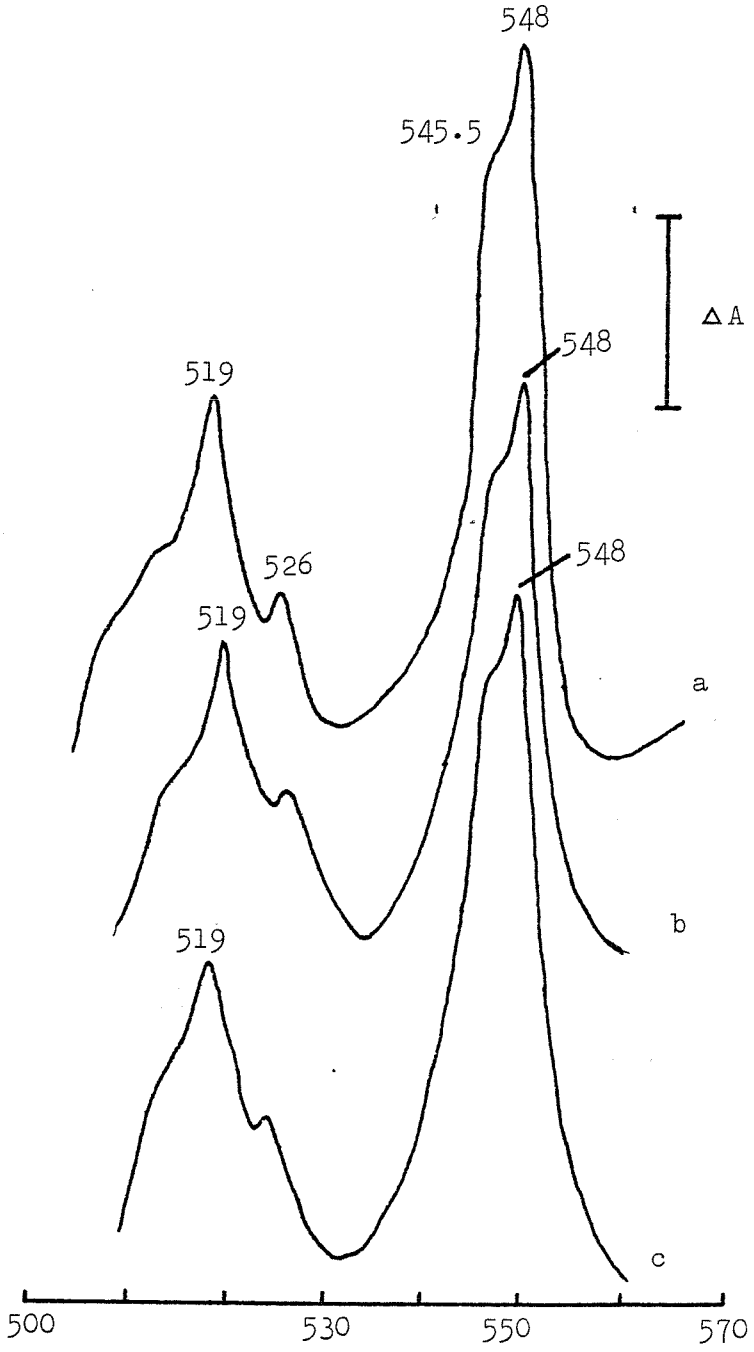
Fig. 4.5 - Reduced-minus-oxidized difference spectra of the soluble fraction of Methylophilus methylotrophus at 77°K

Details of the methods used for the growth of bacteria and for recording spectra are given in Sections 2.6; 2.7 and 2.12. All spectra were recorded as (dithionite-reduced)-minus-(ferricyanide-oxidized) difference spectra; the light path was 2mm.

a. Cell-free extract from methanol-limited bacteria; 6.95mg protein/ml. Absorbance marker: 0.1.

b. Cell-free extract from methanol-excess bacteria (nitrogen-limited); 5.2mg protein/ml. Absorbance marker: 0.1.

c. Cell-free extract from methanol-excess bacteria (oxygen-limited); 7mg protein/ml. Absorbance marker: 0.1.


548
545.5
ΔA
519
548
526
519
548
a
519
b
c
500
530
550
570
Wavelength (nm)

from nitrogen- and methanol-limited bacteria. The wavelength pairs used were 550-540nm for cytochrome c; 559-570nm for cytochromes b and c; and 563-570nm for cytochromes b. The results of the titrations are shown in Figs. 4.6, 4.7, 4.8 and 4.9, and are summarized in Table 4.2. The absorption changes due to cytochrome c contribution to the cytochrome b titration (563-570nm) were not analysed (i.e. changes above 300mV) and thus are not shown in Figs. 4.6 and 4.7. The contributions of the b cytochromes to the cytochrome c titration (550-540nm) were very small and are also not shown (Figs. 4.8 and 4.9).

From the results of these titrations (Figs. 4.6 - 4.9; Table 4.2) it can be seen that under conditions of methanol-excess (nitrogen-limitation) there were 3 cytochromes b with midpoint redox potentials of 260mV, 109mV and 60mV which contributed to the absorption changes at 563-570nm in the proportion 25%, 37% and 39% respectively. By contrast, under conditions of methanol-limitation only two components were found (Fig. 4.7) with midpoints of 110mV and 61mV contributing 16% and 84% respectively. Thus under conditions of methanol-excess and extra b cytochrome was present with a midpoint potential of 260mV. This b cytochrome is almost certainly the cytochrome o shown to be present under these conditions (methanol-excess) to a much greater extent than in conditions of methanol-limitation where cytochrome a/a_3 was the terminal oxidase (Section 4.2). Cytochromes o typically have high redox potentials (greater than 100mV) which are consistent with their oxidase function. Low temperature spectra (Fig. 4.4) indicate that this cytochrome (cytochrome o) is the cytochrome absorbing at 558nm at 77°K. The percentage contribution to the absorbance changes during the redox titration only reflect the relative amounts of each component if the absorption maxima are very similar and if the molar absorption coefficients are the same. In the case of the cytochromes b considered here these conditions do not apply. The change in percentage contributions in membranes from bacteria grown in different growth conditions do, however, reflect changes in the relative proportions of the cytochromes b with changing growth conditions. Thus the proportions of the two cytochromes b with midpoint redox potentials of 60 and 110mV are clearly different in membranes of bacteria grown in different growth conditions; in bacteria grown in methanol-excess conditions (nitrogen-limited) the proportion of the lower (60mV) to higher potential (110mV) cytochrome b is relatively lower than in

Fig. 4.6 - Potentiometric titration of the membrane bound cytochromes b of methanol-excess (nitrogen-limited) Methylophilus methylotrophus

The methods used for the growth of bacteria and for potentiometric titrations as described in Sections 2.6; 2.7 and 2.14. The wavelength pair used were 563-570nm.

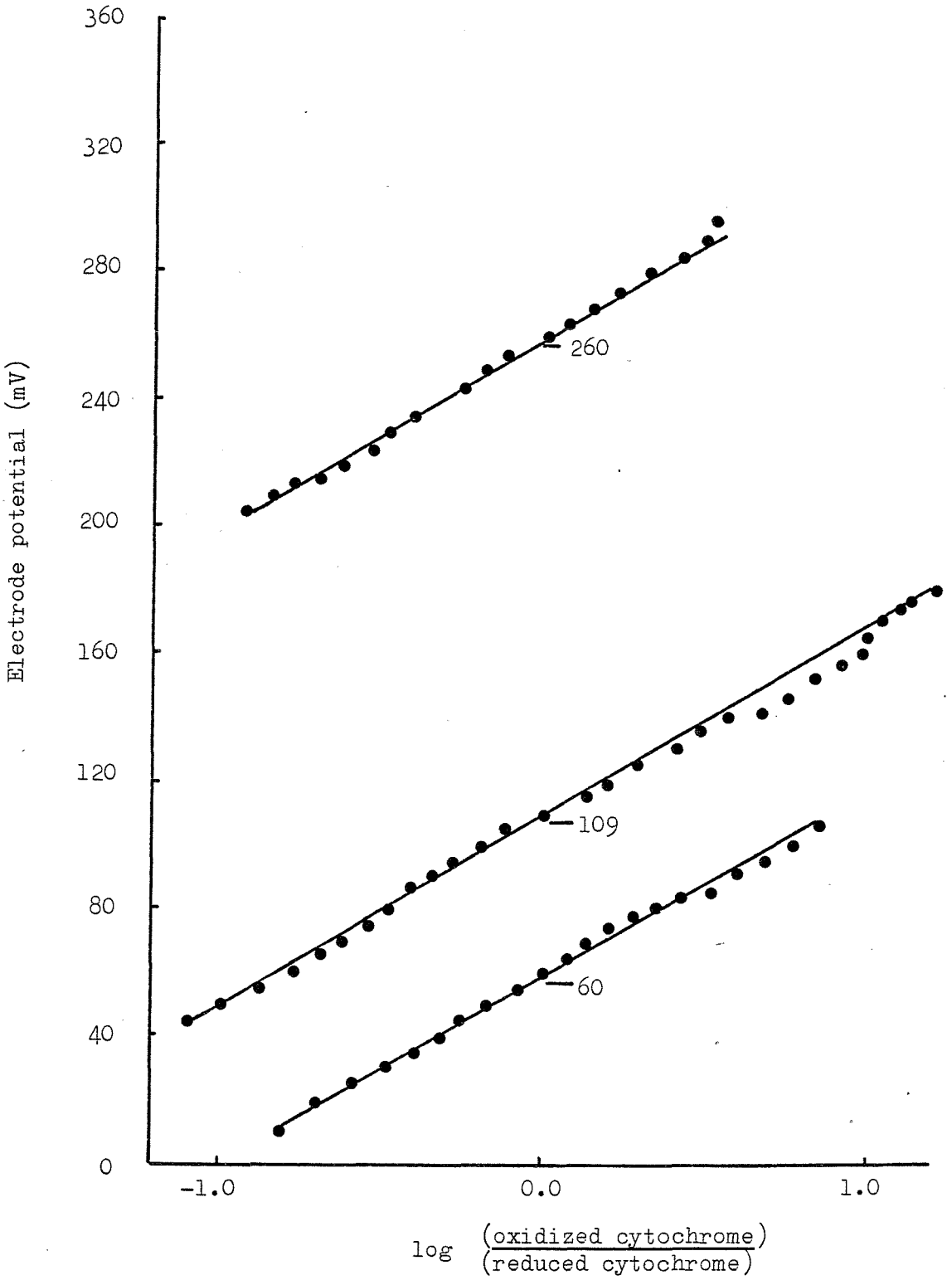


Fig. 4.7 - Potentiometric titration of the membrane-bound cytochrome b of methanol-limited Methylophilus methylotrophus

The methods used for the growth of bacteria and for potentiometric titrations are described in Sections 2.6; 2.7 and 2.14. The wavelength pair used were 563-570nm.

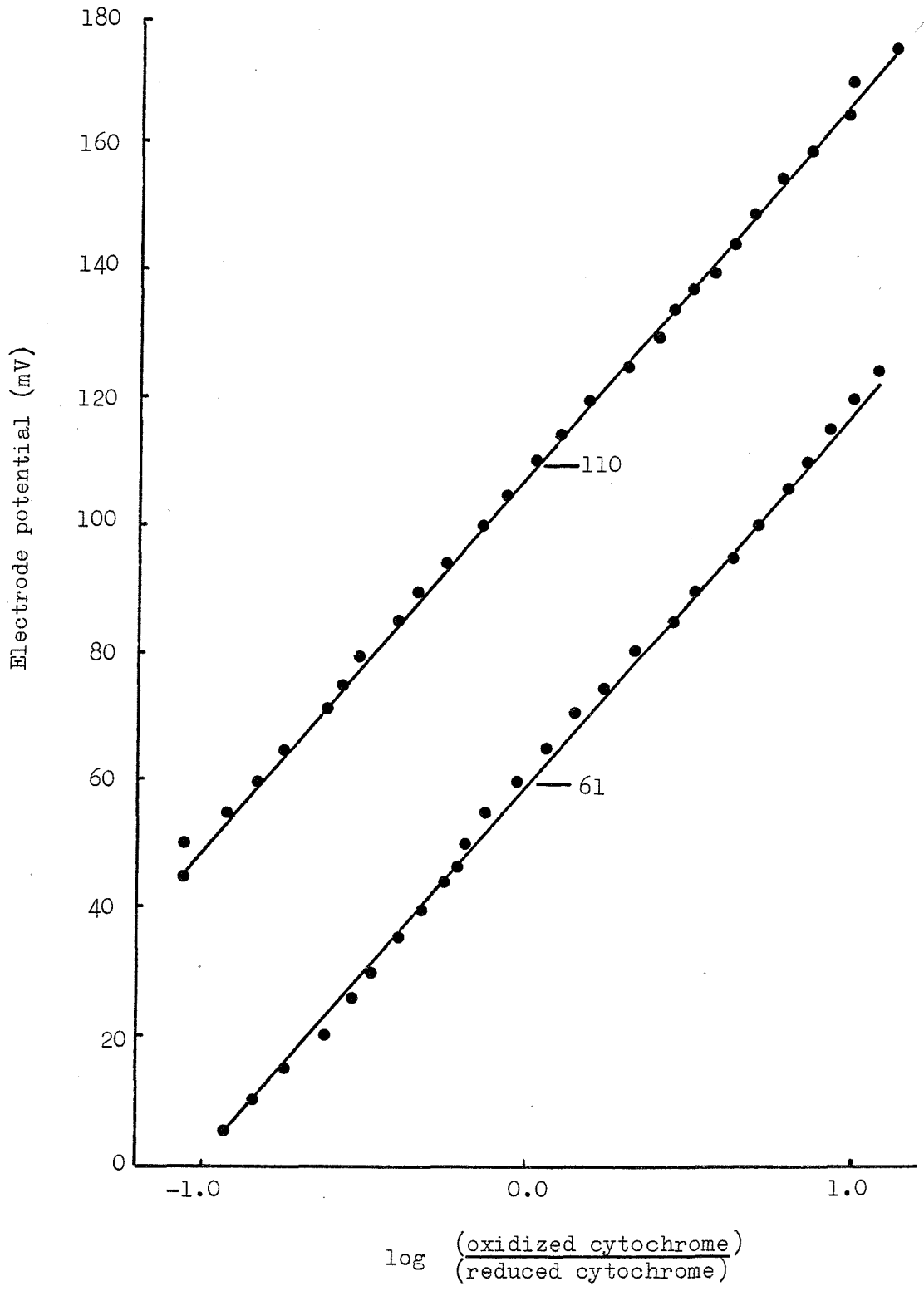


Fig. 4.8 - Potentiometric titration of the membrane-bound cytochromes c of methanol-excess (nitrogen-limited) Methylophilus methylotrophus

The methods used for the growth of bacteria and for potentiometric titrations are described in Sections 2.6; 2.7 and 2.14. The wavelength pair used were 550-540nm.

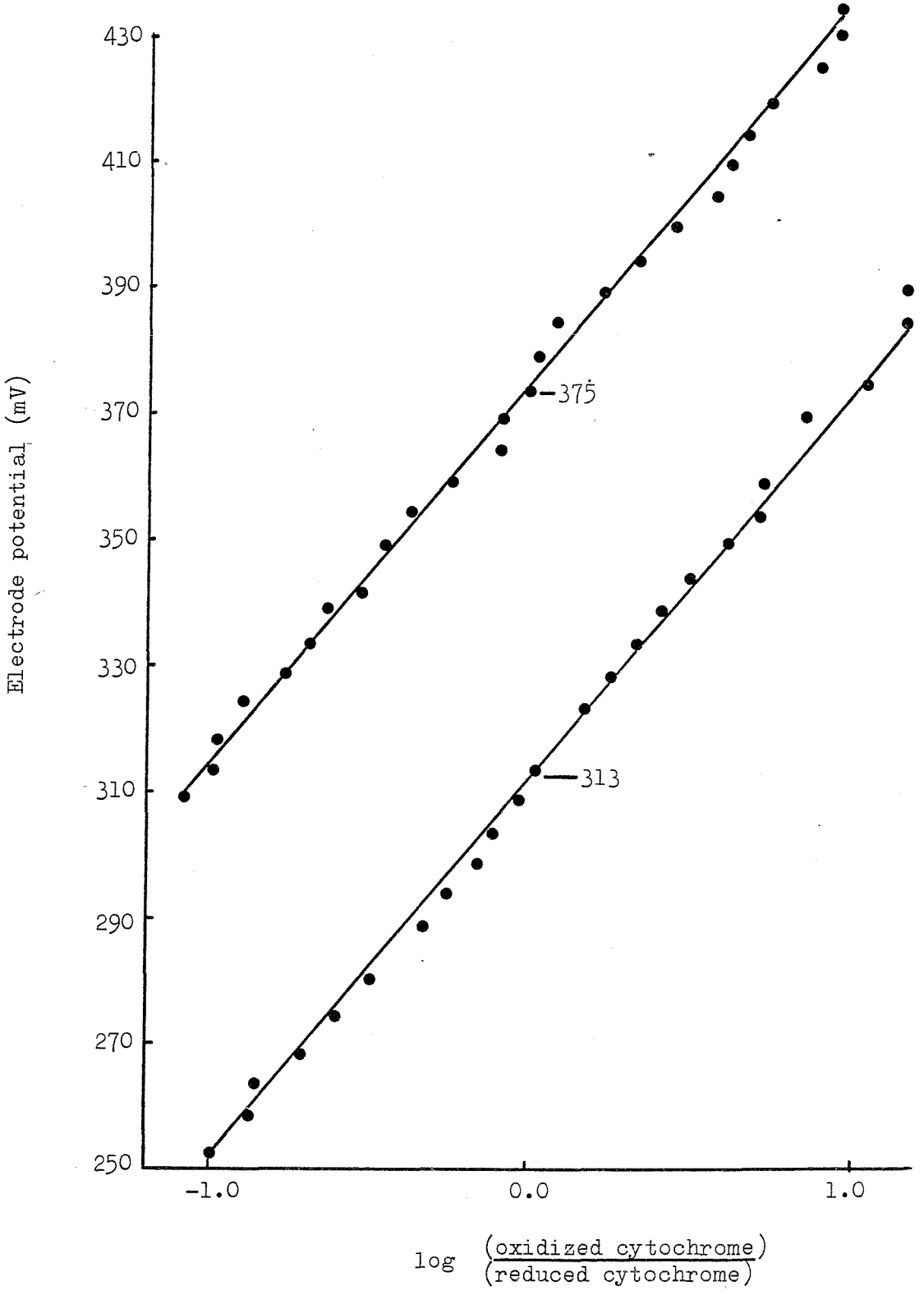
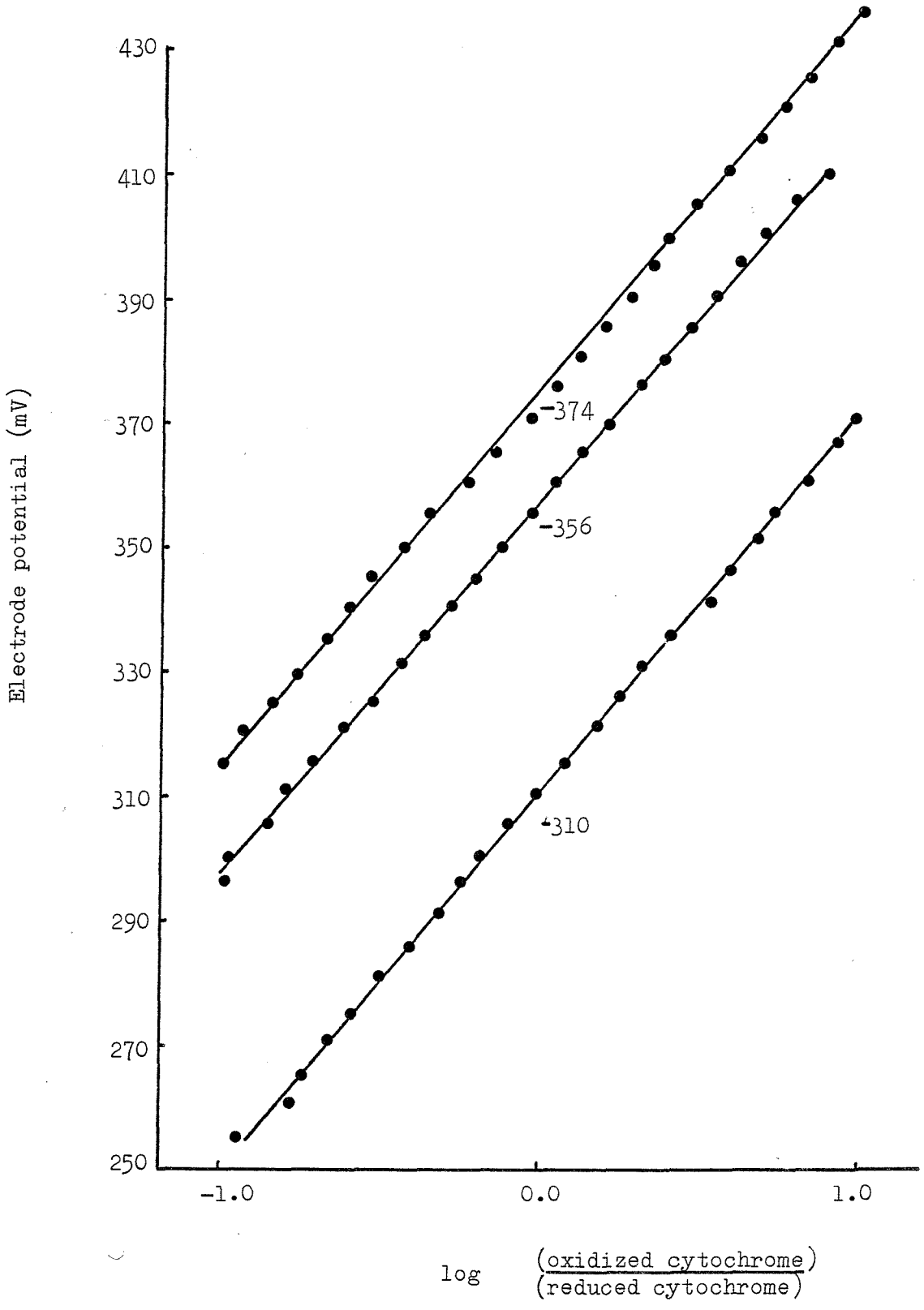


Fig. 4.9 - Potentiometric titration of the membrane-bound cytochromes c of methanol-limited Methylophilus methylotrophus

The methods used for the growth of bacteria and for potentiometric titrations are described in Sections 2.6; 2.7 and 2.14. The wavelength pair used were 550-540nm.



bacteria grown methanol-limited. Although it is not possible to measure accurately the total amount of cytochrome b, the total was not markedly different in bacteria grown in different growth conditions. Spectra of samples reduced with hydroquinone suggested that cytochrome b_{563} had the lowest midpoint redox potential (Section 4.4) and thus the most likely assignment of wavelengths to the b-type cytochromes is as follows :-

	Midpoint redox potential
Cytochrome o (b_{558})	260mV
Cytochrome b_{554}	110mV
Cytochrome b_{563}	60mV

Resolution of the data for the cytochrome c titrations was difficult due to the proximity of the midpoints but the best fit was obtained with 2 components in the membranes from methanol-excess (nitrogen-limited) bacteria (Fig. 4.8), and 3 components in membranes from methanol-limited bacteria (Fig. 4.9). Two components appeared to be the same in both samples, with midpoint redox potentials of 375mV and 310mV; it is probable that these are cytochromes c_H and c_L respectively since the midpoint redox potentials were the same as those found for the purified cytochromes (Chapter 5). The third component (midpoint redox potential 356mV) which was present only in membranes from methanol-limited bacteria was probably cytochrome c_K (Chapter 5), which has a midpoint redox potential of 337mV; similar small changes between soluble and membrane-bound cytochromes have been found for some other cytochromes c (Dutton et al., 1974). The total amount of cytochrome c was not markedly different in bacteria grown in different conditions and the results in Table 4.2 indicate that about 70% of the membrane-bound cytochrome c was cytochrome c_H while the proportion of cytochrome c_L might be lower in methanol-limited conditions.

