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A STUDY OF ELECTRON TRANSPORT IN  
PROPIONIBACTERIUM  
SHERMANII

A THESIS  
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requirements for the degree of  
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## ABSTRACT

Propionibacteria possess a membrane-bound electron transport system coupling oxidation of several substrates to reduction of fumarate or oxygen. Published work has indicated that the electron transport system consists of the following: dehydrogenases for NADH, lactate, glycerol phosphate and succinate; menaquinone (II,III-tetrahydro-menaquinone-9); cytochromes b, a, d and possibly o; terminal oxidase(s) and fumarate reductase. Menaquinone has been demonstrated to be an obligate component of electron transport to fumarate but there is disagreement in the literature concerning the role of the b-cytochrome components in the electron transport pathways. In the present study, the effect of a number of inhibitors on electron transport from NADH, D- and L-lactate and succinate to oxygen and to fumarate was investigated with the aim of elucidating the relationship between the aerobic and anaerobic pathways for oxidation of these substrates and the role of the cytochrome components in these systems.

Membranes were prepared by breaking lactate-grown cells of *P. shermanii* ATCC 9614 in a Hughes press and separating membranes from soluble components by differential centrifugation with a final centrifugation over a stepwise sucrose gradient.

Spectrophotometric analysis of pyridine hemochromogen derivatives showed the presence of significant quantities of cytochromes b and d, but did not detect the presence of cytochrome a or c. The cytochromes were also characterized



by redox titrimetry at room temperature and by analysis of low temperature spectra of redox-poised membranes. These experiments indicated the presence of four b-type cytochromes with  $\alpha$ -absorption peaks (subscript) and approximate midpoint redox potentials at pH 7 (bracketed figures) as follows:  $b_{562-3}$  (+120 mV);  $b_{556-7}^{HP}$  (+90 mV);  $b_{556-7}^{LP}$  (-20 mV);  $b_{553-4}$  (-20 mV). A midpoint potential at pH 7 for cytochrome d of approximately +150 mV was determined from room temperature titrations.

Of several inhibitors surveyed for effects on electron transport activities of membranes, pCMB, dicumarol, UV light and cyanide were selected for detailed study. Dicumarol was particularly effective as an inhibitor of L-lactate dependent activities. L-lactate-dependent reduction of oxygen, DICPIP and  $Fe(CN)_6$  was inhibited by a concentration of dicumarol  $10^3$  times less than that required for comparable inhibition of the corresponding D-lactate and NADH dependent activities. This indicates either that the L-lactate dehydrogenase has distinctive dicumarol-sensitive components or that it is integrated in the membrane in a manner which enhances its accessibility to dicumarol. The fact that the D-lactate-coupled activities are very stable while the L-lactate dependent activities are unstable also indicates that the two lactate dehydrogenase systems have different properties.

Ultraviolet light irradiation caused a rapid inactivation of NADH- and D-lactate-fumarate oxidoreductases but a slower inactivation of NADH and D-lactate oxidases. This was interpreted to indicate that electron transport to

fumarate has an absolute requirement for menaquinone while a portion of the oxidase activity did not require menaquinone.

All electron transport activities were inhibited by pCMB at a concentration around  $10^{-4}$  moles per gram protein but NADH-dependent reduction of oxygen,  $\text{Fe}(\text{CN})_6$  and DICPIP was inhibited to a maximum of only 50%.

The pCMB titration curves for inhibition of NADH-dependent activities and the significantly higher reduction of cytochrome b by NADH than by D- or L-lactate in the presence of inhibitory concentrations of pCMB suggest the presence of two independent dehydrogenases for NADH. However the complete inhibition of NADH-fumarate oxidoreductase by pCMB suggests that only the pCMB-sensitive dehydrogenase is involved in electron transport to fumarate.

Cyanide inhibited D- and L-lactate- and NADH- dependent oxygen consumption with an  $I_{50}$  of about 2 mM. The relatively low sensitivity to cyanide suggests that cytochrome d is the major terminal oxidase. This is consistent with the evidence from difference spectra of cyanide-inhibited aerobic steady state membranes indicating that cyanide binds to cytochrome d and inhibits oxidation of cytochrome b. Although a CO-binding cytochrome b is present, the low cyanide sensitivity and absence of CO inhibition of oxygen uptake suggest that an o-type oxidase does not contribute significantly to oxygen uptake.

The effect of inhibitors on reduction of  $\text{Fe}(\text{CN})_6$  and DICPIP by all four substrates was also systematically investigated. The results obtained from these studies

suggest multiple sites of interaction of these acceptors with the electron transport pathways, the points of interaction depending on both the nature of the acceptor and of the reducing substrate.

Addition of fumarate caused a decrease of 20-30% in the rate of oxygen consumption and in total oxygen consumption at concentrations between 0 and 1 mM. No further inhibition occurred with increasing fumarate concentrations up to 10 mM, but concentrations above 10 mM inhibited first the rate of oxygen consumption and then the total oxygen consumption until both were completely inhibited at 40 mM fumarate. Inhibition at low concentrations of fumarate is probably due to diversion of a portion of the electron flow from oxygen to reduction of fumarate. This competition between oxygen and fumarate for electrons indicates that the pathways to these two acceptors share common initial steps.

Comparison of cytochrome difference spectra of membranes in the aerobic and anaerobic state in the presence and absence of inhibitors indicated that UV, dicumarol and pCMB inhibited electron transport at sites on the reducing site of the cytochrome b components. Concentrations of pCMB which completely inhibited fumarate reduction appeared to leave cytochrome  $b_{553-4}$  largely in the reduced state in the anaerobic steady-state, but not in the aerobic steady state suggesting that this cytochrome is not a component of electron transport to fumarate. The other low potential b type cytochrome  $b_{556-7}^{LP}$  was oxidized by fumarate in the presence of pCMB, suggesting that it may be located

specifically on the anaerobic path to fumarate. Since fumarate oxidized at least part of the cytochrome b in UV-irradiated membranes, a previous scheme for electron transport in this organism in which cytochrome b can only be oxidized by fumarate via menaquinone was not supported by the present study.

Spectra of membranes in the aerobic steady state indicated a greater degree of reduction of cytochrome  $b_{562-3}$  when succinate was reducing substrate than when D- or L-lactate or NADH was reducing substrate. This suggests that there was a close relationship between succinate dehydrogenase and cytochrome  $b_{562-3}$ . Since succinate oxidase activity was low despite the presence of high succinate dehydrogenase activity, it is improbable that  $b_{562-3}$  is part of an oxidase.

The present study did not resolve the question as to whether there was a single succinate dehydrogenase which functions *in vivo* as the fumarate reductase. Most evidence is consistent with there being only one enzyme system but the finding of two distinct  $K_m(\text{succinate})$  values for succinate-DICPIP and succinate- $\text{Fe}(\text{CN})_6$  oxidoreductase may indicate the presence of two distinct systems.