It was not possible to determine the midpoint redox potential of the cytochrome a/a_3 as the potential required to oxidize the cytochrome was greater than could be satisfactorily reached with potassium ferricyanide (above 400mV).

4.6 Reduction of Cytochromes in Membranes and Whole Bacteria by NADH and Methanol

In methanol-limited bacteria the cytochromes did not become

Table 4.2 - Midpoint redox potentials of the membrane-bound cytochromes

Growth conditions	cytochrome	E_{m7} (mV)	% contribution to absorbance change
Methanol-excess (nitrogen-limited)	c_H	375	69%
	c_L	313	31%
	o_{558}	260	25%
	b_{556}	109	37%
	b_{563}	60	39%
Methanol-limited	c_H	374	69%
	c_K	356	15%
	c_L	310	16%
	b_{556}	110	16%
	b_{563}	61	84%

The midpoint redox potentials were taken from the results given in Figs. 4.6 - 4.9. The wavelength pairs were 550-540nm and 563-570nm. Because the cytochromes c have virtually the same α absorption maxima at room temperature, these percentage contributions are a measure of the amount of each cytochrome c present (assuming similar molar absorption coefficients). This does not apply to the b-type cytochromes (Section 4.5).

reduced in the absence of added substrate. Addition of NADH to an aerobic suspension of Methylophilus methylotrophus caused a rapid reduction of all the cytochromes; the addition of methanol to aerobic suspensions of bacteria caused a rapid reduction of cytochrome(s) c, a/a_3 and some of the b-type cytochromes. The remainder of the cytochromes b became reduced only after a period of several minutes. Similar results were obtained with bacteria grown in batch cultures, or under conditions of methanol-excess, except that there was sufficient endogenous substrate present to fully reduce all the cytochrome c, although the rate of reduction was always slower than in the presence of added substrate. Membrane preparations did not oxidize methanol and the cytochromes were not reduced by methanol in spite of the fact that at least 60% of the methanol dehydrogenase was bound to the membranes (Chapter 6). By contrast, membrane preparations did oxidize NADH and this substrate rapidly reduced all the cytochromes present on the membranes.

4.7 Summary and Discussion

The results of work presented in this chapter are summarized for convenience in Table 4.3; the most important conclusions are as follows :-

1. The potential oxidase, cytochrome a/a_3, was only present in bacteria grown in methanol-limited cultures, while the total quantity of cytochromes b and c were not markedly affected by the growth conditions.

2. There are 3 b-type cytochromes. Two of these, with α-absorption maxima of about 557nm and 563nm (measured at room temperature) were always found present on membranes and their midpoint redox potentials were 110mV and 60mV respectively. There appeared to be relatively more of the cytochrome b_{563} in methanol-limited bacteria.

3. The third b-type cytochrome, reacted with CO, had an α-absorption maximum at 558nm (at 77°K; the absorption maximum at room temperature is not known) and a high midpoint redox potential (260mV). This cytochrome is thus a potential oxidase of the cytochrome o type and is referred to as cytochrome o throughout the rest of this thesis.

Table 4.3 - Summary of the properties of membrane-bound cytochromes of Methylophilus methylotrophus

Growth Conditions	Cytochrome	E_{m7} (mV)	α-Absorption max (at 77°K)	% contribution to abs. change
Methanol excess	cytochrome c_H	375	split 548	69
	cytochrome c_L	313	545.5(sh)	31
	cytochrome o	260	558	25
	cytochrome b	109	554	37
	cytochrome b	60	563	39
Methanol limitation	cytochrome c_H	374		69
	cytochrome c_K	356	548	15
	cytochrome c_L	310		16
	cytochrome b	110	554	16
	cytochrome b	61	563	84
	cytochrome a/a_3	nd	601	

nd = not determined.

4. The cytochrome o is detectable (by CO-binding) in methanol-limited bacteria and is present in relatively much larger amounts in bacteria grown in methanol-excess conditions.

5. Redox potentiometry demonstrated that 2 cytochromes c were always present on membranes; the major component (about 70% of the total cytochrome c) had a high midpoint redox potential (375mV) and was possibly the cytochrome c_H described in Chapter 5. The second component had a midpoint redox potential of 310mV and probably corresponds to the cytochrom c_L described in Chapter 5. A third minor cytochrome c (midpoint redox potential 356mV) was only detected on membranes of methanol-limited bacteria and probably corresponds to the cytochrome c_K described in Chapter 5. It is possible that the function of this cytochrome is to donate electrons to cytochrome a/a_3 since both were present in membranes of bacteria grown in methanol-limitation but were absent in membranes from bacteria grown in conditions of methanol-excess.

6. Splitting of the α-band of the membrane-bound cytochrome c at 77°K was only detected in bacteria grown in methanol-excess conditions. It was not possible to determine whether or not the failure to detect α-band splitting in membranes from methanol-limited bacteria was significant.

7. The concentrations of the cytochromes bound to the bacterial membrane are similar to those which have been determined in other bacteria (Gel'man et al., 1975).

8. There is a pathway for electron transport from NADH to all the cytochromes a/a_3, b and c in membranes of Methylophilus methylotrophus. All the cytochromes were reduced when NADH replaced dithionite as electron donor in reduced-minus-oxidized difference spectra.

9. There is a pathway for electron transport from methanol to all the cytochromes c and a/a_3 in whole bacteria. All the cytochromes c and a/a_3 were rapidly reduced by the addition of methanol to bacterial suspensions.

The purification and properties of the soluble cytochromes c of Methylophilus methylotrophus are described in the following chapter. An investigation of the role of the potential oxidases, cytochrome o and cytochrome a/a_3, is described in Chapter 6.

CHAPTER 5

PURIFICATION AND PROPERTIES OF THE SOLUBLE CYTOCHROMES c OF METHYLOPHILUS METHYLOTROPHUS

5.1 Introduction

The properties of many cytochromes c have been studied from bacterial sources, including some from methylotrophic bacteria such as the facultative organisms Pseudomonas AM1 (Anthony, 1975b; O'Keeffe and Anthony, 1979), Pseudomonas extorquens (Higgins, et al., 1976b), Paracoccus denitrificans (Scholes, et al., 1971), and the obligate methane utilizer Methylosinus trichosporium OB3b (Tonge et al., 1977a). The properties of methylotrophic cytochromes c are of particular interest in view of their involvement with the enzymes methanol dehydrogenase and methane mono-oxygenase (see Chapter 1 and Table 1.1), and bacterial cytochromes c are of interest in a more general sense in relation to growth efficiency, since it has been suggested that the presence of a high potential cytochrome c is a prerequisite for energy coupling at Site 3 (Jones et al., 1975).

This chapter describes the purification and properties of 3 distinct, soluble cytochromes c from Methylophilus methylotrophus; these are called cytochrome c_H, cytochrome c_K, and cytochrome c_L. Numerical subscripts have been avoided in order to prevent confusion with mitochondrial cytochrome c_1, cytochrome c_2 of photosynthetic bacteria and cytochromes c_4 and c_5 of Azotobacter etc. The subscripts H and L are used to denote cytochromes c with high and low isoelectric points respectively; the minor cytochrome component, cytochrome c_K also has a low isoelectric point.

5.2 Purification of the Cytochromes c

The method used to purify the soluble cytochromes c of Methylophilus methylotrophus is described in Section 2.16; this was based on a method devised by O'Keeffe and Anthony (1980). Three soluble cytochromes c have been purified; tables illustrating the yield and purification are given in Table 5.1. The total recovery of pure cytochromes was 24.8% of that present in the cell-free extract, 15.4% was cytochrome c_H, 1.7% was cytochrome c_K and 7.8% was cytochrome c_L. If it is assumed that the proportion of each cytochrome after the first DEAE step was the same as

Table 5.1 - Purification of the soluble cytochromes c from Methylophilus methylotrophus

Details of the method are given in Section 2.16. Cytochrome c_H is the cytochrome having a high isoelectric point; (thus not binding to DEAE cellulose), cytochrome c_K and cytochrome c_L have low isoelectric points and thus bind to DEAE cellulose. The cytochrome concentrations were calculated using a molar absorption coefficient of 19.1 litre/mmol/cm for the reduced-minus-oxidized difference spectrum, this was very close to the values found for the difference spectra of the purified cytochromes. Because accurate cytochrome estimations in crude extracts were difficult, the yield and purification factors for the later purification stages were calculated from the supernatant after acid treatment. This initial treatment gave a purification of about 3 fold in about 90% yield. The purification table assumes that the proportion of each cytochrome (c_H, c_K and c_L) recovered after the DEAE cellulose step was the same as the proportion of each cytochrome in the initial cell free extract, i.e. c_H constituted 49.6%, c_K 7.8% and c_L 42.5%.

Purification Fraction	Specific activity (nmol cyt/mg prot.)	Volume (ml)	Total cytochrome (nmols)	Yield (%)	Purification
Cytochrome c_H					
Acid supernatant	1.79	300	5372	89.5	1
DEAE cellulose eluate	2.53	190	3669	61	1.43
G-150 eluate	23.0	150	2463	41	12.8
CM cellulose (I) eluate	103.3	42	2304	38	57.4
CM cellulose (II) eluate	115.8	170	1864	31	64.4
(plus 216 nmol partially pure oxidized cytochrome)					

/Cont'd...

Table 5.1 (Cont'd.)

Purification Fraction	Specific activity (nmol cyt/mg prot.)	Volume (ml)	Total cytochrome (nmols)	Yield (%)	Purification
Cytochrome c_K					
Acid supernatant	0.282	300	844.7	89.5	1
DEAE cellulose eluate	0.55	80	577	61	2.1
G-150 eluate	17.5	84	521	55	62
DEAE cellulose (II) eluate	47.9	130	206	22	170
Cytochrome c_L					
Acid supernatant	1.534	300	4603	89.5	1
DEAE cellulose eluate	11.46	490	3140	61	7.44
G-150 eluate	21.2	180	3108	60.4	13.8
DEAE cellulose (II) eluate	29.33	295	2423	47	19.0
G-75 eluate:					
cytochrome c (21,000MW)	22.3	10	71.5	1.4	14.5
cytochrome c (17,000MW)	41.5	14	285	5.5	27.0
cytochrome c (mixed)	25.8	80	905	17.6	16.7
Isoelectric focussing of:					
cytochrome c (mixed)	43.7		581	11.2	28.4
Total recovery of cytochrome c_L			937 nmol	18%	

that in the cell-free extract, the original proportions of the cytochromes would have been 49.6% cytochrome c_H, 7.8% cytochrome c_K and 42.5% cytochrome c_L. Because it is impossible to distinguish between the 3 cytochromes in mixtures, and because the overall yield in the first 2 purification steps is only 60% the proportions in crude extracts before separation could not be determined.

5.3 Purity of the Cytochromes, their Molecular Weights and Isoelectric Points

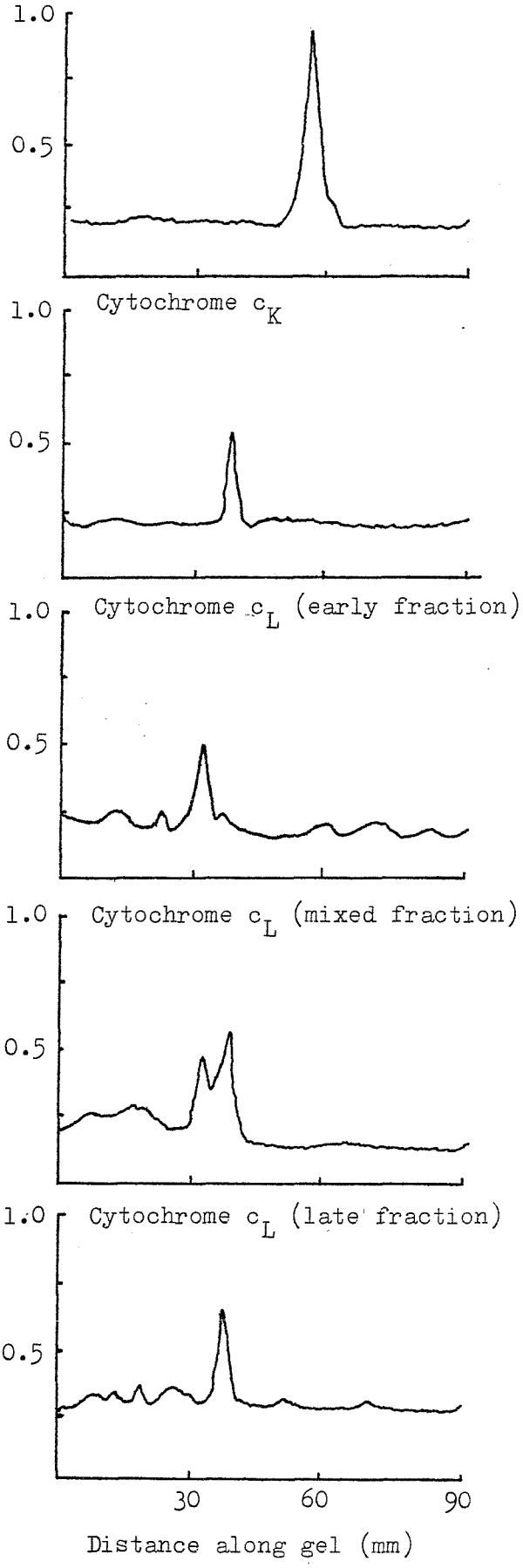
Cytochrome c_H gave a single band on SDS polyacrylamide gels after elution from a CM cellulose column, corresponding to a molecular weight of 8,500 (2 determinations); a scan of the gel stained for protein is shown in Fig. 5.1, the method is described in Sections 2.17 and 2.18. The cytochrome also gave one band after analytical isoelectric focussing; the isoelectric point of the protein was about pH 8.85 (2 determinations) (Fig. 5.2).

Cytochrome c_K gave one band on SDS gels corresponding to a molecular weight of 16,800 (2 determinations) after linear gradient elution from a DEAE cellulose column (Fig. 5.1). The cytochrome also gave one band after analytical isoelectric focussing; the isoelectric point was about pH 4.55 (2 determinations), (Fig. 5.2).

The properties of cytochrome c_L were more complex; apparently 3 distinct components were obtained by gel filtration on Sephadex G-75. The cytochrome fraction which eluted first (less than 10% of the total) gave one band on SDS polyacrylamide gels corresponding to a molecular weight of 21,000, the cytochrome fraction which eluted last (about 30% of the total) also gave one band but of 17,000 molecular weight. On SDS gel electrophoresis the intermediate cytochrome fractions gave two major cytochrome bands of approximately equal intensity (which could be seen in the unstained gel) and a number of other minor protein bands were detected by staining (see Fig. 5.1). The cytochrome bands from this intermediate fraction corresponded exactly with the bands on the gels of the first and last fractions and thus appeared to be a mixture of two cytochrome species of 17,000 and 21,000 molecular weights. When the cytochrome fractions were subjected to analytical isoelectric focussing they all gave a number of cytochrome bands (see Fig. 5.2) of similar isoelectric point (pH 4 - 4.35). The cytochromes from the first and last fractions had identical spectra and midpoint redox

Fig. 5.1 - S.D.S. polyacrylamide gel electrophoresis of the soluble cytochromes of Methylophilus methylotrophus

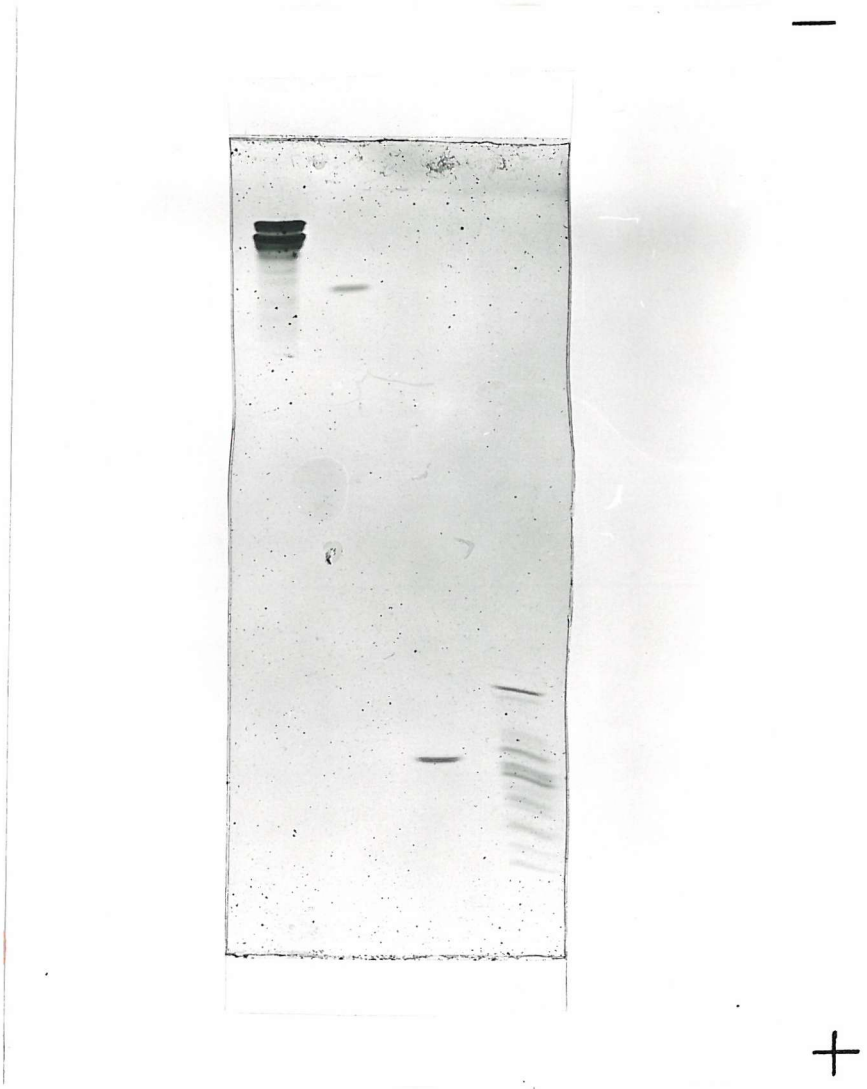
The methods for purification and electrophoresis of the cytochromes are described in Sections 2.16; 2.17 and 2.18.



	Absorption maxima (nm) (reduced form) before electrophoresis		
	α	β	γ
Cytochrome c_H	551.25	520.75	416.25
Cytochrome c_K	550.25	521.5	416.25
Cytochrome c_L (early fraction)	549.75	520.25	415.75
Cytochrome c_L (mixed fraction)	549.75	520.25	416
Cytochrome c_L (late fraction)	549.75	520.5	416

Fig. 5.2 - Analytical isoelectric focussing of the purified cytochromes c_H and c_K of Methylophilus methylotrophus

The methods used to purify the cytochromes and for analytical isoelectric focussing are given in Sections 2.16 and 2.20.



potentials (Sections 5.4 and 5.5). The mixed fraction (containing both species) was further purified by preparative isoelectric focussing (described in Section 2.19) ; this resulted in 7 cytochrome bands of similar isoelectric points (pH 4.0-4.35). Because of the proximity of the bands, some of these had to be collected together (see Fig. 5.3). Samples of the individual bands from the isoelectric focussing were run on SDS polyacrylamide gels as before (Fig. 5.4). Two cytochrome species were found of molecular weights 17,000 and 21,000 (as found in the original fractions from the Sephadex G-75 column); they were spectrally indistinguishable and had the same mid-point redox potentials (see Sections 5.4 and 5.5). The proportion of the 21,000 molecular weight species was less than in the original mixed fraction indicating that the lower molecular weight cytochrome may be a breakdown product of the higher molecular weight cytochrome. This proposal is substantiated by the similarity of the midpoint redox potentials and of the spectral properties of the two species. Furthermore, the samples after isoelectric focussing gave an additional minor band on SDS gel electrophoresis corresponding to a protein of molecular weight of about 4,000 which could be the fragment formed in the conversion of the higher molecular weight cytochrome into the lower molecular weight cytochrome. The larger cytochrome c_L (17,000 - 21,000) is unlikely to be a dimer of cytochrome c_H (8,500) because the molecular weights were determined by SDS polyacrylamide gel electrophoresis which dissociates dimers (Swank and Burris, 1969).

It is not clear why multiple bands were formed during isoelectric focussing, although this has been the case with a number of other pure cytochromes; multiple band formation is sometimes due to deamidation (Smith, 1961); the other possibilities are :-

(i) The formation of polymers;

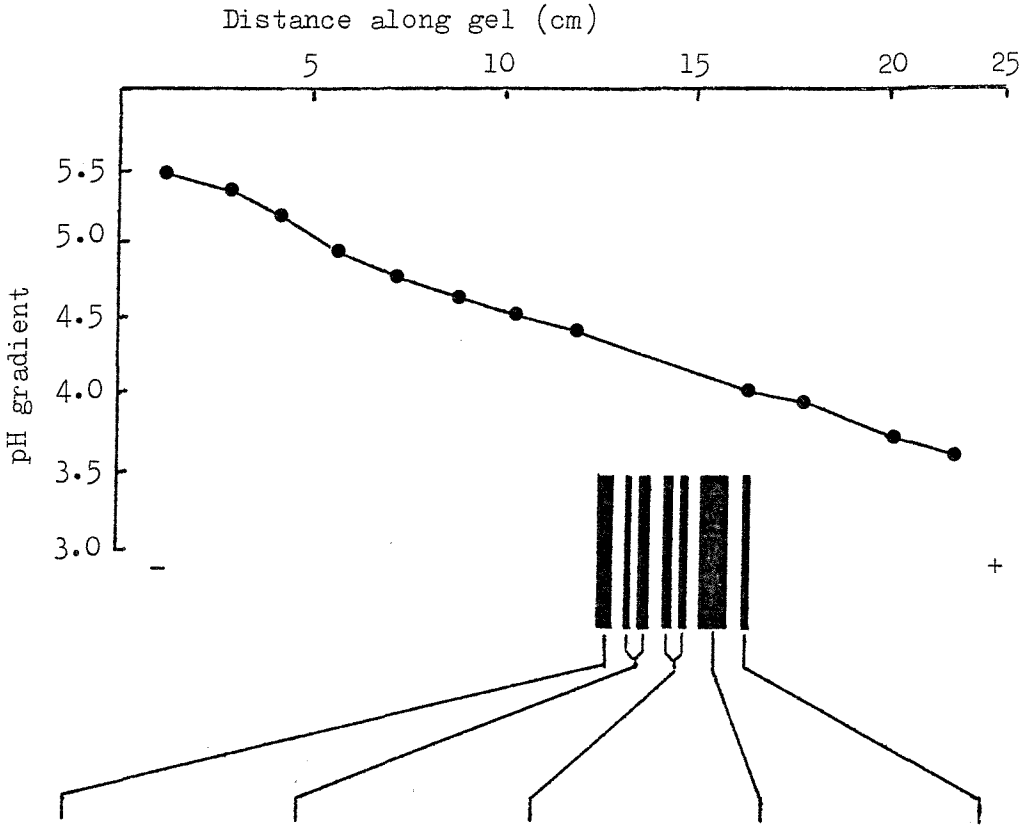
(ii) the presence of reduced and oxidized forms; or,

(iii) the presence of isocytochromes.

It is not known whether polymers are formed during isoelectric focussing but it is unlikely that they are normally present since they would have separated during the gel filtration steps. The presence of reduced and oxidized forms may account for one of the bands; when the sample was applied the cytochrome was oxidized, after isoelectric focussing all

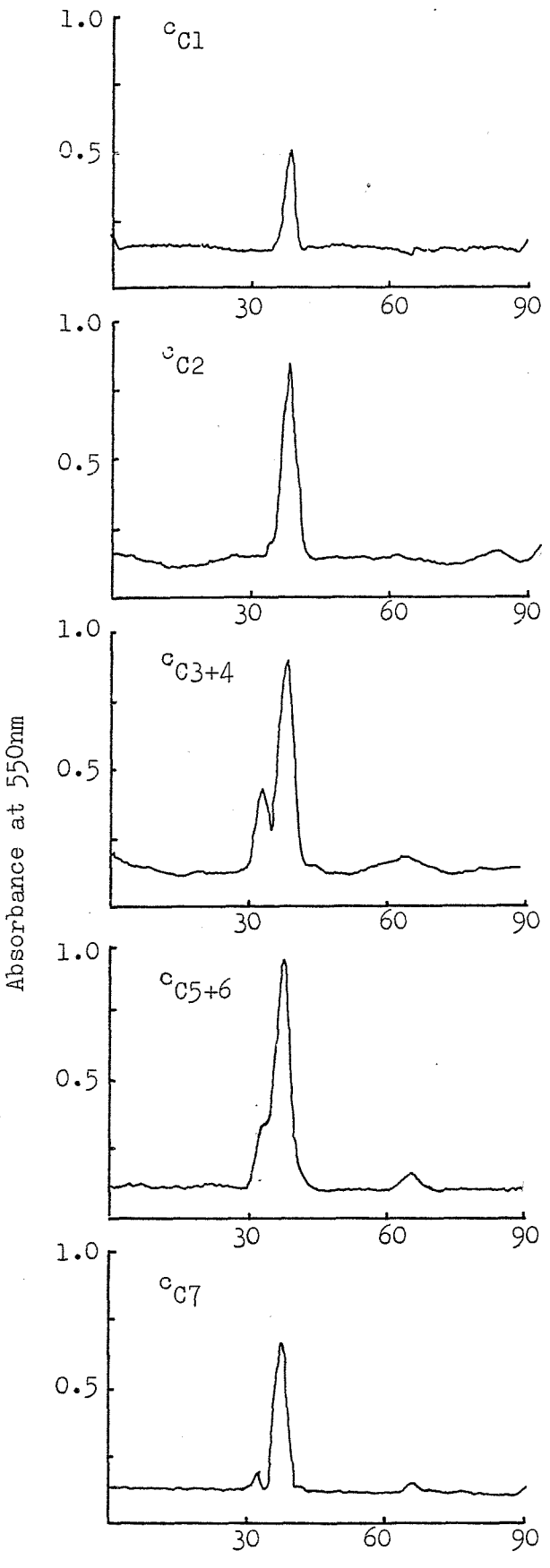
Fig. 5.3 - Preparative isoelectric focussing of cytochrome c_L

The method is described in Section 2.19



cytochrome fractions	c_L7	c_L6+5	c_L4+3	c_L2	c_L1
pI	4.35	4.30 -4.27	4.22 - 4.19	4.13	4.04
nmols cytochrome	35.5	167.1	118.4	231.6	28.9
% of total cytochrome	6.1	28.7	20	40	5

Fig. 5.4 - SDS gel electrophoresis of cytochrome c_L fractions after isoelectric focussing



	Absorption maxima of cytochrome in the reduced form before electrophoresis (nm)		
	α	β	γ
$^{c}C1$	549.75	520.25	416
$^{c}C2$	550	520.25	416
$^{c}C3+4$	549.75	520	416
$^{c}C5+6$	549.75	520.25	416
$^{c}C7$	549.75	520.25	416

The methods used for purification and electrophoresis of the cytochrome as described in Sections 2.16; 2.17; 2.18 and 2.19.

bands were oxidized except for band 1. The presence of isocytochromes having one or more amino acids substituted or absent would also account for minor differences in isoelectric point without necessarily altering other properties of the protein.

The simplest interpretation of this analysis of the soluble cytochromes c of Methylophilus methylotrophus is that the organism produces 3 different soluble cytochromes c, (cytochromes c_H, c_K and c_L) and that cytochrome c_L is in at least 2 forms; one of which may arise by the 'loss' of a 4,000 dalton component from the other.

5.4 Spectral Properties of Cytochromes c

The absorption spectra of the purified cytochromes were recorded as described in Section 2.12 at room temperature (about 22°C) and at the temperature of liquid nitrogen (77°K), (Figs. 5.5-5.7). At low temperature the absorption maxima were shifted slightly to the blue, and in the case of cytochromes c_H and c_L (both 17,000 and 21,000 M.W. forms) the alpha peak was split, in common with many other cytochromes c (Eastabrook, 1961), see also Chapter 4. These 3 cytochromes appear to be the same as those bound to the membranes of Methylophilus methylotrophus (Chapter 4).

At room temperature the absorption maxima (in nm) in the reduced form are :-

Cytochrome	α	β	Soret	77°K
Cytochrome c_H	551.25	520.75	416.25	550/546 (split)
Cytochrome c_K	550.75	521.75	416.5	548
Cytochrome c_L 21,000	549.75	520.5	415.75	547.5/545 (split)
Cytochrome c_L 17.000	549.75	520.5	416	547.5/545 (split)

The values for both species of cytochrome c are the same as those found for the isoelectric forms described in Section 5.3

None of the cytochromes c was rapidly autoxidizable. During the separation procedures cytochrome c_K and cytochrome c_L became partially oxidized; cytochrome c_K was normally about 60% oxidized, cytochrome c_L was about 85% oxidized, but after vigorous aeration the amount of cytochrome in the oxidized form could be increased. On standing, the proportion of oxidized cytochromes decreased to their original values.

Fig. 5.5 - Absorption spectra of the purified cytochrome c_H if Methylophilus methylotrophus

The method used to purify the cytochrome is described in Section 2.16 and absorption spectra were recorded as described in Section 2.12. The light path was 10mm. The spectra were recorded at 22°C.

——— Absorption spectrum of cytochrome c_H (1.99 nmol/ml) reduced with sodium dithionite.

----- Absorption spectrum of cytochrome c_H (1.99 nmol/ml) oxidized with H_2O_2.

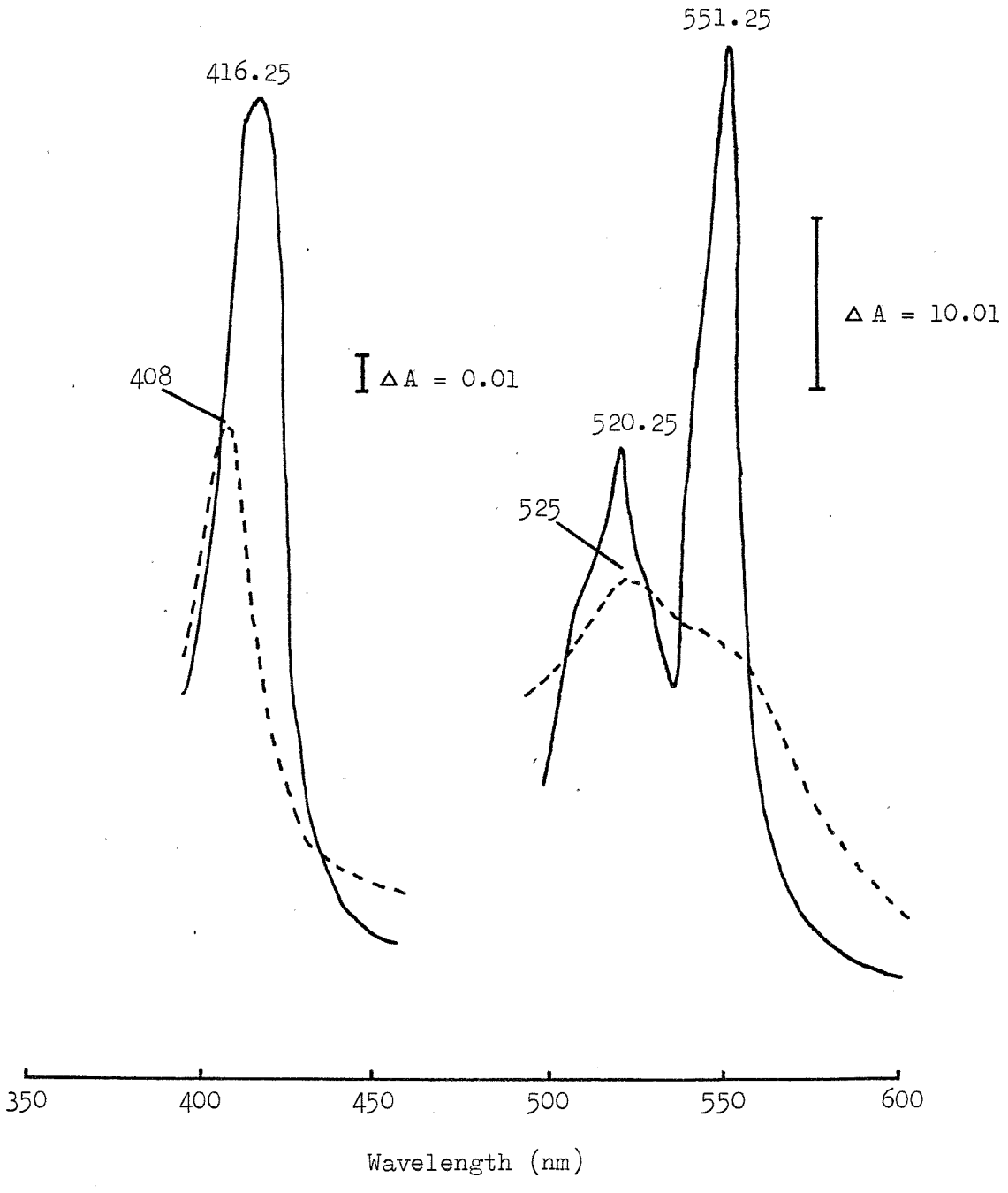

551.25
416.25
ΔA = 10.01
ΔA = 0.01
408
520.25
525
350
400
450
500
550
600
Wavelength (nm)

Fig. 5.6 - Absorption spectra of the purified cytochrome c_K of Methylophilus methylotrophus

The method used to purify the cytochrome is described in Section 2.16 and absorption spectra were recorded as described in Section 2.12. The light path was 10mm. The spectra were recorded at 22°C.

——— Absorption spectrum of cytochrome c_K (1.75 nmol/ml) reduced with sodium dithionite.

----- Absorption spectrum of cytochrome c_K (1.75 nmol/ml) oxidized with H_2O_2.

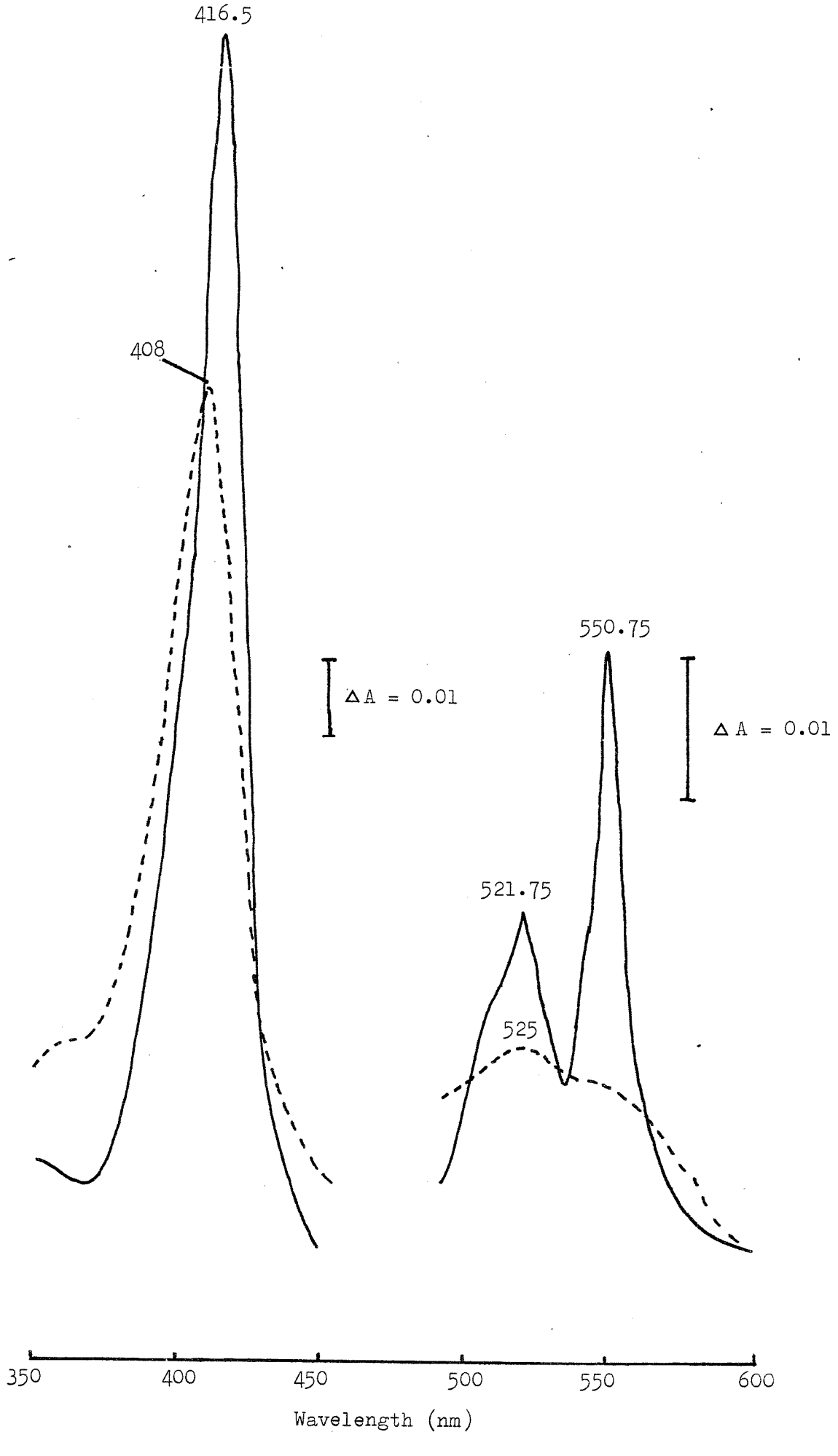

416.5
408
550.75
ΔA = 0.01
ΔA = 0.01
521.75
525
350
400
450
500
550
600
Wavelength (nm)

Fig. 5.6a - Absorption spectra of the purified cytochrome c_L of Methylophilus methylotrophus

The method used to purify the cytochrome is described in Section 2.16 and the absorption spectra were recorded as described in Section 2.12. The light path was 10mm. The spectra were recorded at 22°C.

——— Absorption spectrum of cytochrome c_L (3.42 nmol/ml) reduced with sodium dithionite.

----- Absorption spectrum of cytochrome c_L (3.42 nmol/ml) oxidized with H_2O_2.

Fig. 5.6a

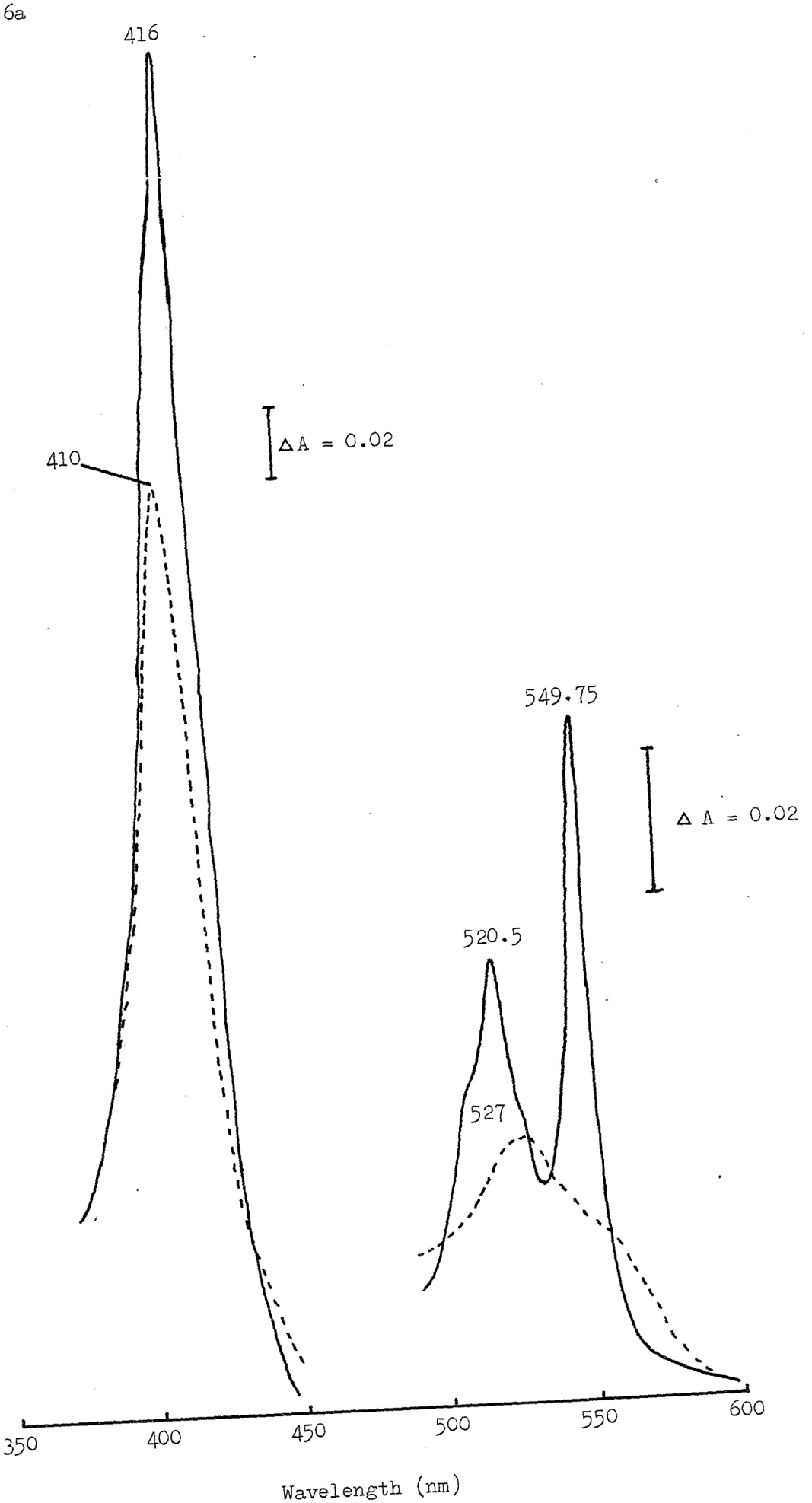
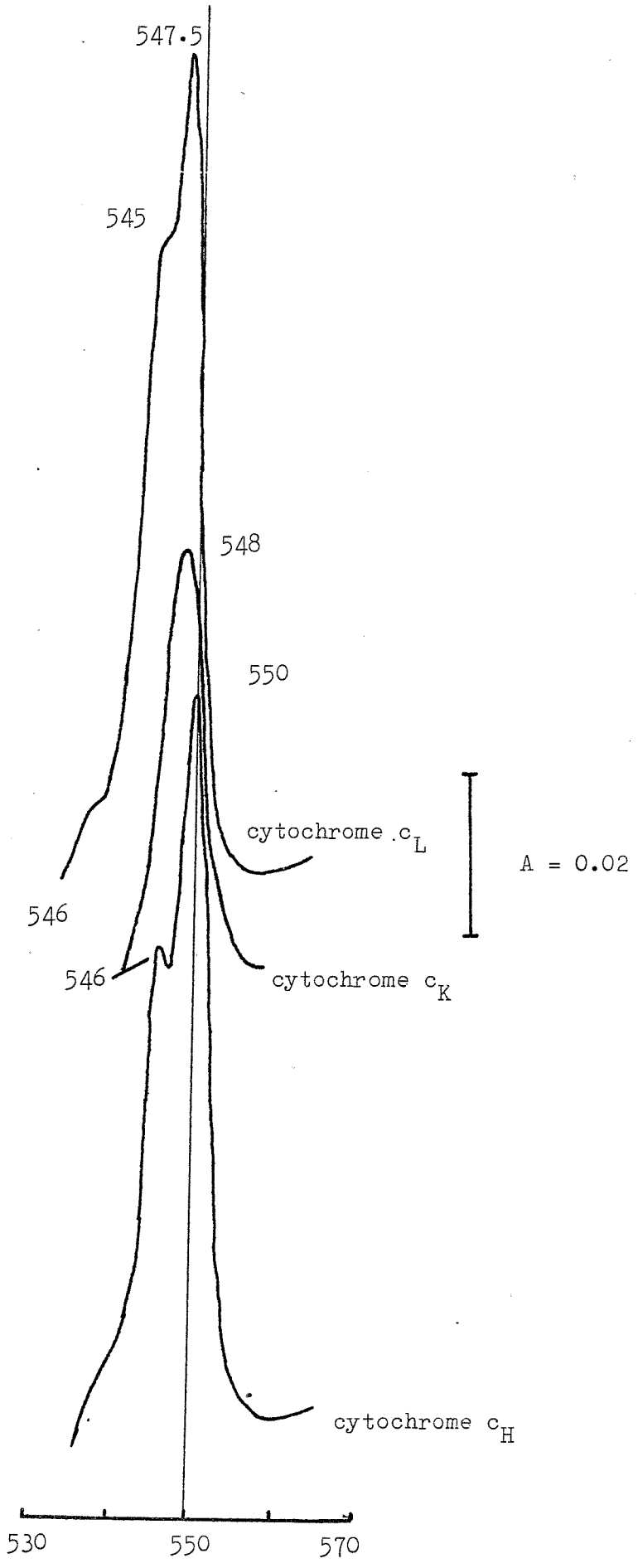

416
410
ΔA = 0.02
549.75
Δ A = 0.02
520.5
527
350
400
450
500
550
600
Wavelength (nm)

Fig. 5.7 - Absorption spectra of the purified cytochromes c_H, c_K and c_L of Methylophilus methylotrophus recorded at 77°K

The method used to purify the cytochromes is described in Section 2.16 and absorption spectra were recorded at 77°K as described in Section 2.12. The light path was 2mm. All samples were reduced with sodium dithionite; cytochrome c_H (2.8 nmol/ml), cytochrome c_K (1.75 nmol/ml), cytochrome c_L (2.5 nmol/ml).

Fig. 5.7


547.5
545
548
550
cytochrome c_L
A = 0.02
546
546
cytochrome c_K
cytochrome c_H
530
550
570
Wavelength (nm)

Cytochrome c_H was normally fully reduced in untreated samples but after vigorous aeration it became about 10% oxidized; if left unstirred it returned to the fully reduced state in about 10 minutes.