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## ABBREVIATIONS

a, b, c or d	Cytochromes a, b, c or d
CN	Cyanide
DIC	Dicumarol
DICPIP	2,6-dichlorophenolindophenol
Fe(CN) <sub>6</sub>	potassium ferricyanide
fp <sub>D</sub>	D-lactate dehydrogenase
fp <sub>FR</sub>	Fumarate reductase
fp <sub>L</sub>	L-lactate dehydrogenase
fp <sub>N</sub>	NADH dehydrogenase
fp <sub>S</sub>	Succinate dehydrogenase
HOQNO	N-heptyl-4-hydroxyquinoline-N-oxide
MQ	Menaquinone
NOQNO	n-Nonyl-4-hydroxyquinoline-N-oxide
pCMB	p-chloromercuribenzoic acid
PMS	Phenazine methosulfate
UV	Ultraviolet light

1. INTRODUCTION

### 1.1: Some General Characteristics of Bacterial Electron Transport Systems

The means by which living organisms conserve and utilize the energy released by catabolism has long been of interest to biologists and chemists. The most commonly used means of coupling energy-releasing processes to energy-requiring processes involves the generation of ATP during catabolism. There are two main mechanisms whereby ATP may be synthesized in living organisms: substrate level phosphorylation (SLP) and electron transport associated phosphorylation (ETP). The former mechanism (SLP) is associated with enzyme-catalysed steps of intermediary catabolism (such as that catalysed by 3-phosphoglycerate kinase). The latter (ETP) is now widely accepted to be associated with the utilization of a potential energy difference across membranes (Mitchell, 1979). This potential energy difference may be utilized by the cell in a number of ways other than ATP synthesis, such as cell motility, nutrient transport, reversed electron transport, and heat production in animals (Skulachev, 1979).

The system which generates this potential energy difference occurs in the membranes of cells and is termed the electron transport system (ETS). The ETS is usually represented as a series of reduction-oxidation (redox) reactions beginning with the reaction of lowest redox potential, the 'reducing substrate' or dehydrogenase end, and proceeding through intervening redox reactions to the reaction with the highest redox potential, the 'oxidizing



substrate' or terminal oxidase end. These intervening reactions involve the oxidation and reduction of iron-sulfur (FeS) proteins, quinones and cytochromes.

In mitochondria (Figure 1.1.A), oxygen may be considered the normal terminal oxidant, whereas in bacteria, a number of alternative oxidants may serve in this role. Besides oxygen, sulfate and nitrate are common exogenous terminal electron acceptors used by bacteria. The major organic compound which has been characterized as a terminal oxidant for bacteria is fumarate (Kroger, 1978), which may be provided externally for the cells or generated by intermediary metabolism as described later in this introduction for propionibacteria. Fumarate may also act as a terminal oxidant in some eucaryotic cells under anaerobic conditions (ibid). Other terminal electron acceptors for certain bacteria are iron ( $\text{Fe}^{3+}$ ), elemental sulfur, carbon dioxide and dimethyl sulfoxide (Zinder and Brock, 1977).

Some bacterial ETS resemble that of the mitochondrion. *Paracoccus denitrificans* and *Alcaligenes eutrophus* have a constitutive ETS (Figure 1.1.B) which is similar to that of mitochondria in its cytochrome, quinone, dehydrogenase and oxidase content and its sensitivity to inhibition by rotenone and cyanide (Jones, 1977). These similarities are such that *P. denitrificans* has been suggested as an evolutionary predecessor of mitochondria (John and Whatley, 1975). However, even in these bacteria there are inducible electron transport components which have no counterpart in the mitochondrial system. Most bacteria have ETS which differ greatly from that of the mitochondrion, e.g. that in

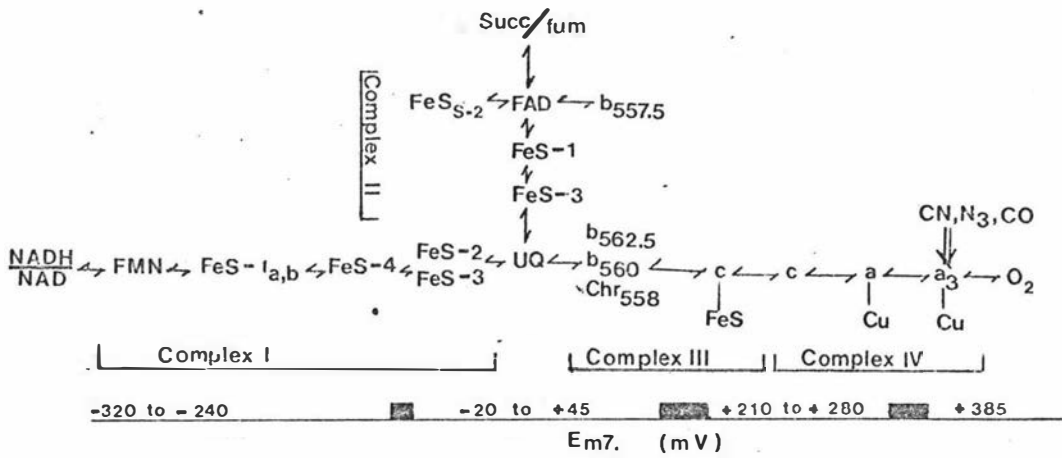
*Escherichia coli* (Figure 1.1.C). These variations may occur in the type(s) of membrane bound dehydrogenase(s) available, the type(s) of iron-sulfur proteins, quinones and cytochromes involved, and the type(s) of terminal oxidoreductase system(s).

Like other components of the ETS, the dehydrogenases are membrane bound, and occur in the 'particulate fraction' of broken cells. Their presence may be detected by substrate dependent reduction of natural terminal electron acceptors such as those listed above, or of artificial electron acceptors such as tetramethylphenylene diamine (TMPD), dichlorophenol indophenol (DICPIP) or potassium ferricyanide ( $K_3Fe(CN)_6$ ). These artificial electron acceptors may react with the dehydrogenase itself, or with some other component of the electron transport system, or both.

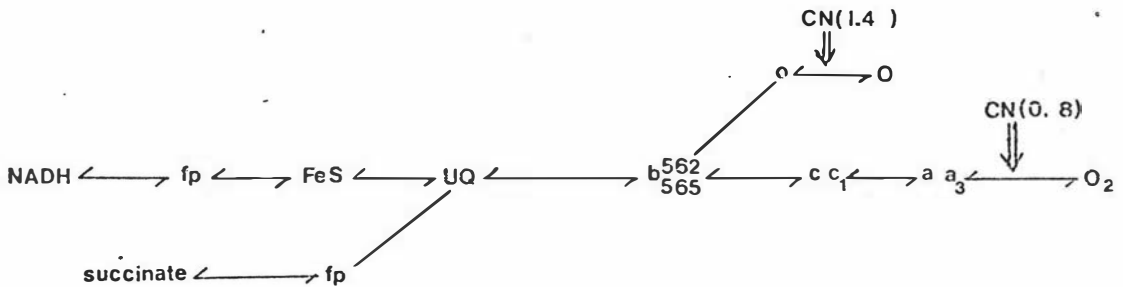
Specific membrane-bound dehydrogenases for several different substrates may occur and these may be present in different isoenzymic forms. In *E. coli*, dehydrogenases for  $\alpha$ -glycerophosphate, succinate, lactate and NADH occur. *E. coli* NADH dehydrogenase has been reported to occur in at least four electrophoretically distinct forms (Thompson and Shapiro, 1981) which are distinguishable by their reactivities with natural and artificial electron acceptors. Two of the NADH dehydrogenases reacted with DICPIP, and of these, one reacted more rapidly with quinone than DICPIP and was composed of a single polypeptide (46,000 daltons) containing one iron atom per polypeptide. Another form from *E. coli* which does not contain iron has been studied in purified form