All the cytochromes bound carbon monoxide. It should be noted that it is very difficult to use values calculated from (reduced plus CO)-minus-reduced difference spectra as a reliable measure of the percentage of cytochrome c binding CO. Only if it is known that complete binding occurs, can absorption coefficients from spectra be calculated and used to estimate partial binding of CO. For the purposes of comparison it is convenient to estimate the percentage of cytochrome c binding to CO in a CO-saturated solution and for this purpose a molar absorption coefficient must be assumed. Bartsch and Kamen (1960) calculated a molar absorption coefficient at 414nm of 165 litre/mmol/cm for the (reduced plus CO)-minus-reduced difference spectrum of the cytochrome c from a Chromatium species which contains 3 haem per mol. This value was modified by Weston and Knowles (1974) to give a value of 55 (165/3) litre/mmol/cm in calculating the percentage cytochrome c binding CO for the monohaem cytochrome c from Beneckea natriegens. The same coefficient (55 litre/mmol/cm) was used by Tonge et al., (1974, 1975, 1977a) for estimation of soluble CO-binding cytochrome c in Methylosinus trichosporium and Pseudomonas extorquens. Assuming the same absorption coefficient of 55 litre/mmol/cm for the 'peak to trough' in the Soret region; 7% of cytochrome c_H combines with CO (this was measured after bubbling with CO for 3 minutes followed by 20 minutes incubation in the dark. 24% of cytochrome c_K and 60% of cytochrome c_L combined with CO after similar treatment. This CO-binding is unlikely to be due to denaturation of the cytochromes during purification since CO-binding cytochrome c constitutes about 30% of the cytochrome c in whole bacteria and cell extracts (Section 4.2). CO-binding cytochromes c seem to be a common feature of methylotrophic bacteria and several have been reported (Tonge et al., 1974; Widdowson and Anthony, 1975); see also Section 1.4; Table 1.1. The cytochromes almost certainly do not function as physiological oxidases and no aerobic methylotrophic bacterium has been reported without a functional a- or o-type terminal oxidase in addition to the CO-binding cytochrome c.

Assuming that the molar absorption coefficient for the haem in

the cytochromes is 27.7 litre/mmol/cm the measured haem/protein ratios are 1.02, 1.1 and 1.03 for cytochrome c_H, cytochrome c_K and cytochrome c_L respectively; this indicates the cytochromes are typical in having only one haem per mol. of cytochrome.

5.5 Midpoint Redox Potentials of the Purified Cytochromes c

Potentiometric titrations of the purified cytochromes were performed as described in Section 2.14, at pH 5.0, 7.0 and 9.0. The titration curves are shown in Figs. 5.8 - 5.12 and the midpoint potentials are summarized below :-

	pH 5.0	pH 7.0	pH 9.0
Cytochrome c_H	385mV	373mV	375mV
Cytochrome c_K	349mV	336mV	325mV
Cytochrome c_L 17,000 MW	312mV	310mV	297mV
Cytochrome c_L 21,000 MW	nd	310mV	nd

The midpoint potentials of the cytochromes were relatively independent of pH over the range examined but they differ from each other in their slight responses to changing pH.

5.6 Reduction of Cytochrome c by Methanol Dehydrogenase

No autoreduction of the cytochromes was observed at pH 9.5 as has been observed in the cytochromes c of Pseudomonas AM1 (O'Keeffe and Anthony, 1980). Partially purified methanol dehydrogenase from Methylophilus methylotrophus or completely purified from Pseudomonas AM1 rapidly reduced cytochrome c_L; it slowly reduced cytochrome c_K but did not reduce cytochrome c_L. Except for cytochrome c_L which was already oxidized, the cytochromes were first oxidized with the minimum quantity of potassium ferricyanide. The significance of this reduction by methanol dehydrogenase is not known, but because it does not require methanol it may not be a reflection of the normal functioning of the enzyme. There is some evidence that methanol dehydrogenase which has been purified anaerobically is capable of methanol-dependent cytochrome c reduction, in extracts of some methylotrophs, whereas aerobically prepared enzyme is not (Duine et al., 1978; O'Keeffe and Anthony, 1980). Similar experiments with extracts of Methylophilus methylotrophus have not been done.

Fig. 5.8 - Potentiometric titration of the purified cytochrome c_H of Methylophilus methylotrophus at pH 7.0

The methods used for the purification of the cytochrome and for potentiometric titrations are given in Sections 2.16 and 2.14. Open circles represent the oxidative titration (the oxidant was 50mM potassium ferricyanide). The closed circles represent the reductive titration (reduced with 50mM ascorbate).

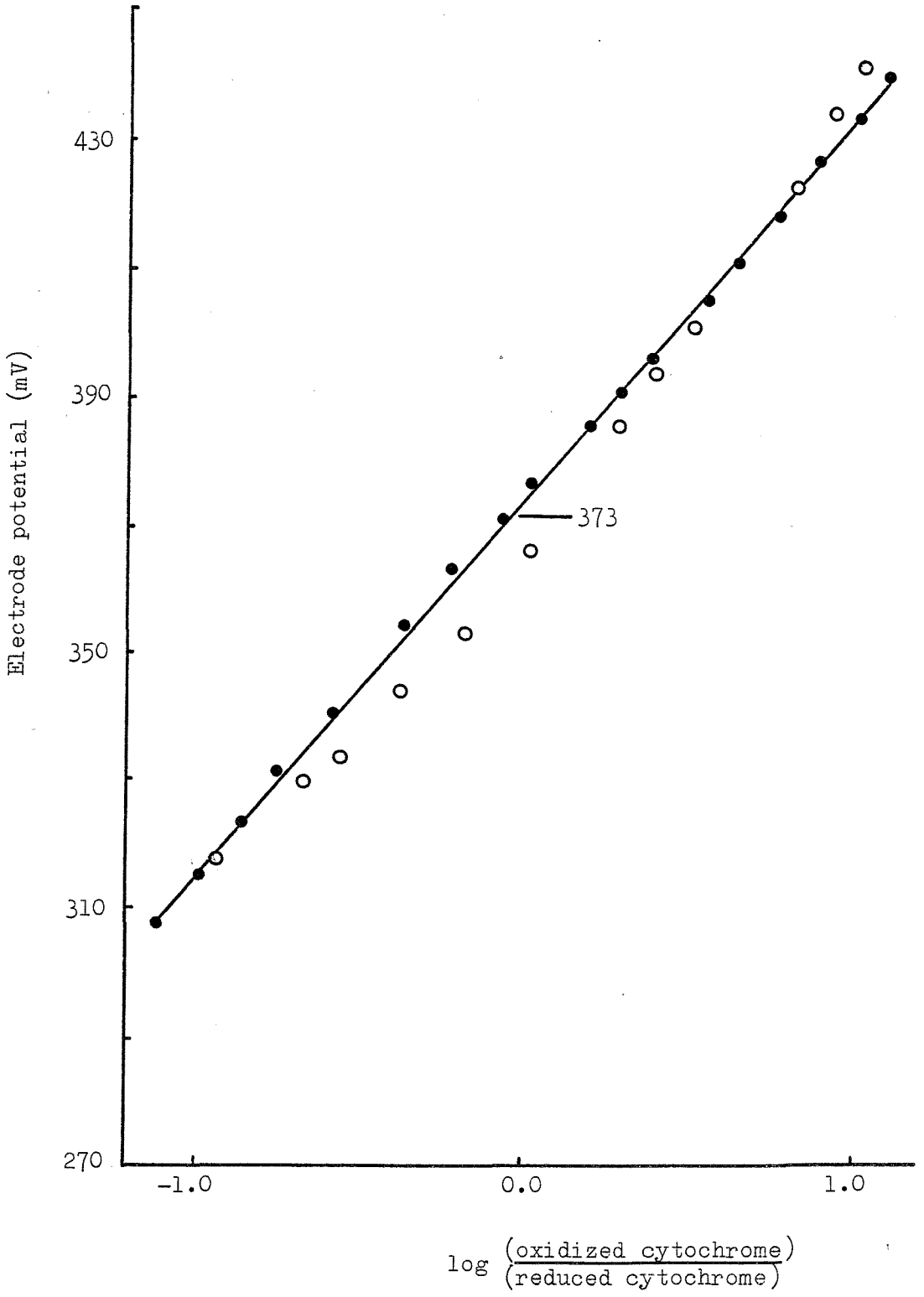


Fig. 5.9 - Potentiometric titration of the purified cytochrome c_K of Methylophilus methylotrophus at pH 7.0

The methods used for the purification of the cytochrome and for potentiometric titrations are given in Sections 2.16 and 2.14. Open circles represent the oxidative titration. The closed circles represent the reductive titration.

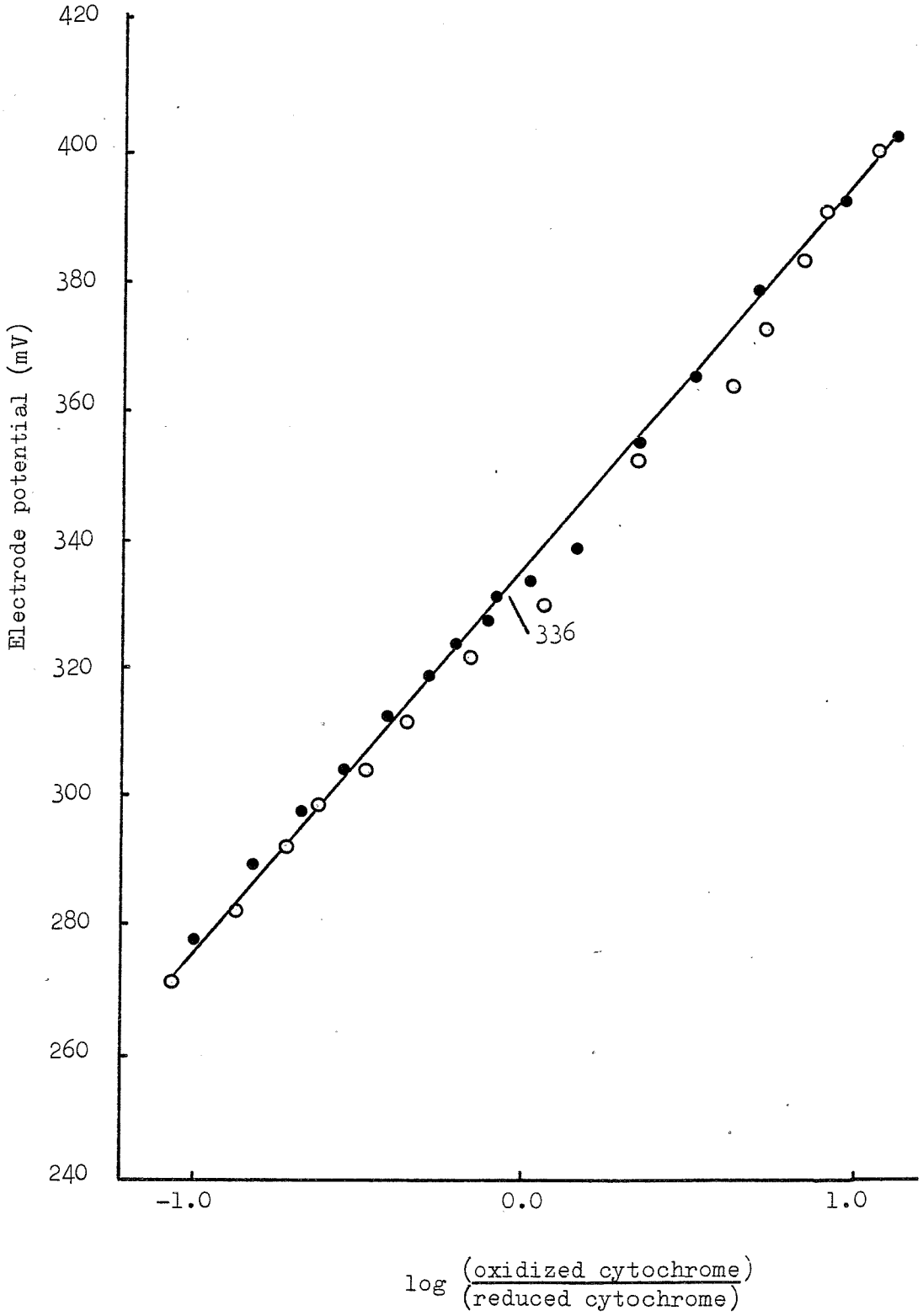


Fig. 5.10 - Potentiometric titration of the purified cytochrome c_L of Methylophilus methylotrophus at pH 7.0

The methods used for the purification of the cytochrome and for potentiometric titrations are given in Sections 2.16 and 2.14. Open circles represent the oxidative titration. The closed circles represent the reductive titration.

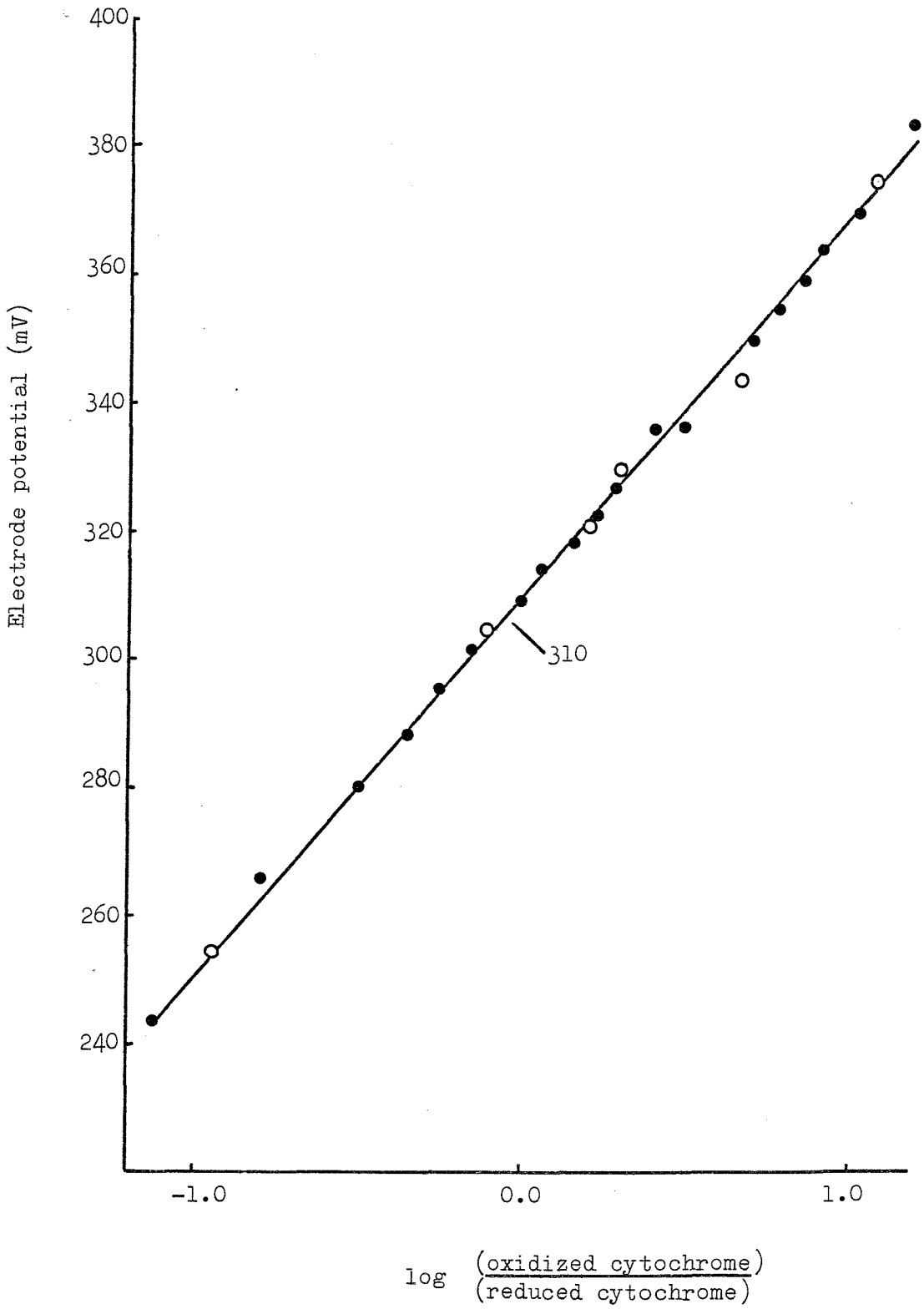


Fig. 5.11 - Potentiometric titration of the purified cytochromes c of Methylophilus methylotrophus at pH 5.0

The methods used for this experiment are in Sections 2.14 and 2.16. The buffer was 50mM Tris-succinate pH 5.0. Open circles represent oxidative titrations. Closed circles represent reductive titrations.

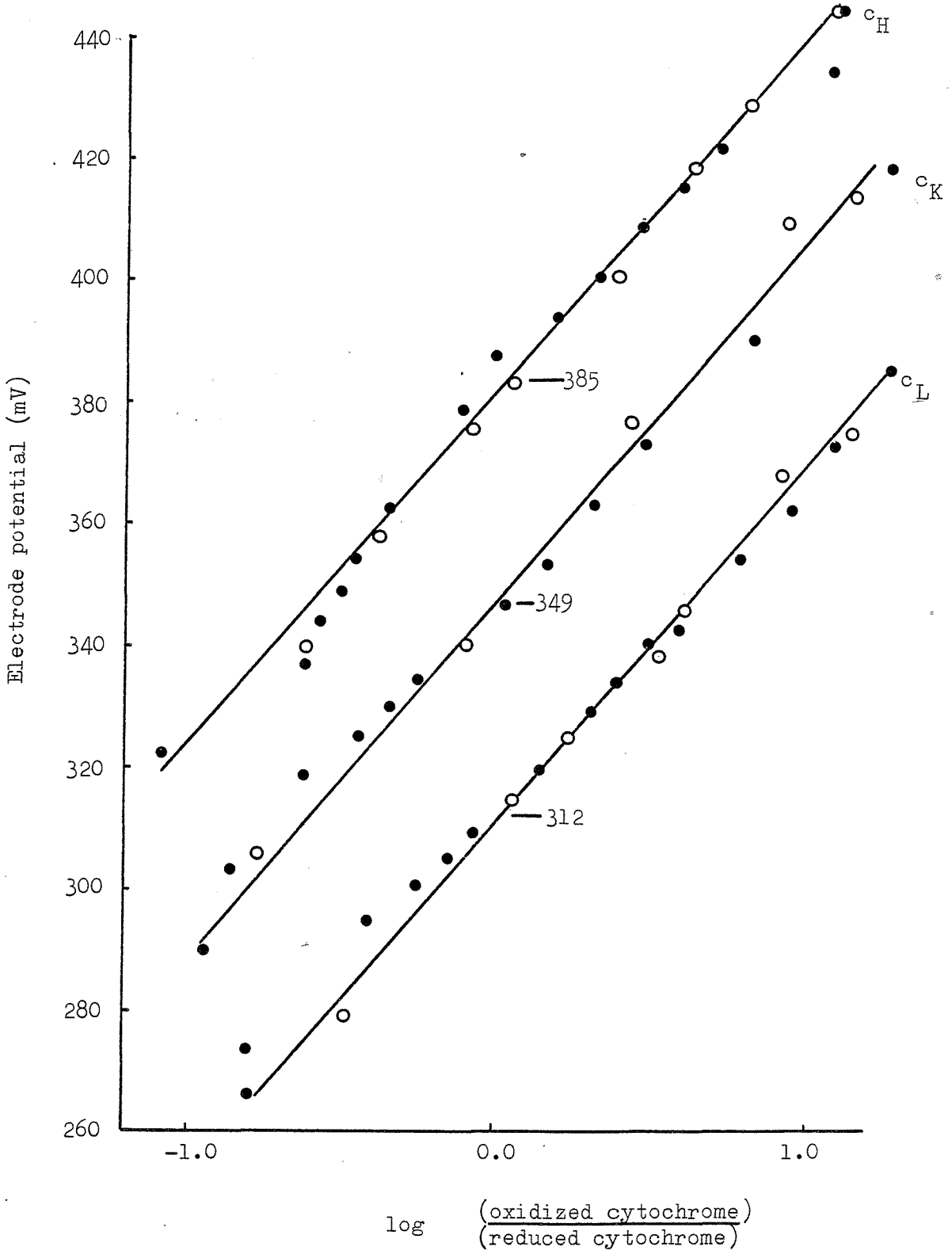
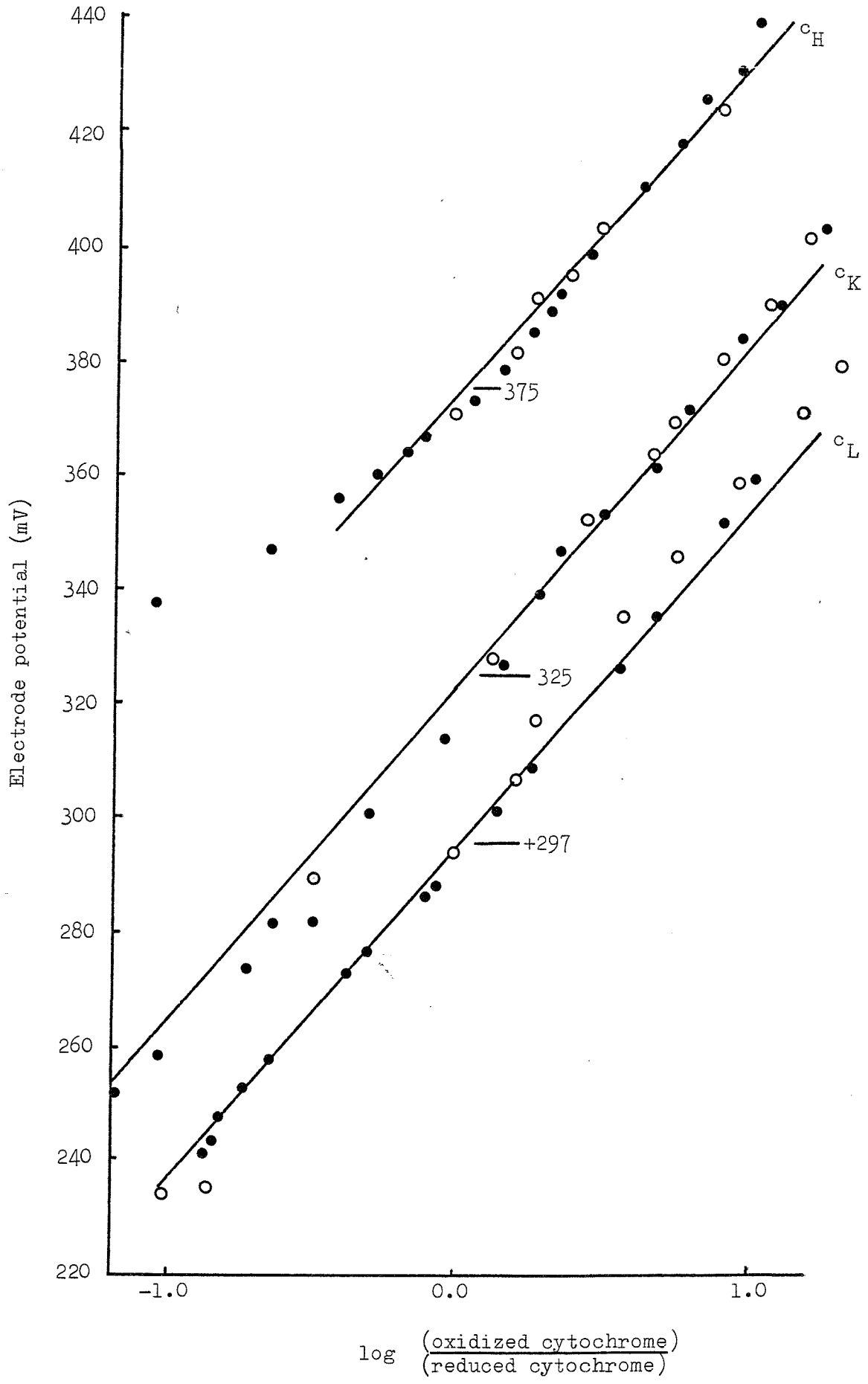


Fig. 5.12 - Potentiometric titrations of the purified cytochromes c of Methylophilus methylotrophus at pH 9.0

The methods used to purify the cytochromes are given in Section 2.16. The methods used for the potentiometric titrations are given in Section 2.14. Open circles represent oxidative titrations (50mm potassium ferricyanide was the oxidant). Closed circles represent reductive titrations (50mm ascorbate was used as reductant). The buffer used was 50mm CHES, pH 9.0.