Figure 1.I: Aerobic Electron Transport

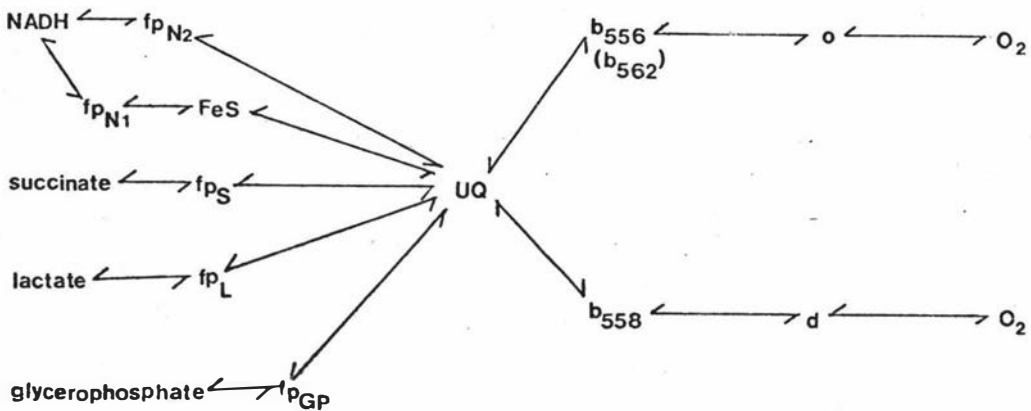
A. Mitochondria (Hatefi & Galante, 1978)



B. *Paracoccus denitrificans* (Stouthamer, 1980)



C. *Escherichia coli* (Poole & Haddock, 1975)

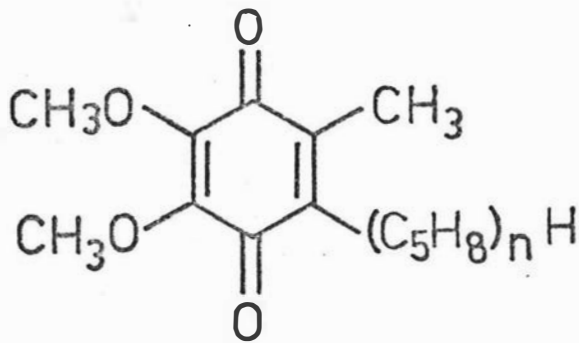


(Young, I.G., pers. comm.). Succinate dehydrogenase also occurs in *E. coli* and has a separate genetic locus from fumarate reductase (Spencer and Guest, 1973), which catalyses the same reaction but acts as a terminal oxidoreductase. Thus the presence of isozymes which may have different functions, must be considered when describing electron transport systems.

The quinones involved in electron transport in bacteria (Fig. 1.2) also vary according to the organism and the growth conditions. Both benzoquinones (such as ubiquinone (UQ)) and naphthoquinones (such as menaquinone (MQ)) occur frequently, but vary between genera in the length and degree of hydrogenation of the isoprenoid side chain (Collins and Jones, 1981). Ubiquinone and menaquinone have different roles in *Proteus rettgeri* in that menaquinone functions in anaerobic electron transport to fumarate while ubiquinone functions in aerobic electron transport to oxygen (Kroger, et al, 1971). The different functions of ubiquinone and menaquinone are reflected in their midpoint redox potentials at pH 7 ( $E_{m7}$ ) since that of ubiquinone (+115 mV) is more positive than the potential of the succinate/fumarate couple (+33 mV) while that of men<sup>a</sup>quinone (-70 mV) is more negative (Thauer, Jungermann and Decker, 1977).

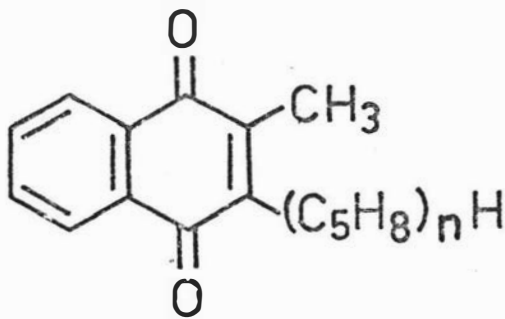
Other types of quinone may occur as electron transport components. *Butyrivibrio fibrisolvens* has been shown to contain  $\alpha$ -tocopherol quinone which is specifically involved in hydrogenation of unsaturated fatty acids (Hughes and Tove, 1980) but may also be involved in ETP, which has been suggested to occur in this organism (Asmundson, 1974;

Figure 1.2: Bacterial Quinones



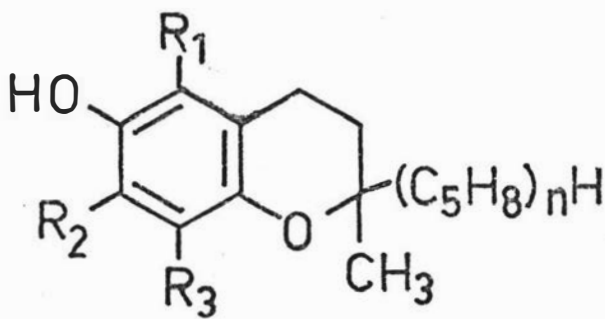
benzoquinone

(ubiquinone, UQ-n)



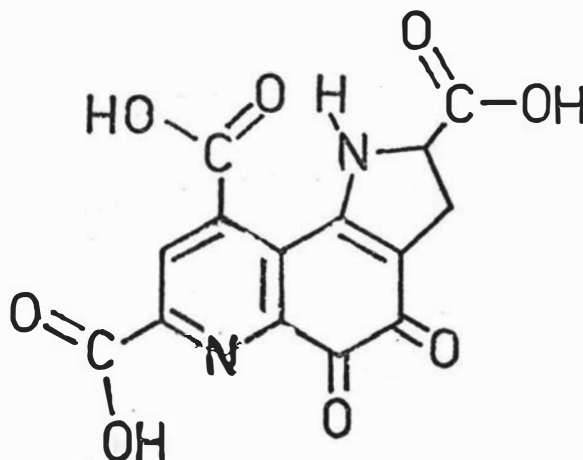
naphthoquinone

(menaquinone, MQ-n)



tocopherol

(vitamin E)



pyrroloquinoline quinone

Russell and Baldwin, 1979). Another type of quinone, pyrroloquinoline quinone, occurs in several bacteria in association with dehydrogenases for methanol, alcohols, glucose, methylamine and lactate (Duine and Frank, 1981; Ameyama *et al.*, 1981). This quinone differs from the others mentioned in the absence of a non-polar side chain and in its highly acidic nature. Duine and Frank (*ibid*) cite an unpublished survey of the occurrence of this type of quinone. Of particular relevance to the present study is the finding that it occurs in association with lactate dehydrogenase of *Propionibacterium pentosaceum*.

There is also considerable variation between bacterial species in cytochrome content, both in the type of cytochrome and relative amounts of the different types. Four groups of bacteria have been identified in terms of content of cytochromes  $a_1$ ,  $aa_3$ ,  $b$ ,  $c$ ,  $d$  and  $o$  (Meyer and Jones, 1973). Propionibacteria fall into a relatively small group which contains cytochromes  $d$  and with cytochrome  $b$  as a major component.

Multiple forms of cytochrome  $b$  may be present in a single species. *E. coli* can produce seven  $b$ -type cytochromes which differ in their absorption maxima, midpoint redox potentials and the conditions under which they appear (Reid and Ingeldew, 1979). Thus the nitrate reductase of *E. coli* is specifically associated with  $b_{556}^{+10}$  (Table 1.1).