Fig. 5.12


440
420
400
380
360
340
320
300
280
260
240
220
Electrode potential (mV)
c_H
c_K
c_L
375
325
+297
-1.0
0.0
1.0
log (oxidized cytochrome)/(reduced cytochrome)

5.7 Summary and Discussion

The results presented in this chapter are summarized in Table 5.2. They clearly show that there are at least 3 soluble cytochromes c in Methylophilus methylotrophus. These differ in isoelectric points, molecular weights, midpoint redox potentials, extent of CO-binding, reaction with methanol dehydrogenase and their absorption spectra. They are similar in having one haem per mole and in all having high midpoint redox potentials. Redox potential measurements of cytochrome c on membranes (Chapter 4) indicated that these same cytochromes are all able to become firmly bound to the membrane. The α- bands of the two major cytochromes c (c_H and c_L) both split at 77°K; this splitting is still seen on membranes of bacteria grown in methanol-excess conditions but it was not detected in membranes from methanol-limited bacteria (although it was not concluded that splitting did not occur). No α-band splitting was observed of the minor cytochrome, cytochrome c_K.

There are few full descriptions of pure cytochromes c from methylotrophs and so extensive comparisons are not possible. One of the cytochromes c that has been characterized is from the obligate methanotroph, Methylosinus trichosporium (Tonge et al., 1975, 1977a). This cytochrome has been completely purified; it probably has a high isoelectric point since it does not bind to DEAE cellulose during the purification procedure. It has a low molecular weight (13,000), one haem per mole, a midpoint redox potential of 310mV, a molar absorption coefficient of 22.5 litre/mmol/cm and an α-absorption maximum at 551nm in the reduced form. In these respects this cytochrome is similar to the cytochrome c_H of Pseudomonas AM1 described below, but differs from it in its extensive and rapid binding to CO and in being rapidly autoxidizable. The cytochrome c of another obligate methylotroph, Methylomonas methicana has been purified to some extent and shown to have a molecular weight of 18,000 and to react with CO (Patel et al., 1979). Unfortunately the very high absorbance at 278nm ($A_{550-575}/A_{278}$ = 0.06) indicates that the preparation was impure and so these results (particularly the amino acid analysis) cannot be relied upon until they are confirmed with pure preparations.

The presence of more than one soluble cytochrome c in non-methylotrophic bacteria is not uncommon (and these cytochromes have

Table 5.2 - The properties of the 3 pure soluble cytochromes c of Methylophilus methylotrophus

The methods used to purify the cytochromes are described in Section 2.16. The properties are described in Chapter 5.

Property	cytochrome c_H	cytochrome c_K	cytochrome c_L
Relative percentage in crude extract.	49.6%	7.8%	42.5%
Isoelectric point	8.85	4.55	4.0-4.35
Molecular weight	8,500	16,800	17,000/21,000
Absorption maxima (nm) in the reduced form :	551.25,520.25, 416.25	550.75,521.75, 416.5	549.75,520.5, 416
in the oxidized form:	408	408	410
Split α-band at 77°K	+	-	+
Absorption coeff. (l/mmol/cm)			
α	27.1	25.2	26.9
γ	128	116	139
γ/α ratio	4.72	4.27	5.16
A_α/A_{280}	0.83	0.95	1.05
Midpoint redox potential (E_{m7})	373mV	336mV	310mV
CO-binding (%)	7%	24%	60%
Autoxidizability	very slight	slight	slow
Reaction with methanol dehydrogenase	none	slight	rapid
Haem/mol protein	1	1	1

sometimes been shown to have different physiological functions) (Lemberg and Barrett, 1973). There are few reports of more than one cytochrome c in methylotrophs; this is not necessarily very significant because if the absorption maxima are close then they would not be detected in whole bacteria or crude cell extracts. Two cytochromes c were described in the obligate methanol-utilizer Methylomonas P11 (Drabikowska, 1977) differing in absorption maxima by 3.5nm and Paracoccus dentrificans may have more than one cytochrome c (apart from cytochrome c_1); during growth on methanol, a CO-binding cytochrome c has been detected which is not present during growth on succinate (van Verseveld and Stouthamer, 1978). If the usual cytochrome c is also present then this organism must have two cytochromes c (at least) during methylotrophic growth. Two soluble cytochromes c have been purified from the facultative methylotroph Pseudomonas AM1 and these have been well characterized (O'Keeffe and Anthony, 1980). The 2 major cytochromes c (cytochrome c_H and cytochrome c_L) from Methylophilus methylotrophus are similar in most respects to the corresponding cytochromes c_H and c_L from Pseudomonas AM1; the properties of the cytochromes from both organisms are summarized in Tables 5.2 and 5.3. The main differences between the two bacteria are that the midpoint redox potentials of the Methylophilus methylotrophus cytochromes are higher than those of the Pseudomonas AM1 cytochromes (although in both bacteria, the cytochrome c_H midpoint redox potential is higher than the cytochrome c_L midpoint redox potential). Another difference is that no α-band splitting of either of the Pseudomonas AM1 cytochromes c occurs at low temperature and both of these react with methanol dehydrogenase, whereas only cytochrome c_L of Methylophilus methylotrophus reacts with the dehydrogenase.

In view of the differences in the properties of the 3 soluble cytochromes c from Methylophilus methylotrophus (isoelectric points, molecular weights, reaction with methanol dehydrogenase etc.) it is unlikely that they are performing identical functions in the cell, although it is not possible to identify a particular role for a particular cytochrome. It is possible that one of the cytochromes may react (not necessarily direct) with methanol dehydrogenase and the others are associated with each of the two alternative oxidases. It would be valuable to know if the relative proportion of each cytochrome changes with the growth conditions in the same manner as cytochrome a/a_3 and o.

Table 5.3 - The properties of the 2 pure soluble cytochromes c of Pseudomonas AM1 (from O'Keeffe and Anthony, 1980)

Property	cytochrome c_H	cytochrome c_L
Relative percentage in crude extract	72%	28%
Isoelectric point	8.8	4.2
Molecular weight	10,960	20,890
Absorption maxima (nm) in the		
Reduced form :	550.5,521.5,416.5	549,520,416
Oxidized form :	409	407
Split α-band at 77°K	no	no
Absorption coefficient (l/mmol/cm)		
α	31	26
γ	162	163
γ/α ratio	5.23	6.25
A_α/A_{280}	1.13	1.0
Midpoint redox potential (E_{m7})	294mV	256mV
CO-binding	36%	72%
Autoxidizability	++ (slow)	+++ (slow)
Reaction with methanol dehydrogenase	2 fold molar excess cyt reduced	54 fold molar excess cyt reduced
Haem/mol protein	1	1

There is some evidence from redox titrations of membrane preparations that this may be the case (Section 4.5). C-type cytochromes with such high midpoint redox potentials (all greater than 300mV) are unusual in non-photosynthetic bacteria and it is surprising to find that all the cytochromes c should have high midpoint redox potentials. The slow rate of autoxidation of the purified cytochromes preclude them from consideration as physiological oxidases (as has been suggested by Tonge et al., 1974 and Ferenci, 1974). It should be noted however, that the autoxidation of the cytochromes c might be altered on binding to the membranes but in the presence of alternative oxidases (cytochromes a/a_3 and o) it was not possible to demonstrate any oxidase function for a membrane-bound cytochrome c.

It is interesting that although Methylophilus methylotrophus is an obligate methylotroph it requires at least 3 cytochromes c whereas in the facultative methylotrophs which have been studied, at the most only 2 c-type cytochromes have been found, although these organisms might be expected to have a more complex electron transport system. Unfortunately at the present time there is not sufficient information about the cytochromes c from methylotrophs to come to any definite conclusions about their physiological roles.

CHAPTER 6

RESPIRATORY ACTIVITIES OF WHOLE CELLS AND MEMBRANE PREPARATIONS OF METHYLOPHILUS METHYLOTROPHUS

6.1 Introduction

To investigate the organization of the electron transport chain of Methylophilus methylotrophus and the changes that occur in different growth conditions, the pattern of repiratory inhibition was studied using the respiratory chain inhibitors cyanide, azide, antimycin A, 2-heptyl-4 hydroxyquinoline-N-oxide and rotenone.

Cyanide and azide are important inhibitors used for studying the terminal electron transport component. Titrating the inhibitory effect of cyanide (and azide) and establishing that inhibition occurs at low concentrations is the most common method of indicating the presence of a terminal cytochrome oxidase (White and Sinclair, 1970).

Antimycin A and HQNO inhibit mitochondrial electron transport at the level of cytochrome b (Chance and Williams, 1956). Inhibition at this level also occurs in some bacterial electron transport chains, but in other bacteria there is also inhibition after cytochrome b (Cox, 1970) and at cytochrome o (Daniel, 1970).

Rotenone inhibits the electron transport chain at the level of NADH-ubiquinone oxidoreductase (Ragan, 1976).

Results

6.2 Respiratory Activities of Whole Bacteria and Membranes

Whole Methylophilus methylotrophus cells were found to oxidize methanol, formaldehyde, formate, propane 1,2 diol, NADH, NADPH and ascorbate/TMPD at good rates (Table 6.1). Oxidation of NADH and NADPH is unusual by whole bacteria but has been observed before in suspensions of Haemophilus parainfluenzae (White and Sinclair, 1970) where the oxidation was attributed to the rapid entry of nucleotides through a permeable cell wall. The rates of oxidation of substrates were similar in all growth conditions with the exception of ascorbate/TMPD oxidation which was greater in both conditions of methanol-excess (oxygen- or nitrogen-limitation), than in bacteria grown in methanol-limited conditions. The inhibition of oxidation of formaldehyde, formate and

Table 6.1 - Respiratory activities of whole Methylophilus methylotrophus bacterial cells grown in continuous culture under various growth limitations

Methods used for the growth of bacteria in continuous culture are given in Sections 2.6 and 2.7 and for the measurement of respiration in Section 2.13.

Respiration rates are expressed as nmols O_2/min/mg dry weight of bacteria. There was no endogenous rate in the absence of substrate. Respiration rates have been corrected for the non-enzymic rate of ascorbate/TMPD oxidation which was always less than 5% of the respiratory rate.

Respiratory substrate	Limiting growth substrate		
	Methanol	Nitrogen	Oxygen
Methanol	251	254	288
Ascorbate/TMPD	47	222	238
NADH	171	202	201
NADPH	62	67	65
Formaldehyde	130	nd	119
Formate	25	nd	28
Propane 1,2 diol.	65	nd	nd

nd = not determined.

propane 1, 2 diol was not studied in detail as it is not known where these substrates donate electrons to the electron transport chain.

Membrane preparations oxidized NADH, NADPH and ascorbate/TMPD (Table 6.2) but methanol was oxidized at very low rates or not at all, although methanol dehydrogenase was present in these preparations; (see Section 6.3); formaldehyde and formate were not oxidized, propane 1, 2 diol was not tested. Ascorbate/TMPD was oxidized at an extremely high rate in membranes prepared from bacteria grown in continuous culture under conditions of methanol-excess. If this oxidation proceeds via cytochrome o and there is assumed to be 200 nmols cytochrome o per mg protein in membranes from bacteria grown in methanol-excess conditions (Section 4.4), then the oxidation of ascorbate/TMPD is probably approaching the turnover rate of the oxidase (218 per sec); this compares with the quoted rates of 120 per sec. for yeast (Chance and Williams, 1955) and 620 per sec. for bacteria (Smith, 1961).

6.3 Methanol Deydrogenase Activities of Membrane Preparations

Although membrane preparations did not oxidize methanol, methanol dehydrogenase was shown to be present when assayed by the method of Anthony (Section 2.22) at pH 9.0 with PMS as the primary hydrogen acceptor and ammonia as activator. The activity was 450nmol O_2/min/mg protein compared to 299nmol O_2/min/mg protein in the soluble fraction. There was no activity at pH 7.0. The activity was also lost during incubation in the assay system in the absence of methanol (Fig. 6.1); it has been shown that the methanol dehydrogenase from Pseudomonas M27 becomes inactivated in the presence of PMS in the absence of methanol (Anthony and Zatman, 1964) and the enzyme from Methylophilus methylotrophus has also been shown to be unstable in the absence of methanol (Ghosh and Quayle, 1978). For this reason 5mM methanol was included in the buffers used to prepare the membrane fractions but without effect. Addition of the soluble fraction, incubation at pH 8.0 or pH 9.0 and the addition of ammonium sulphate failed to give methanol-dependent respiratory activity, unless PMS was present. Ribbons and Wadzinski, (1976); Wadzinski and Ribbons, (1975) during work on Methylococcus capsulatus obtained methanol oxidation in membrane preparations but found that this was only a proportion of the total activity of methanol dehydrogenase when assayed with PMS.

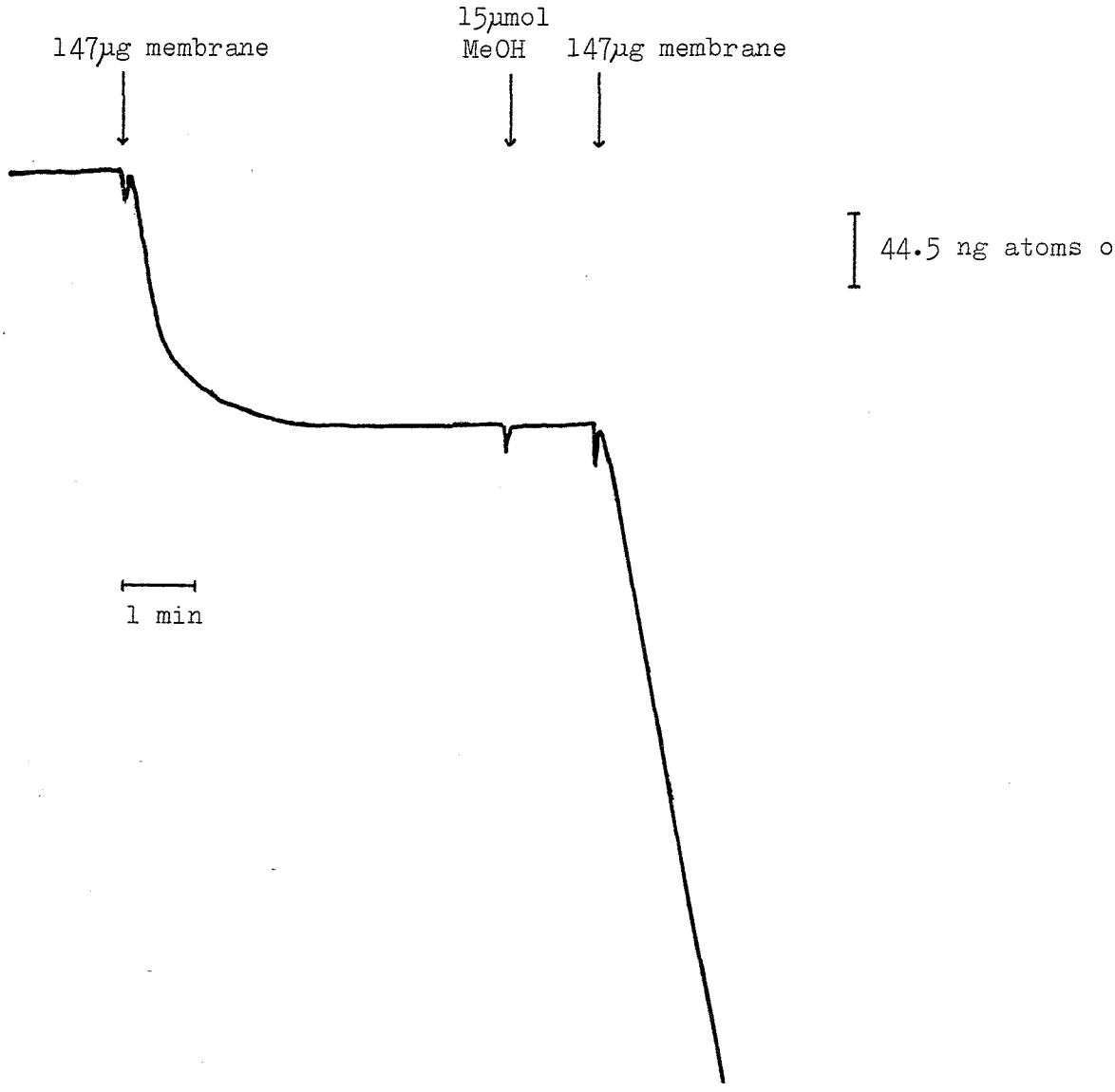
Table 6.2 - Respiratory activities of membrane preparations from Methylophilus methylotrophus grown in continuous culture under various growth limitations

The methods used for the growth on bacteria and preparation of membranes are described in Sections 2.6, 2.7 and 2.10. Respiration rates were measured as described in Section 2.12 and are expressed as nmols O_2/min/mg protein. There was no endogenous rate in the absence of substrate. Respiration rates have been corrected for the non-enzymic rate of ascorbate/TMPD oxidation which was always less than 5% of the respiratory rate.

Respiratory substrate	Limiting growth substrate		
	Methanol	Nitrogen	Oxygen
Methanol	3.7	-	-
Ascorbate/TMPD	245	2615	2361
NADH	66	59	73
NADPH	31	26	36

Fig. 6.1 - Loss of methanol dehydrogenase activity in the absence of methanol

Oxygen electrode recording showing the effect of the addition of membrane (147μg protein) in a methanol dehydrogenase assay in the absence of methanol, giving a transitory methanol dehydrogenase activity. After this activity is lost the addition of 15μmols of methanol (7.5mM final concentration) gives no increase in oxygen uptake. The further addition of membrane (147μg protein) causes a further persistent oxygen uptake. The assay system is described in Section 2.22.



The oxidase activity was also less stable to heat than was the methanol dehydrogenase activity and they suggested that in vivo the site of methanol oxidation is in the membrane and during disruption of the bacteria methanol dehydrogenase is released. It is possible that in Methylophilus methylotrophus the coupling of methanol dehydrogenase to the electron-transport system is damaged during the membrane preparation while not substantially altering the methanol dehydrogenase enzyme itself.

6.4 Respiratory Inhibition by Cyanide and Azide in Bacteria grown in Methanol-excess Cultures

In conditions of methanol-excess (oxygen- or nitrogen-limitation) no cytochrome a/a_3 was present and spectral measurements (Section 4.3) indicated the presence of a CO-binding membrane-bound cytochrome b. The results below suggest that this is probably a functional oxidase in these bacteria and it will be referred to as cytochrome o.

The characteristics of this oxidase were best demonstrated in membranes of oxygen or nitrogen-limited bacteria in which cytochrome a/a_3 was absent and the concentration of the CO-binding cytochrome o was high (Figs. 4.3 and 4.4). This cytochrome probably corresponds to the cytochrome b absorbing at 558nm seen in low temperature spectra of these membranes which was absent from membranes of methanol-limited bacteria (Fig. 4.4). It was previously concluded (Chapter 4) that the additional b-type cytochrome, with a midpoint redox potential of 260mV, on the membranes of methanol-excess bacteria was probably cytochrome o. In these membranes the rate of ascorbate/TMPD was very high (about 2,500nmol O_2/min/mg protein) (Table 6.2) and was 10 times higher than in membranes from bacteria grown under methanol-limitation which have a lower concentration of this cytochrome.

The oxidation of ascorbate/TMPD was inhibited powerfully by cyanide and azide. The inhibition was monophasic (suggesting one oxidase), 50% inhibition occurred at 0.9μM KCN and 2μM azide (Figs. 6.2, 6.3). That the same oxidase was involved in whole cell oxidation of ascorbate/TMPD was indicated by the similar pattern of inhibition by cyanide and azide which was obtained with membranes, being monophasic with 50% inhibition at 0.8μM KCN and 2μM azide (Fig. 6.4). The much lower rate of ascorbate/TMPD oxidation in whole bacteria compared to that

Fig. 6.2 - Inhibition by cyanide of respiration in membranes and whole bacteria

a. ▲—▲ Ascorbate/TMPD oxidation by membranes from methanol-excess bacteria (oxygen-limited) (which do not contain cytochrome a/a_3). The uninhibited oxidation rate was 2307 nmol O_2/min/mg protein.

b. ●—● NADH oxidation by membranes from methanol-excess bacteria (oxygen-limited) (no cytochrome a/a_3). The uninhibited oxidation rate was 63 nmol O_2/min/mg protein.

c. O—O NADH oxidation by membranes from methanol-limited bacteria (cytochrome a/a_3 present). The uninhibited oxidation rate was 54 nmol O_2/min/mg protein.

The inhibition was determined as described in Section 2.13.