Table 1.1: Cytochromes of the b type present in *E. coli*.

<u><math>\alpha</math> peak</u> (nm)	<u><math>E_{m7}</math></u> (mV)	<u>Culture Conditions</u>
555	+40	anaerobic, NO <sub>3</sub> , fum
556	-50	aerobic
556	+10	anaerobic, NO <sub>3</sub>
556	+80	aerobic
556	+260	aerobic
558	+250	anaerobic, NO <sub>3</sub> , fum
563	+260	aerobic

from Reid and Ingledew, 1979.

Several different cytochromes appear to function as terminal oxidases in bacteria, the particular ones present again varying according to the organism and the growth conditions. These are cytochromes  $a_1$ ,  $aa_3$ , o, d, c (Jurtschuk *et al*, 1975) and bd (Watanabe *et al*, 1979). In *E. coli* and other bacteria the terminal oxidase depends on the growth phase such that in the log phase of growth, cytochrome o functions as the main terminal oxidase whereas cytochrome d functions as terminal oxidase in stationary phase (Meyer and Jones, 1973).

Thus there is a wide diversity of composition and organisation of the electron transport chain in the bacteria, not only between the different groups and species of bacteria, but also within any one organism, depending on growth conditions. In view of this wide diversity, a thorough review of bacterial electron transport chains is beyond the scope of this introduction. The following section will review what is known of the composition and organization of the electron transport chain in propionibacteria.



## 1.2: The Electron Transport System of Propionibacteria

Propionibacteria are chemo-organotrophic bacteria which anaerobically metabolise carbohydrates, polyols and organic acids such as lactate. Fermentation products characteristically include combinations of propionic and acetic acids with lesser amounts of mono- and dicarboxylic acids and carbon dioxide (Moore and Holdeman, 1975). The intermediary metabolism of propionibacteria has been extensively studied by Wood and coworkers (Wood, 1981).

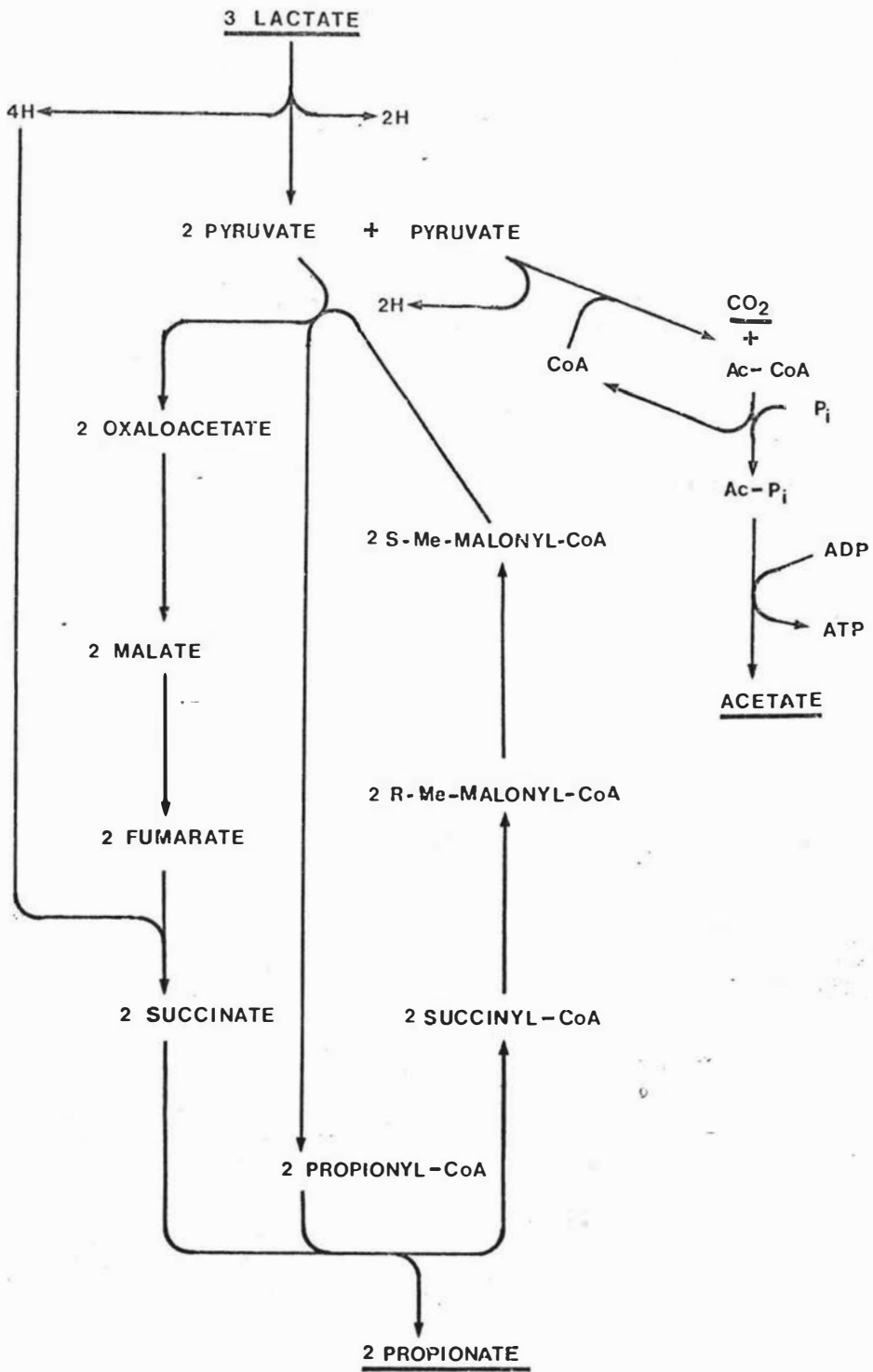
Propionibacteria are generally characterized as aerotolerant anaerobes (Moore and Holdeman, 1975), meaning that while their metabolism is normally anaerobic, they survive exposure to oxygen but derive no benefit from it. It has been shown that propionibacteria can adapt to fairly high levels of aeration in liquid culture provided a certain limit is not exceeded (Pritchard *et al*, 1977). The different species of propionibacteria differ in their sensitivity to inhibition by oxygen (Schwartz, 1973; deVries *et al*, 1972). *P. shermanii*\* and *P. freudenreichii* are somewhat less aerotolerant than *P. pentosaceum*.

Early evidence that electron transport occurred in propionibacteria was the observation that cytochromes occurred in these bacteria (Chaix and Fromageot, 1942). Studies of molar growth yields indicated that SLP could not account for the mass of cells produced and consequently it was proposed that ETP dependent on lactate-fumarate oxidoreductase could

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\* In the most recent revision of the taxonomy of propionibacteria (Moore and Holdeman, 1975) *P. shermanii* has been renamed *P. freudenreichii* subsp. *shermanii*. The name *P. shermanii* will be retained in this thesis since it is referred to by this name in all the literature on this organism.

Figure 1.3: Lactate Metabolism in Propionibacteria



provide additional ATP (Bauchop and Elsdon, 1960).

1.2.1: Electron transport activities and components in membranes from propionibacteria

Membranes prepared from all species of propionibacteria which have been studied contained electron transport systems which coupled oxidation of lactate, NADH and glycerol-3-phosphate to reduction of fumarate or oxygen. Specific activities of some of these electron transport reactions are shown in Table 1.1.