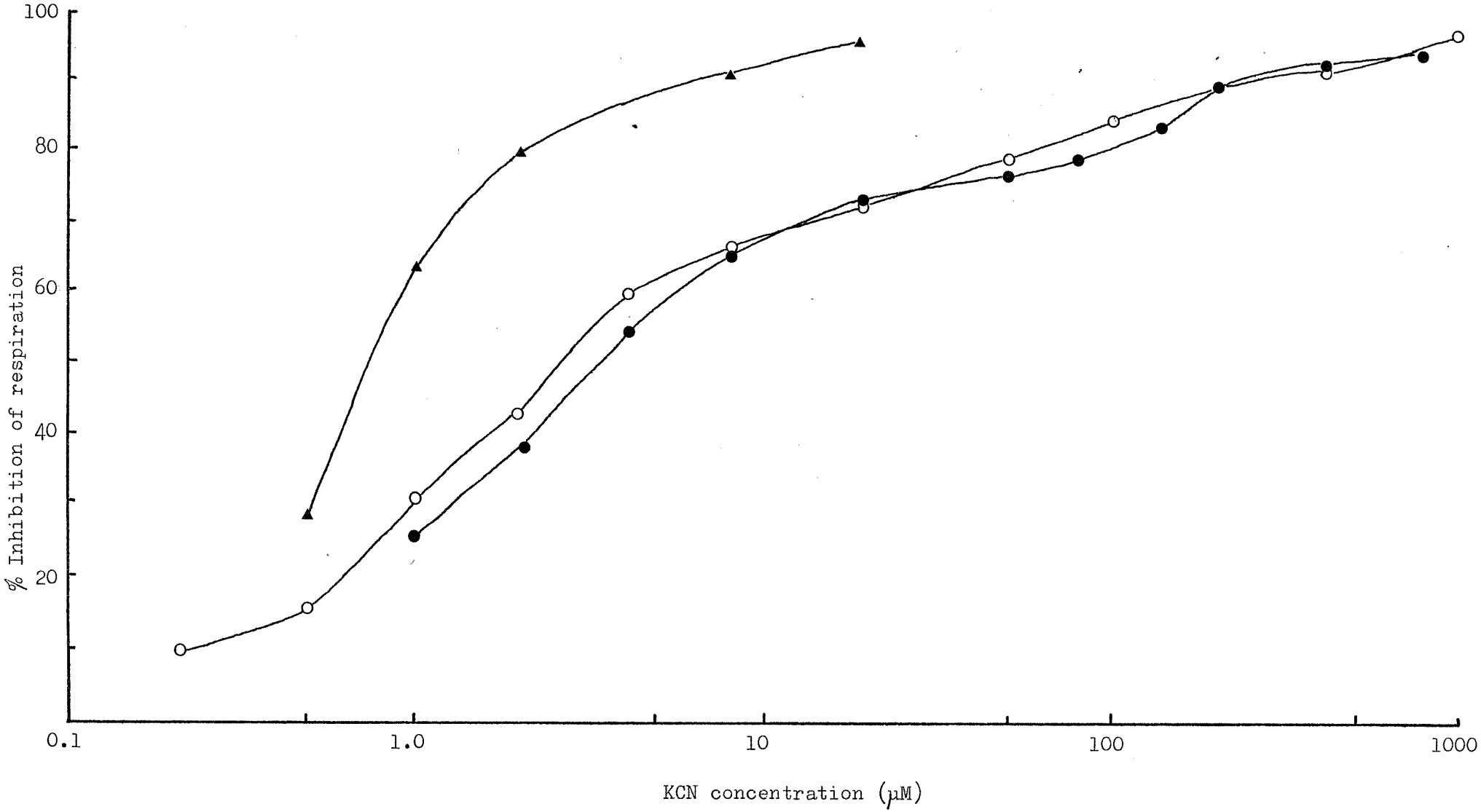

100
80
60
40
20
% Inhibition of respiration
0.1
1.0
10
100
1000
KCN concentration (μM)

Fig. 6.3 - Inhibition by azide of respiration in membrane preparations

a. ▲—▲ Ascorbate/TMPD oxidation by membranes from methanol-excess bacteria (nitrogen-limited) (which contain no cytochrome a/a_3). The uninhibited oxidation rate was 2604 nmol O_2/min/mg protein.

b. △—△ Ascorbate/TMPD oxidation by membranes from methanol-limited bacteria (cytochrome a/a_3 present). The uninhibited oxidation rate was 170 nmol O_2/min/mg protein.

c. ●—● NADH oxidation by membranes from methanol-excess bacteria (oxygen-limited) (no cytochrome a/a_3). The uninhibited oxidation rate was 59 nmol O_2/min/mg protein.

d. ■—■ NADPH oxidation by membranes from methanol-excess bacteria (nitrogen-limited) (no cytochrome a/a_3). The uninhibited oxidation rate was 26 nmol O_2/min/mg protein.

e. O—O NADH oxidation by membranes from methanol-limited bacteria (cytochrome a/a_3 was present). The uninhibited oxidation rate was 61 nmol O_2/min/mg protein.

The inhibition was determined by the method described in Section 2.13.

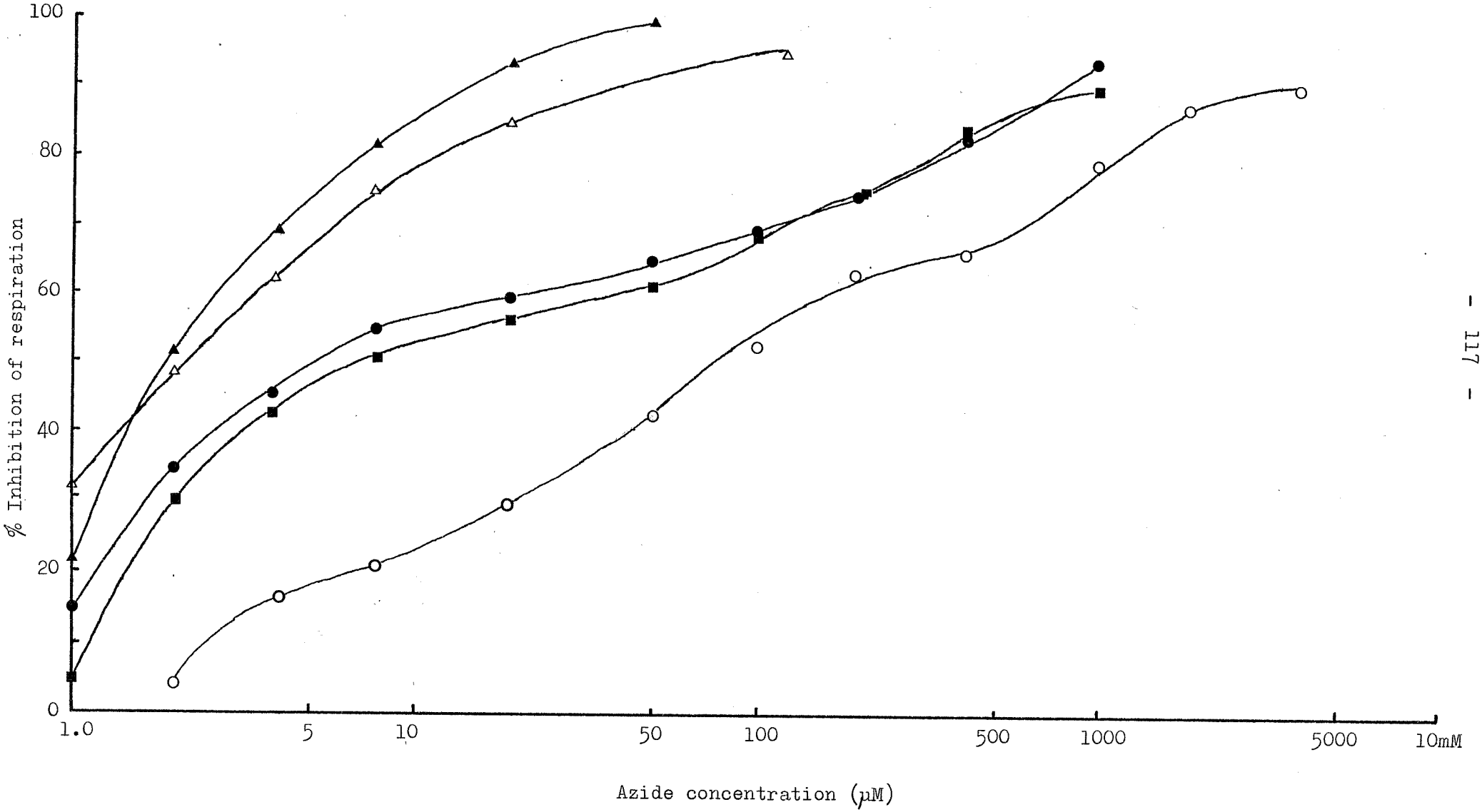

100
80
60
40
20
0
% Inhibition of respiration
1.0
5
10
50
100
500
1000
5000
10mM
Azide concentration (μM)

Fig. 6.4 - Inhibition by cyanide of respiration in whole bacteria and soluble bacterial extracts

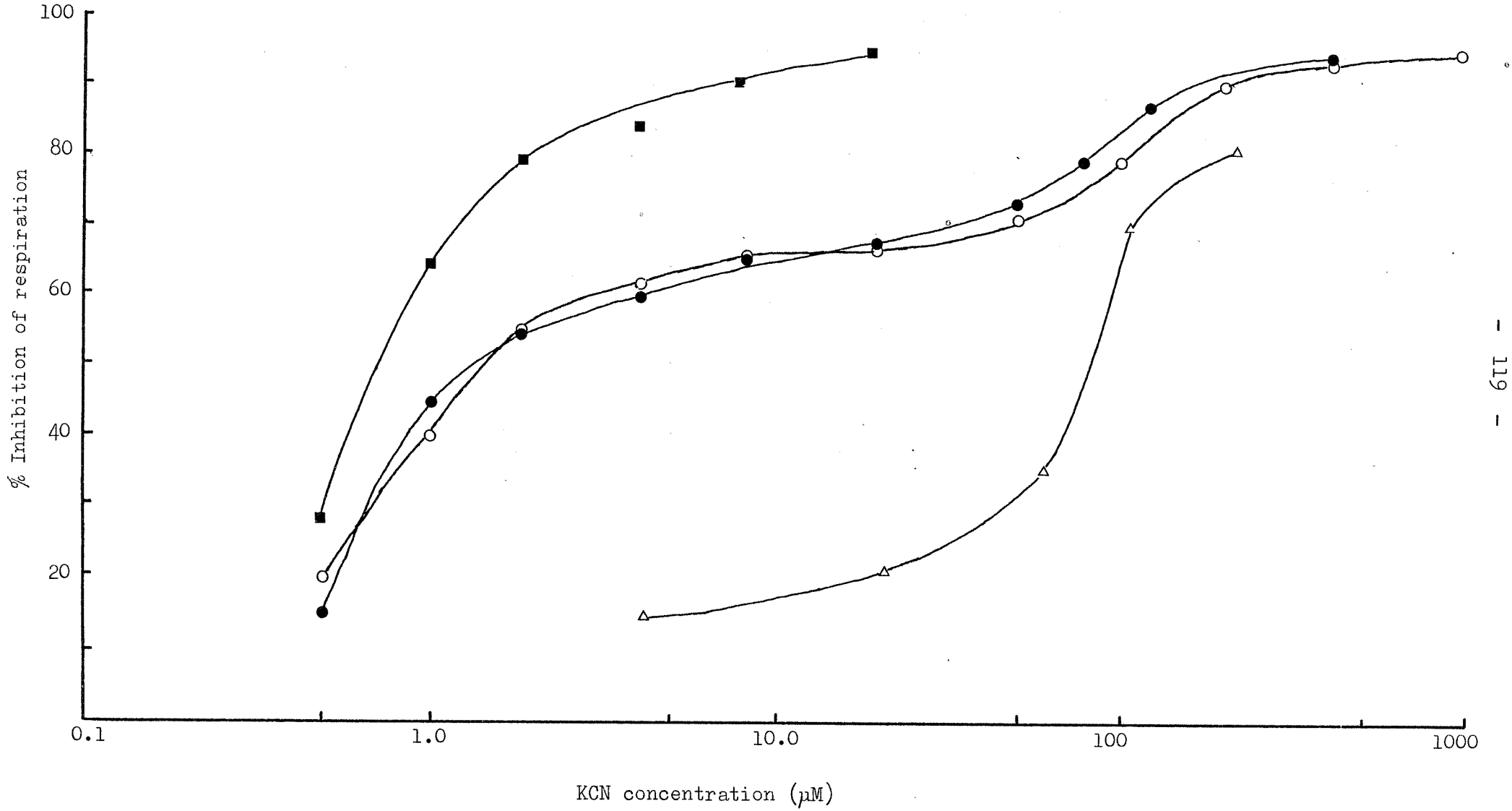
a. ■—■ Ascorbate/TMPD oxidation by methanol-excess (oxygen-limited) bacterial (no cytochrome a/a_3). The uninhibited oxidation rate was 238 nmol O_2/min/mg dry weight of bacteria.

b. ●—● Methanol oxidation by methanol-excess (oxygen-limited) bacteria (no cytochrome a/a_3). The uninhibited oxidation rate was 288 nmol O_2/min/mg dry weight of bacteria.

c. O—O NADH oxidation by methanol-excess (oxygen-limited) bacteria (no cytochrome a/a_3). The uninhibited oxidation rate was 225 nmol O_2/min/mg dry weight of bacteria.

d. Δ—Δ Methanol oxidation by the soluble cell fraction of methanol-excess (oxygen-limited) bacteria. The uninhibited oxidation rate was 9.7 nmol O_2/min/mg protein.

The inhibition was determined by the method described in Section 2.13.


100
80
60
40
20
% Inhibition of respiration
0.1
1.0
10.0
100
1000
KCN concentration (μM)

of membranes was probably due to a lack of permeability to ascorbate/TMPD.

The oxidation of NAD(P)H differed from that of ascorbate/TMPD in that inhibition by cyanide and azide is biphasic (Figs. 6.2, 6.3). The inhibition pattern for NADH and NADPH oxidation was always very similar (Fig. 6.3). In membrane preparations about 75% of respiration was by way of an oxidase which was very sensitive to cyanide and azide, the concentrations required for 50% inhibition (1.9μM KCN; 5μM azide) indicating that the cytochrome o responsible for the oxidation of ascorbate/TMPD was also involved in this oxidation of NAD(P)H. The same pattern of inhibition was seen in whole bacteria (Figs. 6.4, 6.5).

Work with whole bacteria showed that cytochrome o was also involved in methanol oxidation; inhibition by cyanide was biphasic, 70% of respiration being cyanide sensitive with 50% inhibition at 0.8μM. (Fig. 6.4).

As stated above, inhibition by cyanide and azide of oxidation of methanol in whole bacteria, and NADH in whole bacteria and membranes, was biphasic after growth in methanol-excess conditions where, that is, in the presence of sufficient inhibitor to prevent respiration by the cyanide-sensitive oxidase (cytochrome o) respiration could continue at up to about 30% of the uninhibited rate. This respiration which was probably non-physiological, was sensitive only to high concentrations of inhibitors, 50% of inhibition being obtained with 90μM KCN (methanol oxidation by whole bacteria, Fig. 6.4), 130μM KCN (NADH oxidation by membranes, Fig. 6.2) and about 1mM azide (NADH oxidation by whole bacteria, Fig. 6.5). It is proposed that this less-sensitive respiration is by way of a slowly autoxidizable cytochrome c (see Chapter 5). It was concluded that ascorbate/TMPD was oxidized exclusively by way of cytochrome o which was very sensitive to cyanide (50% inhibition at less than 5μM cyanide) and azide (50% inhibition at less than 5μM azide) and that this oxidase was also involved in the oxidation of methanol and NAD(P)H. The question arises; why, if the cytochrome c was reduced by ascorbate/TMPD was the inhibition of oxidation of ascorbate/TMPD not biphasic? The answer may be that the maximum rate of cytochrome c autoxidation is very low compared with the high rate of ascorbate/TMPD oxidation by way of cytochrome o and hence is not readily observed in curves of inhibition concentration against percentage inhibition of respiration.

Fig. 6.5 - Inhibition by azide of respiration in whole bacteria and soluble bacterial extracts

a. ▲—▲ Ascorbate/TMPD oxidation by methanol-excess (oxygen-limited) bacteria (no cytochrome a/a_3). The uninhibited oxidation rate was 290 nmol O_2/min/mg dry weight of bacteria.

b. △—△ Ascorbate/TMPD oxidation by methanol-limited bacteria (cytochrome a/a_3 present). The uninhibited oxidation rate was 62 nmol O_2/min/mg dry weight of bacteria.

c. ■—■ Methanol oxidation by methanol excess (oxygen-limited) bacteria (no cytochrome a/a_3). The uninhibited oxidation rate was 251 nmol O_2/min/mg dry weight of bacteria.

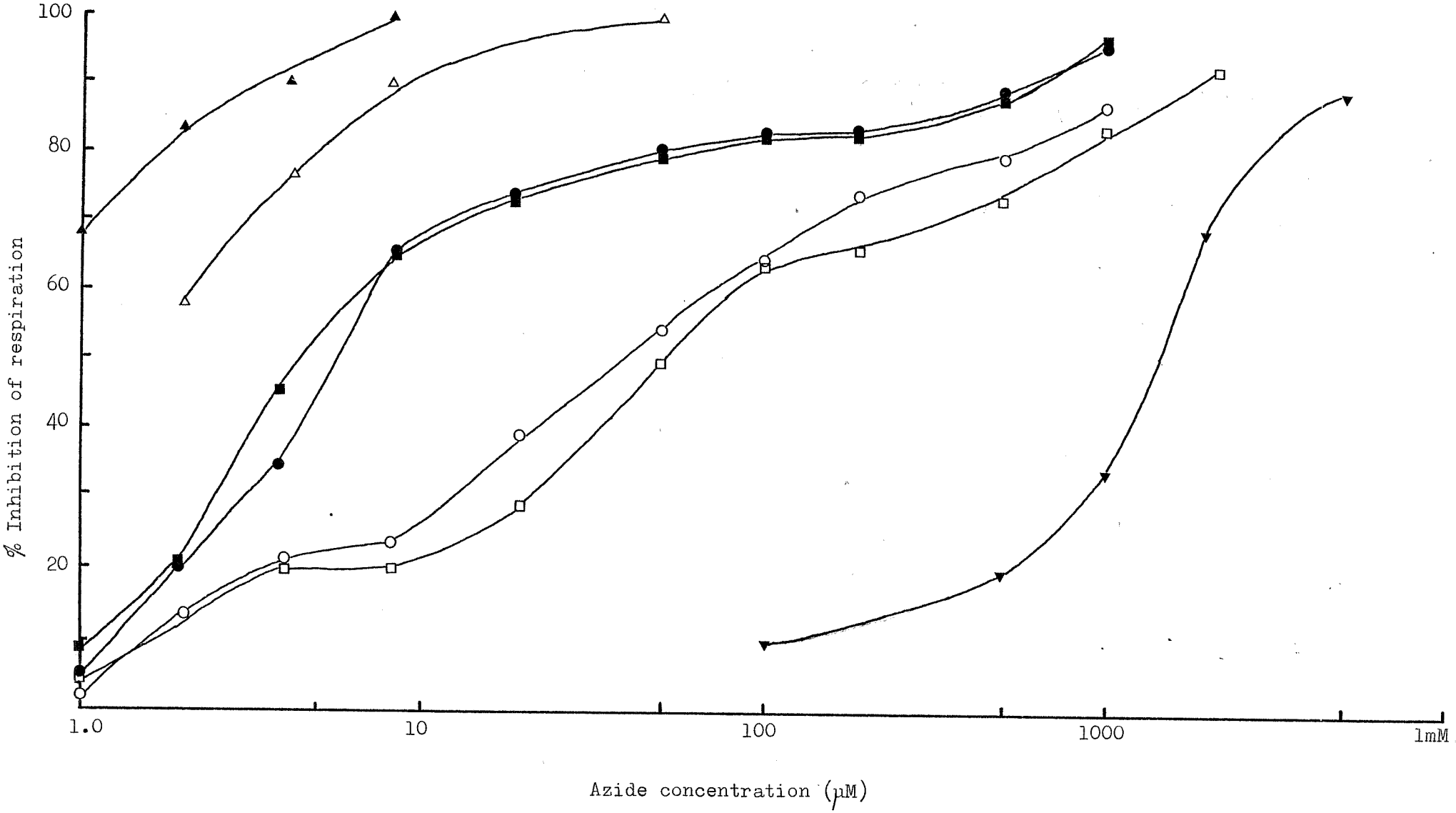
d. □—□ Methanol oxidation by methanol-limited bacteria (cytochrome a/a_3 present). The uninhibited oxidation rate was 225 nmol O_2/min/mg dry weight of bacteria.

e. ●—● NADH oxidation by methanol-excess (oxygen-limited) bacteria (no cytochrome a/a_3). The uninhibited oxidation rate was 225 nmol O_2/min/mg dry weight of bacteria.

f. ○—○ NADH oxidation by methanol-limited bacteria (cytochrome a/a_3) present. The uninhibited oxidation rate was 222 nmol O_2/min/mg dry weight of bacteria.

g. ▼—▼ Methanol oxidation by the soluble cell fraction from methanol-excess (oxygen-limited) bacteria. The uninhibited oxidation rate was 3 nmol O_2/min/mg protein.

The inhibition was determined as described in Section 2.13.


100
80
60
40
20
% Inhibition of respiration
1.0
10
100
1000
1mM
Azide concentration (μM)

6.5 Inhibition by Cyanide and Azide of Respiration in Bacteria grown in Methanol-limited Cultures

Bacteria grown in methanol-limited cultures differ from those grown under conditions of methanol-excess (oxygen or nitrogen-limitation) by having an extra potential oxidase, cytochrome a/a_3 as seen in Fig. 4.3 and Fig. 4.4. Methanol-limited cells contained much less cytochrome o as indicated by the lower rate of ascorbate/TMPD oxidation by membranes, spectra and redox titrations (Chapter 4); that this oxidation was due to cytochrome o and not the cytochrome a/a_3 also present was indicated by the monophasic inhibition pattern with azide (50% inhibition at about 2μM azide, in both membranes (Fig. 6.3) and whole bacteria (Fig. 6.5).

During oxidation of methanol and NAD(P)H by whole cells and NAD(P)H by membranes, a second oxidase was clearly involved. This is shown by the inhibition pattern with azide (Figs. 6.3 and 6.5). In Figs. 6.3 and 6.5 it can be seen that inhibition by azide of ascorbate/TMPD was monophasic (50% inhibition at 2.2μM); that there was usually some inhibition of methanol and NAD(P)H by this low concentration of azide suggests that cytochrome o was also usually involved in the oxidation of these substrates. The second phase of inhibition (50% inhibition at about 50μM azide) was probably due to cytochrome a/a_3 while the third phase of inhibition occurring in the presence of high concentrations of azide corresponded to that due to the same non-physiological autoxidation of cytochrome c as was found in methanol-excess bacteria (50% inhibition at 1.5mM) which have no cytochrome a/a_3 to confuse interpretation of results.

Whereas the pattern of inhibition by azide of methanol and NAD(P)H oxidation was triphasic, the pattern with cyanide was biphasic with respiration being almost completely inhibited by low concentrations of cyanide (50% inhibition at 0.4-2μM cyanide, Fig. 6.2; Fig. 6.6). In the presence of high concentrations of cyanide some respiration occurred by way of cytochrome c with similar characteristics to those found in methanol-excess preparations (50% inhibition at 80-150μM KCN; Figs. 6.2; 6.6). The oxidation of ascorbate/TMPD was completely inhibited by 2μM cyanide and the lack of cyanide-resistant respiration suggests that either there is no cytochrome c-dependent respiration from this substrate, or that the rate is too low to be detected (Section 6.4).

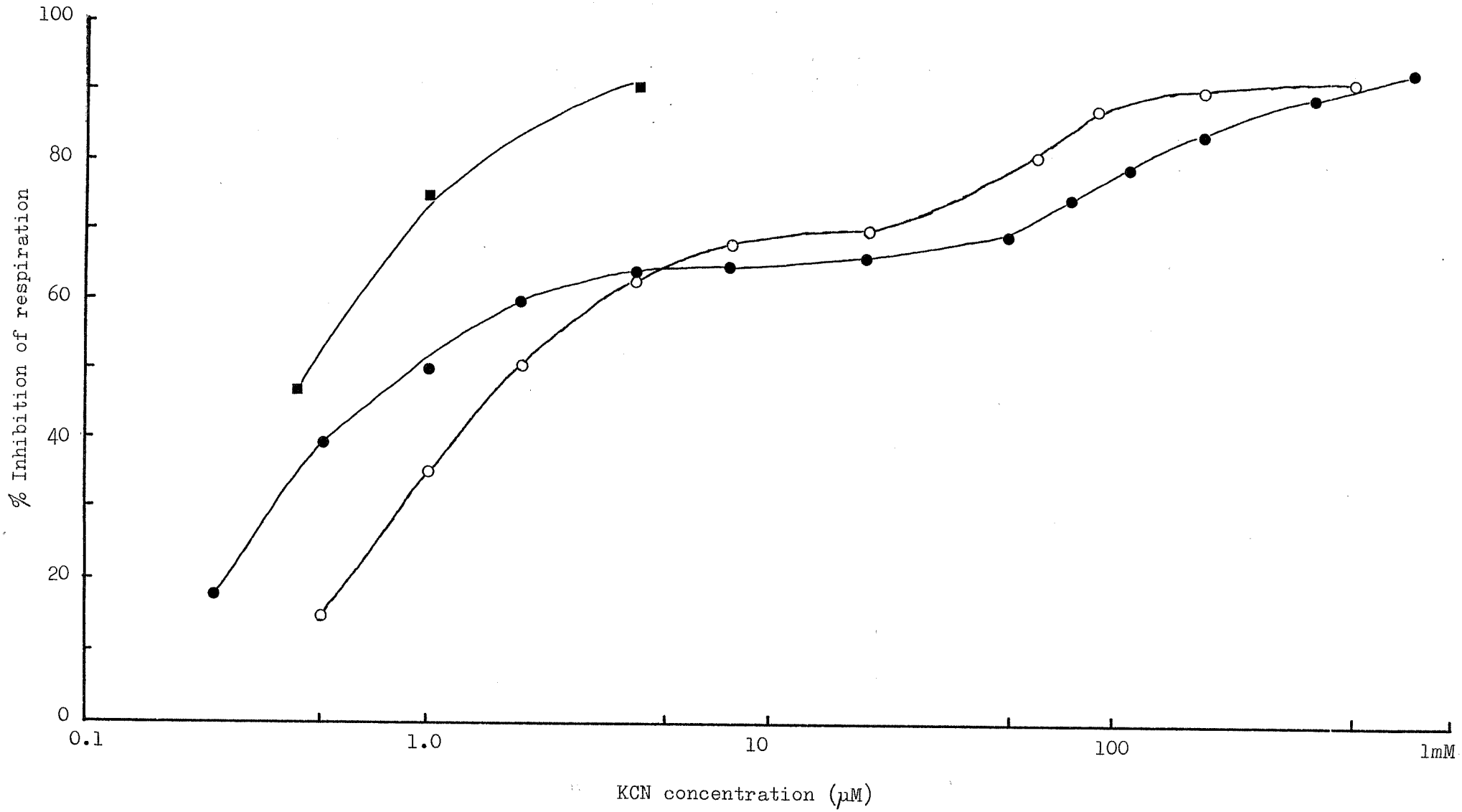
Fig. 6.6 - Inhibition by cyanide of respiration in bacteria grown in methanol-limited cultures

a. ■—■ Ascorbate/TMPD oxidation by methanol-limited bacteria (cytochrome a/a_3 present). The uninhibited oxidation rate was 160 nmol O_2/min/mg dry weight of bacteria.

b. ●—● Methanol oxidation by methanol-limited bacteria (cytochrome a/a_3 present). The uninhibited oxidation rate was 253 nmol O_2/min/mg dry weight of bacteria.

c. o—o NADH oxidation by methanol-limited bacteria (cytochrome a/a_3 present). The uninhibited oxidation rate was 225 nmol O_2/min/mg dry weight of bacteria.

Respiratory inhibition was determined by the method described in Section 2.13.


100
80
60
40
20
0
% Inhibition of respiration
0.1
1.0
10
100
1mM
KCN concentration (μM)

These results with cyanide taken together with those for azide were consistent with the cytochrome a/a_3 having the same sensitivity to cyanide as the cytochrome o, but different sensitivities to azide.

6.6 Methanol Oxidation in Soluble Preparations

Methanol was slowly oxidized by the soluble cell fraction after the removal of the membrane fraction. The only detectable cytochrome was cytochrome c. The pattern of inhibition was monophasic (Fig. 6.4) with 50% inhibition at 90μM cyanide, 1.1mM azide, consistent with the suggestion that the cyanide and azide resistant oxidation of methanol and NAD(P)H in whole cells and membranes could be due to an autoxidizable cytochrome c. This oxidation was probably by way of cytochrome c_L which is the most autoxidizable cytochrome c and the cytochrome which is reduced most rapidly by methanol dehydrogenase.

6.7 The Effect of n-Heptyl-4-hydroxyquinoline-N-oxide, Antimycin A and Rotenone

80% of NADH oxidation by membranes was inhibited by low concentrations of HQNO and antimycin A (Fig. 6.7) with 50% of the inhibition at 3.5μM HQNO and 5μM antimycin A. 20% of the oxidation was insensitive to these inhibitors and may be going through ainhibitor-resistant site. Unexpectedly ascorbate/TMPD was also inhibited by HQNO and antimycin A (Fig. 6.8) but at higher concentrations than those required to inhibit NADH oxidation, with 50% inhibition occurring at 22μM HQNO and 45μM antimycin A. HQNO inhibited virtually all ascorbate/TMPD oxidation but antimycin A inhibited only 70% at the highest concentration used. Neither inhibitor was effective with whole bacteria. In view of the different concentrations required to inhibit NADH and ascorbate/TMPD oxidation it is likely that they are reacting at two sites; at low concentration between cytochromes b and c, (inhibiting NADH oxidation) and at higher concentrations probably at cytochrome o inhibiting ascorbate/TMPD oxidation.

Rotenone was effective in inhibiting NADH oxidation, 50% inhibition occurring at 0.5mM; the effect of rotenone on NADPH oxidation was not tested.

Fig. 6.7 - Inhibition by antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide of respiratory activity in bacterial membranes

a. ●—● The effect of antimycin A on NADH oxidation by membranes. The uninhibited oxidation rate was 76 nmol O_2/min/mg protein.

b. o—o The effect of HQNO on NADH oxidation by membranes. The uninhibited rate was 43 nmol O_2/min/mg protein.

Respiratory inhibition was determined by the method described in Section 2.13.

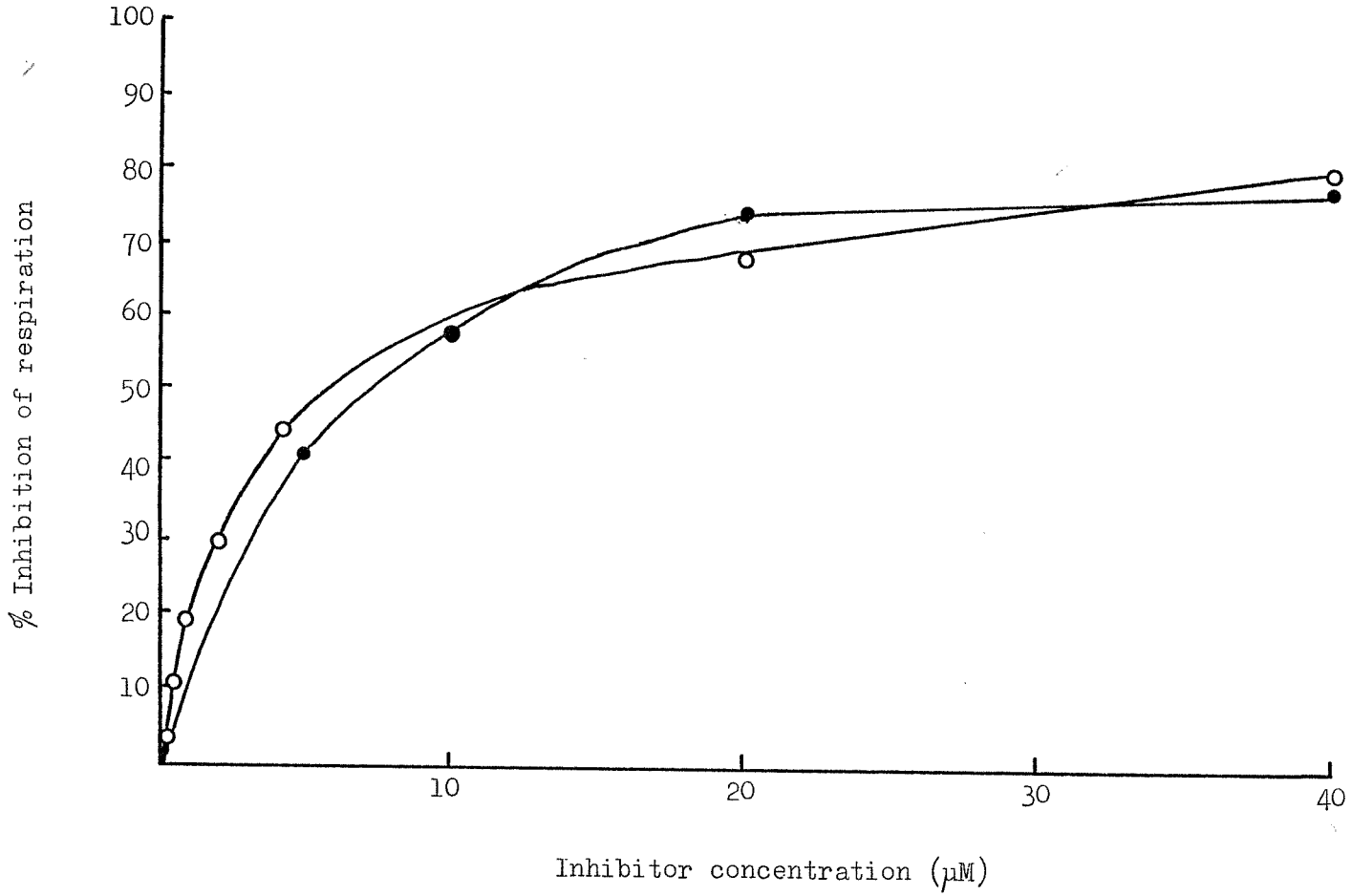
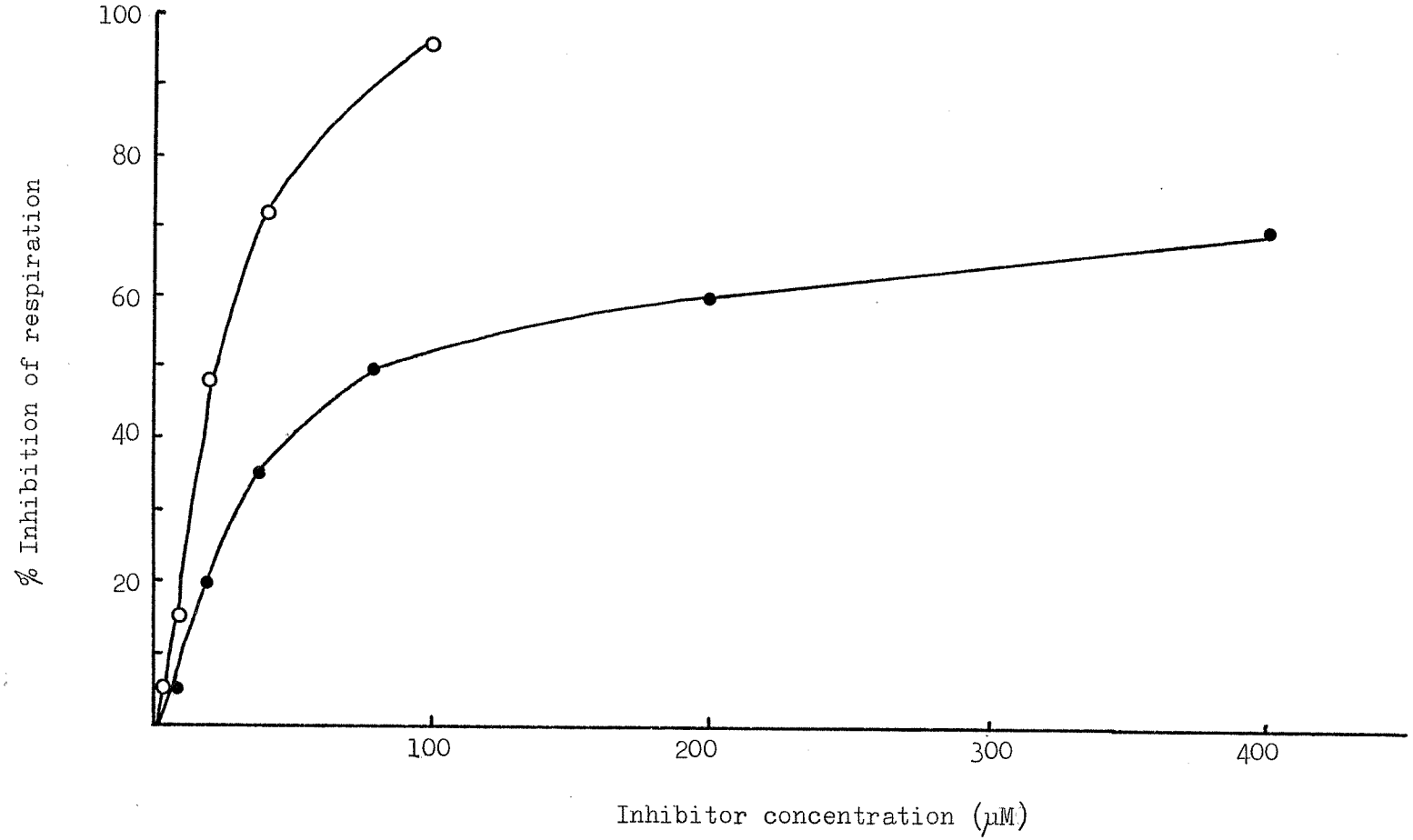

% Inhibition of respiration
100
90
80
70
60
50
40
30
20
10
10
20
30
40
Inhibitor concentration (μM)

Fig. 6.8 - Inhibition by antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide of respiratory activity in bacterial membranes

a. ●—● The effect of antimycin A on Ascorbate/TMPD oxidation by membranes. The uninhibited oxidation rate was 2361 nmol O_2/min/mg protein.

b. ○—○ The effect of HQNO on ascorbate/TMPD oxidation by membranes. The uninhibited oxidation rate was 2767 nmol O_2/min/mg protein.

Respiratory inhibition was determined by the method described in Section 2.13.


100
80
60
40
20
% Inhibition of respiration
100
200
300
400
Inhibitor concentration (μM)

6.8 Summary and Discussion

The main conclusions drawn from the work described in this chapter are as follows :-

1. Whole bacteria oxidized methanol, NADH, NADPH and ascorbate/TMPD; the oxidation of NAD(P)H by whole bacteria is unusual but not unique.

2. Bacterial membranes no longer oxidized methanol although methanol dehydrogenase was present.

3. Bacterial membranes oxidized ascorbate/TMPD at much greater rates than whole bacteria and this rate was much higher using membranes from bacteria grown in methanol-excess conditions compared to those grown under methanol-limited conditions.

4. NADH oxidation by bacterial membranes was inhibited by rotenone, antimycin A and HQNO.

5. Oxidation of ascorbate/TMPD was unexpectedly inhibited by HQNO and antimycin A although at higher concentrations than those required to inhibit NADH oxidation.

6. It was concluded that the high rate of HQNO- and antimycin A-sensitive oxidation of ascorbate/TMPD was by way of cytochrome o.

7. It is proposed that 3 cytochromes are potential oxidases; a cytochrome a/a_3 (present only in methanol-limited conditions); a cytochrome o (induced 10-fold in methanol-excess conditions); and cytochrome c which is unlikely to be a physiological oxidase.

For cytochrome a/a_3 50% inhibition of activity is found at 0.4-2.0μM KCN and at about 50μM azide. For cytochrome o 50% inhibition of activity is found at 0.4-2.0μM KCN and at about 2.5μM azide. For cytochrome c 50% inhibition of activity is found at 80-150μM KCN and at about 1mM azide (Table 6.3).

The most important general conclusions from this chapter relate to the various oxidases in Methylophilus methylotrophus which vary in amount with varying growth conditions. During growth in excess methanol there was no cytochrome a/a_3 and so an alternative oxidase must be

Table 6.3 - Inhibitor concentrations required to cause 50% inhibition of oxidase activity in whole bacteria and membrane preparations

The methods used to measure inhibition of respiratory activity are described in Section 2.13. The substrates used were NADH, NADPH, Ascorbate/TMPD (for whole bacteria and membranes) and methanol (whole bacteria only).

Potential oxidase	KCN concentration (μM)	Azide concentration (μM)
cytochrome a/a_3	2	50
cytochrome o	2	4
cytochrome c	120	1000

operating. In these conditions an alternative oxidase was induced 10-fold, as estimated by the increase in the rate of ascorbate/TMPD oxidation. This increase was concomitant with the appearance in membranes of an extra b-type cytochrome as indicated by a peak at 558nm in reduced-minus-oxidized difference spectra measured at 77°K (Chapter 4). Also concomitant with the increased rate of ascorbate/TMPD oxidation was the appearance of a new component having a midpoint redox potential of 260mV; this was observed in redox titrations using the wavelength pairs corresponding to the b-type cytochromes (563-570nm, 559-570nm) but not using the cytochrome c wavelength pairs (550-540nm) (Chapter 4). It was thus concluded that this inducible b-type cytochrome was the alternative oxidase cytochrome o.

The cytochrome o reacts directly with oxygen using ascorbate/TMPD as electron donor; the turnover number of around 200/second lies within the range found for other oxidases (Chance and Williams, 1955; Smith, 1961). This indicates that either the cytochrome reacts directly with ascorbate/TMPD or that a cytochrome c is present in the membrane capable of rapid reaction with ascorbate/TMPD and thence cytochrome o. That this cytochrome c is not the autoxidizable' cytochrome c observed in inhibition studies with methanol and NADH is shown by the monophasic inhibition pattern by cyanide and azide of ascorbate/TMPD oxidation. The cytochrome o is unusual in being inhibited by HQNO and antimycin A; in this respect it resembles the cytochrome o of Acetobacter suboxydans (Daniel, 1970).

The results in Table 6.4 indicate the proportions of electron transport able to go by way of each potential oxidase. It should be noted that the percentage of respiration able to go through a particular route shown by inhibition does not mean that the route is usually active, since this will be determined by the K_m for oxygen of each potential oxidase, by the redox potential of the oxidase and by the concentration of oxygen present. Furthermore it should be noted that respiration by way of cytochrome c could only be demonstrated unequivocally in the presence of sufficient cyanide or azide to inhibit oxidation by way of cytochromes a/a_3 and/or o. In these conditions the cytochrome could be more reduced than in the absence of inhibitors and the rate of its autoxidation could have been higher than ever might occur in the absence of inhibitors. Because membranes always had the oxidases cytochrome o

Table 6.4 - Estimated electron transport to oxygen via each oxidase

It should be noted that the percentage of respiration able to go through a particular route shown by inhibition does not mean that the route is usually active, since this will be determined by the K_m for oxygen of each potential oxidase, by the redox potential of the oxidase and by the concentration of oxygen present. Furthermore it should be noted that respiration by way of cytochrome c could only be demonstrated unequivocally in the presence of sufficient cyanide or azide to inhibit oxidation by way of cytochromes o and/or a/a_3.

The methods used to measure respiratory inhibition are described in Section 2.13. The results are discussed in Section 6.8.

Growth conditions	Cytochrome		
	a/a_3	o	c
Methanol-limitation	60%	15%	25%
Methanol-excess	0	75%	25%

and/or cytochrome a/a_3 the rate of autoxidation of the membrane-bound cytochrome c could not be measured. It is not known if the slightly autoxidizable soluble cytochrome c_L (Chapter 5) is the cytochrome c responsible for oxidation on membranes but the similarity in sensitivity to inhibition by KCN and azide of methanol oxidation by the soluble fraction suggest that it is. It is concluded that respiration by way of cytochrome c is not likely to be very physiologically significant.

It is not known whether NADPH is oxidized by way of a trans-hydrogenase but in view of the similarity of the responses of NADPH and NADH oxidation to inhibitors it is unlikely that electron transport from these substrates follows different routes on the oxygen side of cytochrome b.

A scheme for electron transport in Methylophilus methylotrophus and for its regulation under various growth conditions is discussed in the following chapter.

CHAPTER 7

GENERAL SUMMARY AND DISCUSSION

7.1 Summary and Conclusions

The main conclusions from Chapters 3 - 6 are summarized and discussed to a certain extent at the end of each chapter. The major conclusions of this thesis which are used to propose an electron transport chain for Methylophilus methylotrophus are summarized below :-

1. Methylophilus methylotrophus contains cytochromes of the a, b, o and c types. All of the a- and b-type cytochromes are membrane bound as is 30% of the total c-type cytochrome produced by the organism. The ratio of the a:b:c cytochromes bound to the membranes is approximately 1:2.5:6, in addition, there is a further similar quantity of cytochrome c in the soluble cell fraction.

2. The cytochrome oxidase; cytochrome a/a_3 was only present in the bacteria grown under conditions of methanol-limitation.

3. Methylophilus methylotrophus always contains 2 b-type cytochromes; with midpoint redox potentials of 60 and 110mV.

4. Methylophilus methylotrophus contains larger quantities of cytochrome o (midpoint redox potential 260mV) during growth under methanol-excess conditions, when cytochrome a/a_3 is absent.

5. The oxidases; cytochromes a/a_3 and o are approximately equally sensitive to inhibition by cyanide. The oxidases are also inhibited by azide, cytochrome o being more sensitive than cytochrome a/a_3. In addition, cytochrome o is also inhibited by antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide. The oxidation of ascorbate/TMPD by cytochrome o is very rapid; the midpoint redox potential of the cytochrome o (260mV) is only 5mV higher than that of TMPD (255mV). The cytochrome is induced 10-fold during methanol-excess growth compared to methanol-limited growth where cytochrome a/a_3 is present.

6. Methylophilus methylotrophus produces two major c-type cytochromes and one minor c-type cytochrome (less than 15% of the total), but only the two major cytochromes appear to be bound to the membrane under conditions of methanol-excess growth.

7. The 3 c-type cytochromes have been purified; their properties are summarized in Table 5.2. They differ from one another in isoelectric point, molecular weight, midpoint redox potential, extent of carbon monoxide binding and reaction with methanol dehydrogenase. They are similar in having one haem per mole and in having high midpoint redox potentials.

8. None of the cytochromes c from Methylophilus methylotrophus particularly resemble those purified from methane utilizing bacteria, but the two major cytochromes c closely resemble the two cytochromes c purified from Pseudomonas AM1; they differ in that they have higher redox potentials and are not auto-reduced at high pH.

9. Inhibitor-insensitive respiration, which continues after cytochromes a/a_3 and o are completely inhibited, is thought to be by way of cytochrome c, but is not thought to be physiologically significant.

10. Respiration-driven proton translocation ratios were measured with Methylophilus methylotrophus cultures, but the results were not consistent enough to determine whether the ratios are altered under growth conditions where cytochrome a/a_3 is the major oxidase or where cytochrome o is the oxidase.

11. The oxidation of NADH and NADPH is by the same route along the electron-transport chain. It is not known if a transhydrogenase is involved.

12. Methanol oxidation proceeds by way of methanol dehydrogenase most of which is bound to the bacterial membrane. Membranes do not oxidize methanol however, and therefore the conformation of the system is altered during preparation or another factor is required.

13. Methanol and NADH rapidly reduce all the cytochromes c and a/a_3 in whole cells.

14. NADH rapidly reduces all the cytochromes in membranes.

15. Inhibition of respiration by cyanide and azide suggest that cytochrome o and a/a_3 can be involved in the oxidation of both methanol and NAD(P)H but that ascorbate/TMPD is oxidized only by way of cytochrome o.