In general, the specific activities reported for *P. shermanii* are greater than those reported for other species of propionibacteria. Although the fumarate reductase activities of *P. shermanii* and *P. arabinosum* are roughly equal, the oxidase activities are very different, especially with NADH and lactate. Lactate oxidase activity in *P. arabinosum* appears to be very low, despite the presence of ample lactate dehydrogenase (lactate(+PMS)-DICPIP oxidoreductase). The large difference in specific activities between membranes from *P. shermanii* and *P. freudenreichii* is surprising since these two species are considered to be closely related (Moore and Holdeman, 1975). However, these differences may be due to differences in the methods of preparation of the membranes.

Some of the dehydrogenases present in propionibacteria have been partially purified and investigated. These are the  $\alpha$ -glycerol phosphate dehydrogenase of *P. arabinosum* (Sone and Kitsutani, 1972; Sone, 1973), the NADH dehydrogenase of *P. shermanii* (Schwartz and Krause, 1975), the

Table 1.2: Specific Activities of Membrane Bound Electron Transport Reactions in Propionibacteria

<u>Oxidant</u>	<u>Reductant</u>	<i>P. arabinosum</i> (a)	<i>P. shermanii</i> (b)	<i>P. freudenreichii</i> (c)
Oxygen	NADH	0.135	1.34	-
	Lac <sup>(4)</sup>	0.009	1.74	-
	GP	0.145	0.24	-
	Succinate	0	0.024	-
Fumarate	NADH	0.39	0.27	-
	Lac	0.27	0.51	0.07
	GP	0.66	0.32	0.14
Other	NADH	0.22 <sup>(1)</sup>	-	-
	Lac	0.33 <sup>(1)</sup>	5.2 <sup>(2)</sup>	0.91 <sup>(3)</sup>
	GP	0.22 <sup>(1)</sup>	0.46 <sup>(2)</sup>	0.36 <sup>(3)</sup>
	Succinate	1.13 <sup>(1)</sup>	1.68 <sup>(2)</sup>	-

Values presented are specific activities in  $\mu$ moles per min per mg protein.

(a) lactate-grown cells (Sone, 1972)

(b) glucose-grown cells (Schwartz & Sporckenbach, 1975)

(c) lactate-grown cells (deVries *et al*, 1973)

(1) DICPIP + PMS

(2) Oxygen + PMS

(3) DICPIP

(4) Schwartz and Sporckenbach (1975) and deVries *et al* (1973) used D-lactate, Sone (1972) did not state whether D- or L-lactate was used.

D-lactate dehydrogenase of *P. pentosaceum* (Molinari and Lara, 1960) and the succinate dehydrogenase of *P. pentosaceum* (Lara, 1959). It should be emphasized that these were only partially purified preparations, especially the D-lactate dehydrogenase (which contained a significant proportion of L-lactate dehydrogenase activity) and the succinate dehydrogenase. While some of the properties of NADH dehydrogenase were different for the particulate and soluble forms, there appeared on the basis of evidence from acrylamide gels to be only one type of NADH dehydrogenase (Schwartz and Krause, 1975) as was also the case with  $\alpha$ -glycerol phosphate dehydrogenase (Sone, 1973).

The menaquinone present in membranes of propionibacteria was isolated and identified by both Sone (1974) for *P. arabinosum* and Schwartz (1973) for *P. shermanii*. It was found by use of chromatography and mass spectrometry to be (II, III)-tetrahydromenaquinone-9 in both species. Schwartz reported the relative concentration of menaquinone to cytochrome b to be 10 to 1. On the basis of the  $R_f$  value of a fluorescent spot in thin layer chromatograms, Schwartz reported the presence of an ubiquinone in *P. shermanii*, but estimated that this composed less than 10% of the total quinone.

Difference spectra of membranes from propionibacteria generally show peaks corresponding to cytochromes b and d. Spectra of membranes from *P. shermanii* and *P. freudenreichii* generally show a small shoulder at 595 to 600 nm which may be indicative of a cytochrome a (deVries *et al*, 1972; Schwartz and Sporckenbach, 1975). The b peak was described

as complex by Schwartz and Sporckenbach (1975) indicating that more than one species of cytochrome b is present.

The terminal oxidoreductases of propionibacteria appear to vary between species. All species investigated possess fumarate reductase activity which may be catalysed by the same enzyme system as that responsible for succinate dehydrogenase activity. Some species possess a nitrate reductase (*P. freudenreichii*, *P. acidipropionici* (synonyms=*P. pentosaceum*, *P. arabinosum*)) whereas others do not (*P. shermanii*, *P. thoenii*, *P. jensenii* (= *P. petersonii*), *P. avidum*, *P. granulorum* (Moore and Holdeman, 1975)). Sone (1972) suggested that the terminal oxidase of *P. arabinosum* is a flavoprotein oxidase on the basis of the absence of cyanide inhibition of oxygen uptake with  $\alpha$ -glycerophosphate and lactate, although his data indicated that NADH oxidase activity can be almost completely blocked by cyanide. The presence of flavin oxidases in *P. shermanii* and *P. petersonii* (*P. jensenii*) has also been suggested by Bonartseva *et al* (1973). However, the presence of cytochromes a, d and possibly o (CO-binding cytochrome (Sone, 1972; Schwartz and Sporckenbach, 1975)) suggests that these may function as components of terminal oxidases (Jurtshuk, *et al* 1975). There have been no direct examinations of terminal oxidases in propionibacteria.

### 1.2.2: Organization of the electron transport system in propionibacteria

The role of menaquinone in electron transport in propionibacteria has been investigated by use of ultra-violet light irradiation of membranes, which destroys quinones. DeVries *et al* (1977) observed a decrease in menaquinone content with time of exposure which paralleled a decrease in L-lactate dependent reduction of both oxygen and fumarate in *P. freudenreichii*, whereas, with *P. pentosaceum*, glycerophosphate-fumarate oxidoreductase activity decreased at a slower rate than did menaquinones.

Sone (1974) also observed a decrease in specific activities of various electron transport processes with exposure to UV light in membrane preparations from *P. arabinosum* (= *P. pentosaceum*). Of the activities tested, oxygen and fumarate reduction decreased most rapidly, being completely inactivated after 15-20 min exposure. Succinate dehydrogenase (DICPIP + PMS) was least sensitive to UV light, only decreasing by 10%, while D-lactate-DICPIP (+ PMS), NADH-DICPIP, and NADH-Fe(CN)<sub>6</sub> oxidoreductases were reduced by only 50% after 30 min exposure to UV light. Sone (1974) also studied the restoration of electron transport activities by addition of menaquinones to membranes which had been irradiated with UV or depleted of menaquinone by use of cholate extraction. Activities of glycerol phosphate-, lactate- and NADH-fumarate oxidoreductase were largely or completely restored to control levels by addition of menaquinones. These results indicated an obligate role for

menaquinone as an intermediate in the membrane-coupled redox reactions from NADH, lactate and glycerol phosphate to fumarate although not to artificial electron acceptors. The reduction of cytochrome b by NADH and glycerol phosphate was also inhibited by irradiation with UV light, indicating a dependence on the presence of menaquinone. However, reduction of cytochrome b by lactate and succinate still occurred in the 1 min reaction time allowed. On the basis of these results and of earlier studies using inhibitors (Sone, 1972), the scheme for electron transport in *P. arabinosum* shown in Figure 1.4.A was presented by Sone (1974). Many of the features of this scheme rest on very tentative evidence which is not very fully discussed in Sone's papers. Noteworthy features are listed below.

1. Menaquinone and cytochrome b are obligate intermediates on the electron transport pathway from NADH, glycerophosphate and lactate to fumarate, although electrons from lactate may be able to bypass the menaquinone step (since lactate can still reduce cytochrome b in UV-treated membranes).
2. Cytochrome b is not an obligate intermediate in electron transport to oxygen. The point at which electron flow to oxygen diverges from that to fumarate is at menaquinone or, in the case of NADH, at an earlier step. The evidence for this latter conclusion rests on the inability to restore NADH-oxygen oxidoreductase by readdition of menaquinone. Sone does not discuss the basis of UV inactivation of NADH-oxygen oxidoreductase activity.



3. DICPIP is considered to interact at different points in the electron transport pathway depending on the reducing substrate used. The evidence for this is not discussed by Sone.

The role of cytochrome b in electron transport to fumarate in propionibacteria has been investigated by two groups with conflicting findings. Both groups have investigated cytochrome b involvement using the alkyl hydroxyquinoline-N-oxide inhibitors HOQNO (deVries *et al*, 1973), and NOQNO (Schwartz and Sporkenbach, 1975).

Using membranes from *P. freudenreichii*, deVries *et al* (1973) showed that cytochrome b reduction by D- or L-lactate or glycerol phosphate and the fumarate oxidoreductases for these same substrates were inhibited by HOQNO. Addition of fumarate to substrate-reduced membrane particles caused a rapid oxidation of cytochrome b, both in the presence and absence of HOQNO. However the steady state reduction level of cytochrome b was lower (46% of the substrate reduced level as opposed to 54% in the absence of HOQNO). This result indicates that while reduction of cytochrome b is inhibited by HOQNO, oxidation by fumarate is not. DeVries *et al* (*ibid*) concluded that cytochrome b functions on the electron transport pathway to fumarate in *P. freudenreichii*.

Schwartz and Sporkenbach, (1975) also studied the effect of a similar inhibitor, NOQNO, on electron transport in membranes from *P. shermanii*. Spectra of membranes in the anaerobic steady state showed an increase in reduction level of menaquinone in the presence of NOQNO over that in

its absence and a decrease in reduction level of cytochrome b, indicating that the site of inhibition of NOQNO was between these two redox components. In sharp contrast to the results of deVries *et al* (1973) they observed no re-oxidation of lactate-reduced cytochrome b by fumarate in the presence of NOQNO, whereas reoxidation did occur when oxygen was added. They concluded from this result that cytochrome b is not involved in electron transport to fumarate but is involved in electron transport to oxygen. The branch point in electron transport to fumarate or oxygen was considered to be menaquinone. Schwartz and Sporckenbach presented a scheme for electron transport in *P. shermanii* as shown in Figure 1.4.B.

In light of these results, deVries *et al* (1977) reinvestigated and expanded their earlier work using membrane preparations of *P. pentosaceum* and *P. freudenreichii* isolated using a sucrose gradient centrifugation. This purification step was included to remove fumarase activity which they found to be present in membranes prepared by the procedure used in earlier studies. In confirmation of their earlier experiments and in contrast to the finding of Schwartz and Sporckenbach (1975), they observed a decrease in the reduction level of cytochrome b in HOQNO-inhibited substrate-reduced membranes upon addition of oxygen-free fumarate. They also examined the effect of UV irradiation on the level of cytochrome b reduction and observed that while the proportional reduction of residual menaquinone remained the same in the anaerobic steady state, the proportion of reduced cytochrome b decreased with increasing exposure. This

result is not consistent with the scheme of Schwartz and Sporckenbach (1975, Figure 1.4.B) on the basis of which the redox state of cytochrome b should be in equilibrium with that of menaquinone during anaerobic electron transport, so that changes in the reduction level of cytochrome b should reflect those of menaquinone. This result is, however, consistent with the idea that cytochrome b is a component of electron transport to fumarate. Since similar results also occurred with membranes in the aerobic steady state, cytochrome b was also considered to be a component of electron transport to oxygen. A scheme representing the findings of deVries and co-workers is shown in Figure 1.4.C.

This controversy must remain unresolved until a more detailed understanding of electron transport in various species of propionibacteria is achieved since the two groups mentioned above have used different species of propionibacteria in their research.

The uncertainty concerning the involvement of cytochrome b in anaerobic electron transport to fumarate is not confined to propionibacteria. Results from studies with other bacteria have also questioned the role of cytochrome b as an obligate intermediate in the anaerobic pathway.

A mutant of *E. coli* which lacks cytochrome b still performs electron transport between glycerol phosphate and fumarate (Singh & Bragg, 1976). However, cytochromeless cells in the presence of glycerol phosphate and fumarate are unable to take up proline, whereas cells containing cytochrome can. It was suggested that cytochrome b was

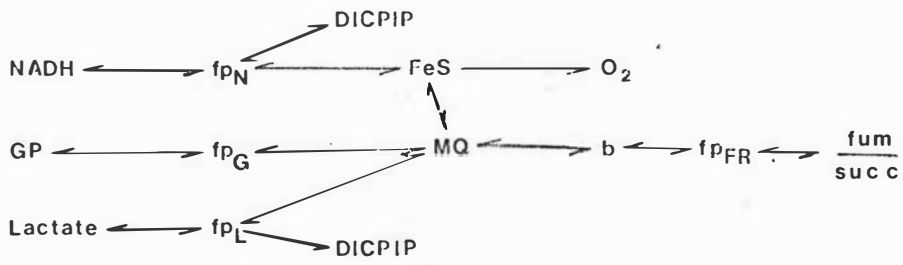
required if membrane energization and energy coupling to active transport were to occur but not for activity of the oxidoreductase systems catalysing electron flow to fumarate.

In *Bacteroides ruminicola*, a strict anaerobe which contains cytochrome b and which has been proposed to derive energy from electron transport to fumarate, the role of cytochrome b in electron transport to fumarate was investigated (Mountfort and Robertson, 1977). In a membrane preparation, they observed that while cytochrome b was reduced by NADH and rapidly reoxidized by oxygen and fumarate, the rate of reduction of cytochrome b by NADH was too slow to account for the rate of NADH-fumarate oxidoreductase observed in these membranes. Also, the rate of reduction of cytochrome b by NADH was more sensitive to HOQNO inhibition than was the NADH-fumarate oxidoreductase. These results were interpreted to indicate that cytochrome b, although associated with fumarate reductase was not an obligatory intermediate on the pathway of electron flow from NADH to fumarate as shown in Figure 1.4.D.

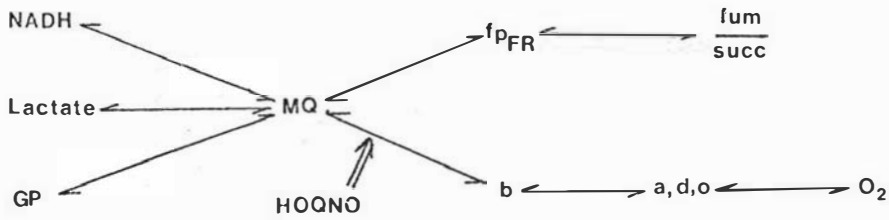
These results conflict with the conclusions of Kroger and co-workers from work with fumarate reductase isolated from *Vibrio succinogenes* (Unden *et al*, 1980). Fumarate reductase was isolated in two forms, one of which contained cytochrome b while the other lacked cytochrome b. Reaction of both forms of the enzyme occurred with viologen dyes or anthrahydroquinone sulfonate, but reaction with naphthoquinones required the presence of cytochrome b. This indicated that cytochrome b was a component of the *in vivo* fumarate reductase since the quinone present in this organism is a naphthoquinone (Figure 1.4.E.).