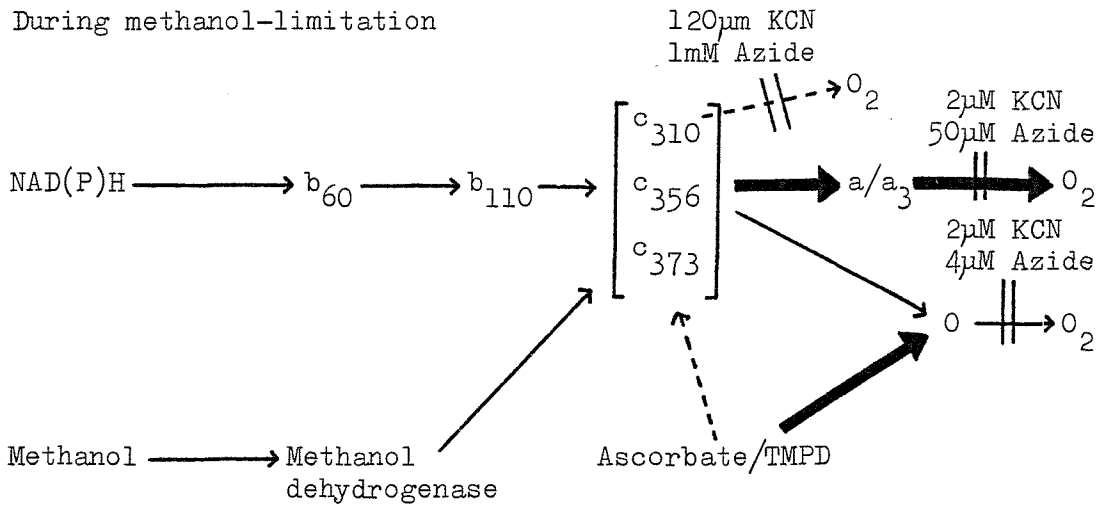
The simplest scheme that is consistent with all the evidence presented in this thesis is shown in Fig. 7.1. It should be noted that in this scheme it is assumed that the electron transport components between NAD(P)H and cytochrome b are similar to those in other electron transport chains; that is, NADH dehydrogenase (an iron/sulphur protein containing FMN) and a quinone/quinol system. It should also be noted that in this scheme, in addition to the membrane-bound components, there is an equal quantity of cytochrome c in solution. The key proposals of this scheme are that the branch point for the entry of electrons from methanol dehydrogenase is at cytochrome c (after cytochromes b_{60} and b_{110}) and that the branch to cytochrome o is from cytochrome c and not from cytochrome b. The proposal that electrons from methanol dehydrogenase enter the electron transport chain at the level of cytochrome c is consistent with the systems which have been proposed for other methylotrophs, and there is no evidence to indicate that Methylophilus methylotrophus differs from other methylotrophs in this respect. The evidence suggesting that the branch point for the entry of electrons from methanol dehydrogenase is at cytochrome c can be divided into two parts; firstly the demonstration that cytochrome c is involved during growth on, and oxidation of methanol, and secondly, the demonstration that cytochrome b is not involved in the oxidation of methanol. The evidence suggesting cytochrome c is involved in the oxidation of methanol in Methylophilus methylotrophus is as follows :-

1. Methanol rapidly reduces all the cytochrome c in whole bacteria.

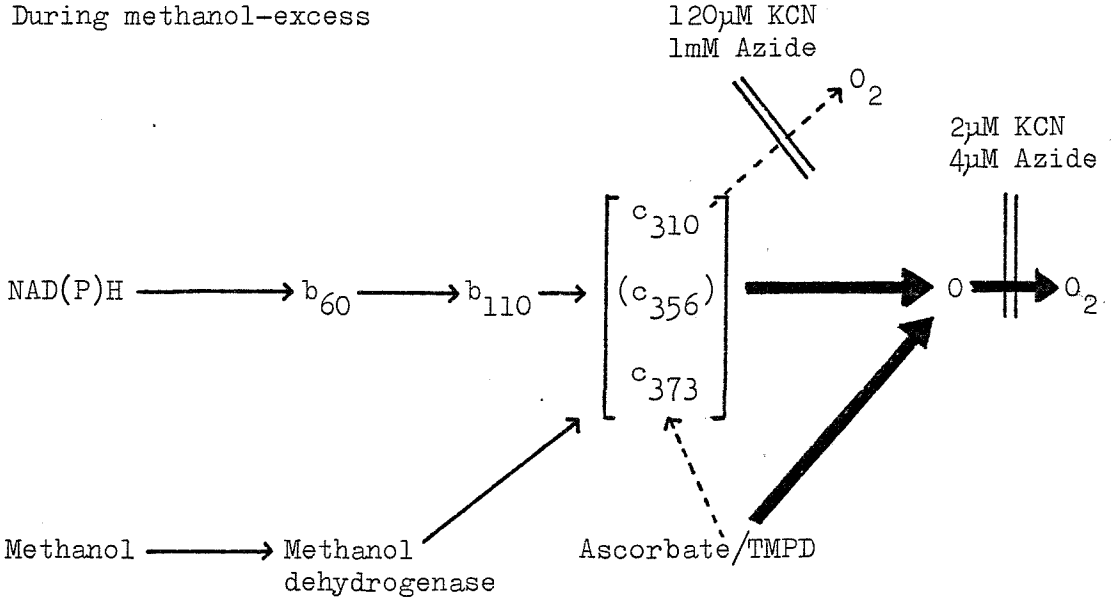
2. The soluble cell fraction (in which the only cytochrome is cytochrome c) oxidizes methanol.

Fig. 7.1 - A proposed scheme for the electron transport chain of Methylophilus methylotrophus

a. During methanol-limitation



b. During methanol-excess



Evidence which suggests cytochrome c is involved in the oxidation of methanol in other methylotrophs is as follows :-

1. Methanol rapidly reduces all the cytochrome c in whole bacterial suspensions of Pseudomonas AM1.

2. Methanol-dependent cytochrome c reduction has been demonstrated in Hyphomicrobium X (Duine et al., 1979) and Pseudomonas AM1 (O'Keefe and Anthony, 1980) cell extracts.

3. Cytochrome c is reduced by addition of methanol to vesicles prepared from Pseudomonas AM1.

4. A mutant of Pseudomonas AM1 lacking cytochrome c (mutant PCT 76) could not grow on, or oxidize methanol, while the ability of the mutant to oxidize other substrates was unimpaired (Anthony, 1975b; Widdowson and Anthony, 1975).

5. The quntities of cytochrome c produced by methylotrophs is increased during growth on methanol or methane (Tonge et al., 1974; Keevil and Anthony, 1979).

6. A carbon monoxide-binding cytochrome c is induced in Paracoccus denitrificans during growth on methanol (van Verseveld and Stouthamer, 1978).

7. In Methylobacter organophilum cytochrome c is induced during growth on methanol, methanol$^-$ mutants have no cytochrome c and the cytochrome c gene is regulated by the same gene responsible for the regulation of the other enzymes required for growth on methanol (O'Connor et al., 1977).

The evidence suggesting that cytochrome b is not involved in methanol oxidation in Methylophilus methylotrophus is that there appears to be some direct interaction between methanol dehydrogenase and the purified cytochromes c. Evidence that cytochrome b is not involved in methanol oxidation in other methylotrophs is summarized below :-

1. There is some evidence that pure methanol dehydrogenase and pure cytochrome c react directly in Pseudomonas AM1 (O'Keeffe and Anthony, 1980).

2. In membrane vesicles prepared from Pseudomonas AM1 respiration-linked ATP synthesis has been measured (Netrusov and Anthony, 1979). The P/O ratio with methanol as the respiratory substrate is the same as that when ascorbate/TMPD is the substrate, and is less than the P/O ratio found when either succinate or NADH is the respiratory substrate.

3. In membrane vesicles prepared from Pseudomonas AM1, cytochrome c is reduced by methanol; the reduction is not sensitive to inhibition by antimycin A. NADH and succinate also reduce cytochrome c; the reduction is inhibited by antimycin A.

4. In membrane vesicles prepared from Pseudomonas AM1 methanol oxidation is not inhibited by antimycin A; NADH and succinate oxidation are inhibited by antimycin A (Netrusov and Anthony, 1978).

5. Oxidation of NADH and succinate by cell free extracts of Pseudomonas extorquens is inhibited by antimycin A and HQNO but oxidation of methanol is not inhibited.

6. Antimycin A inhibits the oxidation of succinate and NADH by whole Paracoccus denitrificans, but does not inhibit methanol oxidation (van Verseveld and Stouthamer, 1978).

7. Antimycin A and HQNO inhibit the oxidation of succinate and NADH by cell free extracts of Pseudomonas extorquens, but these inhibitors do not affect methanol oxidation (Higgins et al., 1976).

8. Antimycin A and HQNO inhibit the oxidation of NADH, but not methanol, by cell free preparations of Methylosinus trichosporium (Higgins et al., 1977).

It should also be noted that the conclusion that cytochrome b was involved in methanol oxidation by Methylomonas P1 1 (Drabikowska, 1977), is probably incorrect for the reasons described in Chapter 1. It is therefore proposed that the branch point for the entry of electrons from methanol dehydrogenase into the electron transport chain is at cytochrome c (after cytochromes b_{60} and b_{110}).

The scheme also proposes that cytochrome c is involved in the oxidation of NAD(P)H; this is suggested from results showing that NADH rapidly reduces all the cytochrome c in whole bacteria, and that in the presence of concentrations of cyanide and azide sufficient to inhibit cytochromes a/a_3 and o a small amount of oxidation continues via cytochrome c.

The involvement of cytochrome c in both methanol and NAD(P)H oxidation places the branch point to cytochrome o at the level of cytochrome c and not at the level of cytochrome b because under methanol-excess conditions the oxidation of these substrates involves both cytochromes c and o.

7.2 A Comparison of the Electron-Transport Chains of Methylophilus methylotrophus with those of other Methylotrophs

The electron transport schemes which have been proposed for Paracoccus denitrificans (a facultative, ribulose bisphosphate pathway bacterium), Pseudomonas AM1 (a facultative, serine pathway bacterium) and Methylosinus trichosporium (an obligate methane-utilizing, serine pathway organism) are shown in Fig. 7.2. All these schemes resemble the mitochondrial electron-transport chain in that they are composed of a NADH dehydrogenase, a quinone/quinol system and cytochromes of the a, b and c types. The electron transport schemes also resemble one another in the following respects :-

1. The branch point for the entry of electrons from methanol dehydrogenase is at cytochrome c.

2. Cytochrome b is not involved in methanol oxidation.

3. Cytochrome c is involved in electron transport from NADH during carbon-limited growth in Methylophilus methylotrophus and Pseudomonas AM1 and in the growth conditions used during the studies made on the other organisms.

4. All the electron-transport chains have the same terminal oxidase for both NADH and methanol.

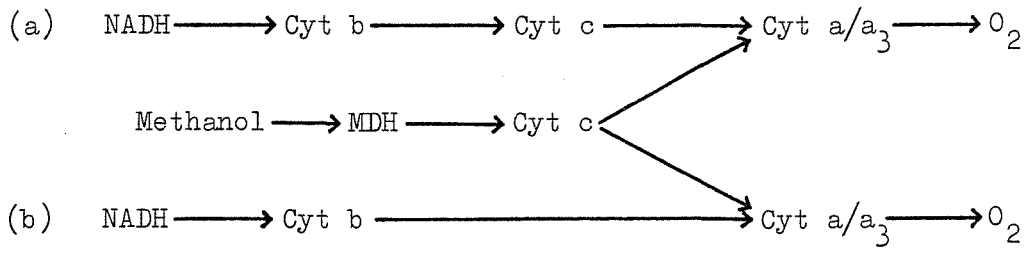
5. All the systems are inhibited by cyanide, azide and antimycin A.

Fig. 7.2 Electron transport in methylotrophs

Pseudomonas AM1 (Keevil and Anthony, 1979b)

(a) Carbon-limited growth conditions.

(b) Carbon-excess growth conditions.



Paracoccus denitrificans (van Verseveld and Stouthamer, 1978)

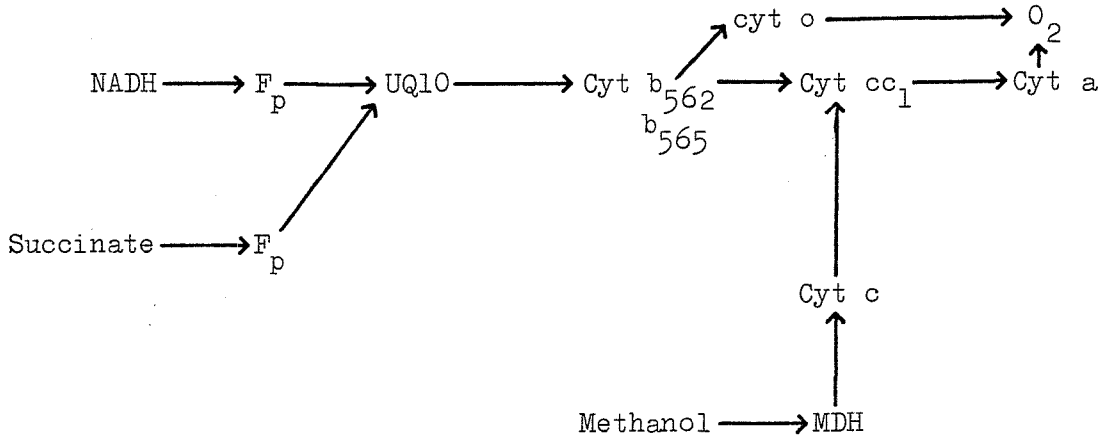
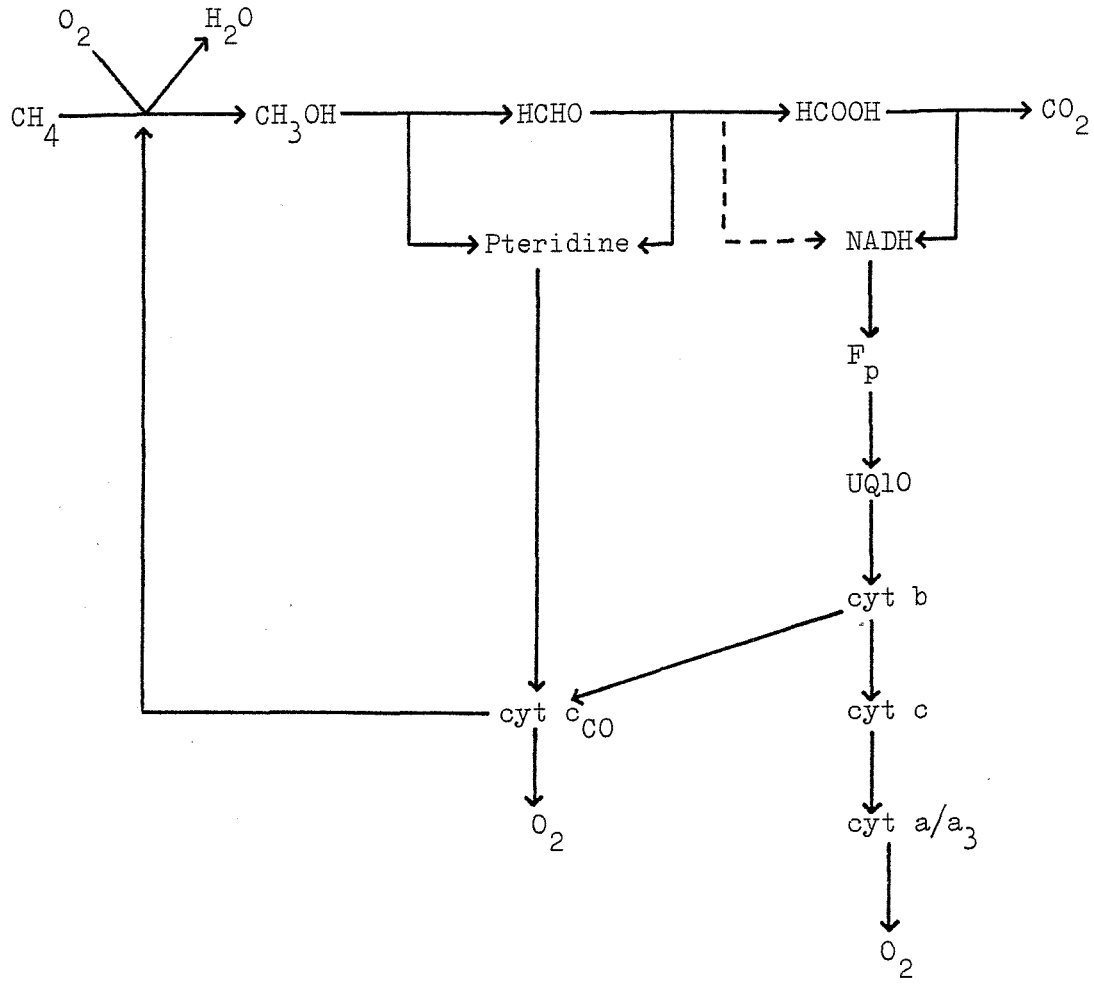


Fig. 7.2 (Cont'd.)

Methylosinus trichosporium (Higgins et al., 1977)



6. All the bacteria have some membrane-bound and some soluble cytochrome c.

Methylophilus methylotrophus differs from the other organisms in that cytochrome o is on the main route for methanol oxidation under conditions of methanol-excess growth. Cytochrome o has not been shown to be involved in methanol oxidation in any other methylotroph. Paracoccus denitrificans contains cytochrome o, but always contains cytochrome a/a_3 during growth on methanol and the quantity of the cytochrome a/a_3 is increased relative to cytochrome o during growth on methanol (van Verseveld and Stouthamer, 1978). It was concluded by these authors that the branch point to cytochrome o is from the b-cytochromes, in contrast to Knobloch (1971) who put the branch point at the level of cytochrome c (although these studies were not performed on methanol-grown bacteria). The evidence presented by van Verseveld and Stouthamer to show that cytochrome o is not involved in methanol oxidation by Paracoccus denitrificans is not conclusive; the authors state, "inhibition of electron-transport pathways will yield straight lines in semi-logarithmic plots". This is not true and must shed doubt on their conclusions as they use straight lines drawn through semi-logarithmic plots to demonstrate the involvement of different oxidases in methanol and endogenous substrate oxidation.

Both Methylophilus methylotrophus and Pseudomonas AM1 alter their electron-transport chains in response to carbon-limitation or excess; but in different ways. Unlike Methylophilus methylotrophus which has alternative terminal oxidases, the oxidase of Pseudomonas AM1 is cytochrome a/a_3 under all growth conditions. Both respiration driven proton translocation ratios and growth yields are reduced in Pseudomonas AM1 during growth in succinate-excess (as compared to succinate-limited growth) and repiration-driven proton translocation ratios were reduced during growth in methanol-excess conditions (compared to methanol-limited conditions). The yield during methanol-limitation would not be expected to rise very much because during growth on methanol relatively little NADH is oxidized (Anthony, 1978b), cell yields on methanol being limited to a large extent by the NAD(P)H rather than the ATP supply (Anthony, 1978a). The alteration in the electron transport chain is thought to be due to cytochrome c only being involved in electron transport from cytochrome b to cytochrome a/a_3 during carbon-

limited growth and not during carbon-excess growth, thus altering the number of proton translocating segments (Keevil and Anthony, 1979). Cytochrome c is known not to be necessary for electron-transport between cytochromes b and a/a_3 in Pseudomonas AM1 as a cytochrome c mutant is capable of normal growth on multi-carbon compounds (Anthony, 1975b; Widdowson and Anthony, 1975).

Methylosinus trichosporium differs from the other methylotrophs in that cytochrome c has been proposed as a physiological oxidase and as electron donor to methane mono-oxygenase (see Chapter 1), but the work of Dalton and his colleagues has cast serious doubt on the latter function of the cytochrome and may necessitate a reappraisal of its proposed oxidase role.

All the methylotrophs which have been studied contains some soluble and some membrane-bound cytochrome c; apparently these cytochromes are of the same type. This phenomenon is not unique to methylotrophs. Clark-Walker and Lascelles (1970) have suggested that the membrane has a limited capacity for binding cytochromes and during the surplus biosynthesis required to provide for certain biochemical functions, some cytochrome remains in the soluble form. Although all the methylotrophs studied have at least one cytochrome with a midpoint redox potential of around 300mV (Table 1.1) one of the major c-type cytochromes from Methylophilus methylotrophus has a midpoint redox potential significantly higher than this; it is not clear why this should be so, but it may be a reflection of a common origin with the photosynthetic bacteria which typically have cytochromes c with mid-point redox potentials above 350mV. Another unusual feature of the cytochrome c from Methylophilus methylotrophus is that considerable quantities are found in the growth medium. Although this is not the case with Paracoccus denitrificans; cytochrome c can be removed from whole bacteria with strong salt solutions, suggestive of a periplasmic location for the cytochrome (Scholes et al., 1971).

Very few whole bacteria will oxidize NAD(P)H; in addition to Methylophilus methylotrophus it has been reported for Acetobacter suboxydans (Daniel, 1970) and Haemophilus parainfluenzae (White and Sinclair, 1970); the latter actively accumulates these nucleotides. Methylophilus methylotrophus cells are relatively fragile and the capacity to oxidize NAD(P)H may be an adaptation to scavenge useful compounds released from lysed cells.

7.3 The Regulation of the Terminal Oxidases of Methylophilus methylotrophus

The regulation of the terminal oxidases cytochromes a/a_3 and o is in response to the methanol concentration experienced by the bacteria. When methanol is the growth-limiting substrate, cytochrome a/a_3 is maximally induced (and cytochrome o is repressed). When nitrogen or oxygen supply become growth-limiting, cytochrome o is induced (approximately 10 fold) and cytochrome a/a_3 is repressed. The regulatory molecule is not known. The response of the bacterium to an excess of methanol may be to oxidize this substrate rapidly to reduce its toxic effects. Unfortunately the results of respiration driven proton translocation experiments were not sufficiently good to determine whether the substitution of one oxidase (a/a_3) with another (o) alters the phosphorylation efficiency of the Methylophilus methylotrophus with a resultant change in yield, but it is known that this organism gives higher yields when grown under methanol-limited conditions than when grown under conditions of methanol-excess (Brooks and Meers, 1973).

7.4 Unanswered Questions concerning the Electron Transport Chain of Methylophilus methylotrophus

The following questions concerning the electron transport chain of Methylophilus methylotrophus have not yet been answered :-

1. What are the characteristics of the dehydrogenases and quinones?

2. What is the midpoint redox potential of the oxidase cytochrome a/a_3?

3. What is the effect of growth rate on the composition of the electron-transport chain?

4. How much does the yield, with respect to oxygen and methanol, change with the growth conditions?

5. Are all three cytochromes c present under all growth conditions and are different c-type cytochromes associated with the alternative oxidases?

6. Can the interaction of methanol dehydrogenase and cytochrome c be demonstrated unequivocally?

7. What are the relative rates of reduction of cytochromes c and o by ascorbate/TMPD?

8. Do whole bacteria accumulate exogenous NAD(P)H?

9. Is the release of cytochrome c into the growth medium significant and what is the mechanism of the cytochrome c photo-oxidation?

By answering these questions, and by studies of other bacteria which must adapt their electron transport chains to cope with unusual bioenergetic problems, we may gain a better understanding of the mechanism of electron transport processes in all organisms.

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