Figure I.4: Anaerobic electron transport

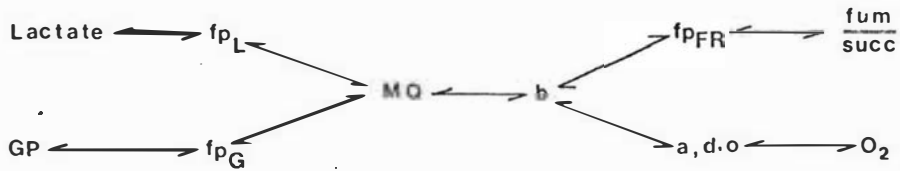
A. *P. arabinosum* (Sone, 1974)



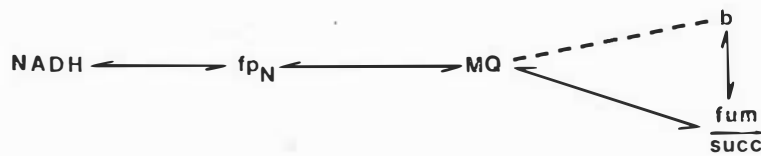
B. *P. shermanii* (Schwartz & Sporkenbach, 1975)



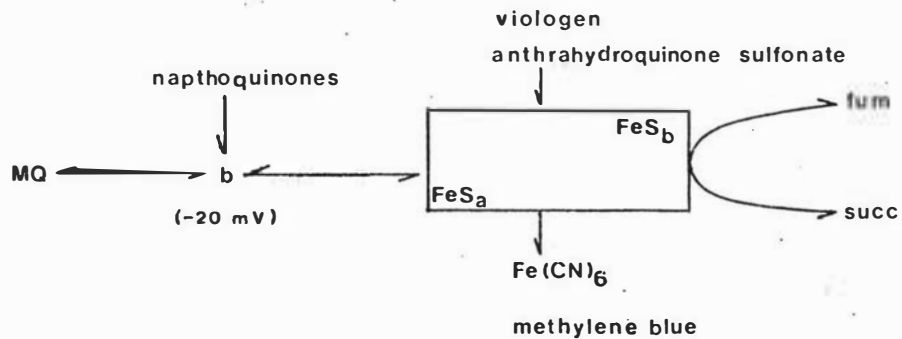
C. *P. freudenreichii* (de Vries et al, 1977)



D. *B. ruminicola* (Mountfort & Robertson, 1977)



E. Fumarate reductase of *V. succinogenes* (Uden et al, 1980)



### 1.3: Aims of the Present Study

Although studies of electron transport in propionibacteria have played an important part in the recognition of the importance of electron transport in the energy metabolism of anaerobes, they have been relatively neglected in recent years in comparison with the more extensive studies that have been carried out on other anaerobes such as *Vibrio succinogenes* (Kroger *et al*, 1975) and *Proteus rettgeri* (Kroger *et al*, 1971). It is clear from the foregoing review of previous work that several aspects of the composition and organisation of the electron transport systems are in need of further investigation before the role of this system in ATP production, active transport and other energy-linked functions in propionibacteria can be evaluated. Particular areas requiring attention are:

1. Characterization of the primary dehydrogenase systems.

Some information is available on the NADH dehydrogenase (Schwartz and Krause, 1975) and glycerophosphate dehydrogenase (Sone and Kitsutani, 1973; Sone, 1974), but the properties of other dehydrogenase systems are virtually unknown. There is no published information on the existence and properties of separate D- and L-lactate dehydrogenases and in some studies it is not even specified which isomer of lactate was used as a reducing substrate. It is not known whether the succinate dehydrogenase activity is due to the same enzyme system as the fumarate reductase or to a distinct dehydrogenase system as in *E. coli*.

2. Characterization of the cytochrome components.

The spectra published by Schwartz and Sporkenbach (1975) suggest that the large cytochrome b peak is complex, but there have been no published reports establishing the existence of multiple forms of cytochrome b species in propionibacteria. In view of the existence of multiple forms of cytochrome b in most other bacteria which have been studied, it is very likely that propionibacteria contain several distinct b type cytochromes. Characterization of the different forms may help to resolve some of the conflicting evidence on the role of cytochrome b in anaerobic and aerobic electron transport.

3. The nature of the terminal oxidases mediating oxygen uptake.

Apart from the suggestion by Sone (1972) and Bonartseva *et al* (1973) that flavoprotein oxidases may be responsible for oxygen uptake by membrane particles from propionibacteria there is no information on the oxidase systems although it is well known that both whole cells and membrane particles have an active respiration (deVries *et al*, 1972; Schwartz, 1973; Pritchard *et al*, 1977).

4. The relationship between the aerobic and anaerobic pathways.

It is not known whether the aerobic and anaerobic systems share common initial steps and if so at what point the two systems diverge. As indicated, previous workers have arrived at very different conclusions concerning the point

of branching of the electron transport system to the two acceptors.

The aim of the present study was to obtain further information on the electron transport processes and components in membrane particles from *P. shermanii* which might clarify some of these areas of uncertainty. Particular attention has been paid to the cytochrome components and their role in the processes of anaerobic and aerobic electron transport.



## 2. METHODS AND MATERIALS

## 2.1: Culture Methods

*Propionibacterium shermanii* ATCC 9614 was obtained from the Department of Food Science, University of Minnesota. The culture was maintained at 30°C by weekly or two weekly subculturing in 20 ml liquid medium composed of 10 g.l<sup>-1</sup>, casein hydrolysate, 10 g.l<sup>-1</sup> yeast extract and 16 g.l<sup>-1</sup> 60-70% sodium, D,L-lactate syrup dissolved in tap water. A stock of freeze dried culture was prepared from the original culture and these were used to restart the culture whenever contamination was noticed.

Bacteria for membrane preparations were grown in 4 to 8 l batches of the above medium. The inoculum used for these larger cultures was a 24 hr old culture in 500 ml of the same medium. The large cultures were stirred with a magnetic stirrer. A mixture of 5% CO<sub>2</sub> and 95% N<sub>2</sub> was passed through a copper reducing column at 350°C to remove traces of oxygen and was bubbled through the medium at least two hours prior to inoculation and during growth in the fermentation vessel. Temperature was controlled at 30°C ( $\pm 1^\circ\text{C}$ ) by infra-red lamps (Phillips IR 133-2B/479\*) powered by a 220 V sine-wave power source controlled by a variable resistance thermistor. Growth was followed by measuring absorbance at 540 nm. Cells were harvested by centrifugation at 2500 g for 25 min when absorbance at 540 nm approached 2.5, which normally occurred 20 hrs after inoculation. Cells were observed by phase contrast microscopy or Gram stain to check for contamination before membranes were prepared.

## 2.2: Preparation of Membranes

Initially, attempts were made to prepare spheroplasts of *P. shermanii* in order to permit osmotic lysis of the cells, a method thought to give least damage to cell membranes. Cells were grown in the medium described above containing 0.3 M mannitol until one doubling of culture growth remained. At that time,  $0.5 \text{ g.l}^{-1}$  sodium benzyl penicillin was added. After 4 hrs, the culture was harvested by centrifugation. The cells were washed and resuspended in 0.3 M mannitol and then treated with lysozyme (40 mg/10 g wet cells) at pH 8 for 4 hrs (Sutherland and Wilkinson, 1971). Some alteration of the cell wall was noted when thin sections were examined by transmission electron microscopy, but no lysis was observed by phase contract microscopy on dilution of the cell suspension. Further attempts using a range of incubation conditions were unsuccessful so this method was abandoned.

In the early stages of the work, membranes were prepared by the following procedure. Harvested cells were diluted to a volume of 20 ml with a buffer composed of 0.1 M Tris, 0.01 M  $\text{MgCl}_2$  and 0.001 M EDTA, pH 7.4, and placed in a Hughes press. The press was cooled to  $-30^\circ\text{C}$  in a  $-70^\circ\text{C}$  freezer and then placed in a hydraulic press. The press usually forced the cells through its aperture at a ram pressure of 22.5 tons per square inch or greater. The frozen broken cells were removed from the press and stirred at room temperature with deoxyribonuclease ( $20 \mu\text{g ml}^{-1}$ ) for 30 min in 120 ml Tris/Mg/EDTA buffer (as above). This

homogenate was then centrifuged at 12000 g for 10 min to remove unbroken cells and large cell debris (cell walls). The pellet was resuspended with 50 ml buffer and again centrifuged at 12000 g for 10 min. The pellet was discarded and the combined supernatants were centrifuged for 1 hr at 100,000 g. The supernatant was discarded and the membrane pellet was resuspended in buffer and recentrifuged at 100,000 g for 1 hr a further two times to free it from soluble enzymes. It was then used without further treatment.

Electron microscopy of the membrane fraction prepared in this way showed large clumps of electron dense material with little evidence of membrane structure. It was eventually realized that this was due to clumping of ribosomes due to the high  $Mg^{2+}$  content of the buffer. The clumped ribosomes sedimented with the membranes. Therefore  $MgCl_2$  was subsequently omitted from the buffer in preparing membranes.

The membranes prepared by the above procedure still had some residual fumarase. Removal of fumarase is important for measurements of fumarate reductase activity. DeVries *et al* (1977) reported that fumarase was completely removed by centrifugation over sucrose layers. Therefore a final centrifugation through a discontinuous sucrose gradient was included in the procedure.

The final procedure adopted for preparing membranes from cells harvested from an 8 l culture was as follows: The cell paste was injected into the Hughes press by syringe with a canula and the press plus cells were frozen overnight at  $-20^{\circ}C$ . The press was then placed in a  $-70^{\circ}C$  freezer for

1 hr before placing it in the hydraulic press where it was placed under continuous pressure (about 26 tons per square inch) until it had warmed sufficiently for the cells to be pressed through. The broken, frozen cell paste was stirred at room temperature for 30 min in 240 ml Tris/EDTA buffer (as above but without  $MgCl_2$ ) containing approximately 2 mg deoxyribonuclease. The suspension of broken cells was centrifuged at 16,000 g for 10 min. The pellet was resuspended in 150 ml Tris/EDTA buffer and recentrifuged as above. The combined supernatants were centrifuged for 2 hr at 140,000 g. The pellet was suspended in 50 ml Tris/EDTA buffer and centrifuged over a discontinuous sucrose gradient (composed of 10 ml each of 60%, 40% and 20% sucrose in Tris/EDTA buffer) for 3 hr at 130,000 g. A portion of the material passed through the 60% sucrose and formed a pellet which appeared under phase contrast microscopy to be composed of whole cells and broken cell walls. The majority of the material formed a loose band between the 60% and 40% layers. Extended centrifugation compressed this layer into a flat disc which could be lifted out with a spatula. Normally the layers of sucrose above the 40%/60% interface were sucked off with a water aspirator and the loose membrane layer was collected with a Pasteur pipette, diluted to 66 ml with Tris/EDTA buffer and centrifuged at 280,000 g for 1 hr. The pellet from this was suspended in about 5 ml Tris/EDTA buffer in a glass homogenizer and stored at 5°C. This procedure resulted in membrane vesicles almost free from fumarase activity.

### 2.3: Electron Microscopy

Membranes were prepared for thin section electron microscopy as follows. Membrane samples suspended in Tris/EDTA buffer (as above) were fixed by adding 0.1 volume of 25% glutaraldehyde. After 2.5 hr on ice, aliquots were centrifuged in 1.5 ml conical propylene tubes, washed with buffer, post-fixed with  $\text{OsO}_4$  and magnesium uranyl acetate, dehydrated and embedded as described by Robertson *et al* (1978). Negative stains of cells were prepared by either a drop wash (1) or spray (2) method as described below:

#### (1) Drop wash method

A drop of diluted cell suspension was placed on a formvar-carbon coated grid and excess liquid was removed after 5 min with a piece of filter paper touched to the edge of the drop. One drop of distilled  $\text{H}_2\text{O}$  was added and removed after 10 sec with filter paper. A drop of 2% phosphotungstic acid was added and removed with filter paper after 10 sec and the grid was allowed to dry 20 to 30 min prior to examination in the electron microscope.

#### (2) Spray method

A fairly turbid cell suspension in 0.3% phosphotungstic acid (pH7) and 0.5% bovine serum albumin was sprayed using an aspirator at a pressure of 20 psi nitrogen from a distance of 40 cm onto formvar-carbon grids, allowed to dry 10 to 20 min and examined in the electron microscope.

The electron microscope used in this work was a Philips EM200 transmission electron microscope.

## 2.4: Analytical Methods

### 2.4.1: Protein determination

The protein content of the membrane preparations was compared using the Folin (Lowry, *et al*, 1951), biuret (Gornal *et al*, 1949), Coomassie dye binding (Bradford, 1976), phenol-hypochlorite (Jaenicke, 1974) and Kjeldahl (Lang, 1958) methods. The values obtained using the Coomassie blue method appeared to agree reasonably well with those determined by the other methods, and, since it is a very convenient and rapid assay, it was routinely used for determination of protein content. Bovine serum albumin was used to prepare standard curves.

### 2.4.2: Lipids

Total lipids were estimated by extracting aqueous suspensions of membranes three times with a mixture of chloroform and methanol (2:1, v/v), drying the extracts in a rotary vacuum evaporator and weighing.

### 2.4.3: Total carbohydrates

Total carbohydrate in membranes was estimated by the sulfuric acid-phenol method (Immers, 1964). Five ml of conc. sulfuric acid containing 1% phenol was rapidly added to 1.0 ml membrane suspension containing 1-100  $\mu$ g of carbohydrate. After a few minutes, the absorbance of the resulting solution was measured at 490 nm. A standard curve was prepared using glucose or a mixture of sugars

prepared in the same ratio as that found in the membranes (see Section 3.1.3).

#### 2.4.4: Gas chromatography

Gas liquid chromatography of carbohydrates was performed by the following method which was developed by D. Fenemore and I.G. Andrew in this laboratory. Briefly, the method consists of acid hydrolysis, acetylation of the resultant monosaccharides and then separation and determination on a gas liquid chromatograph. Specific details of the method are given below.

To 1.0 ml membrane suspension, 0.57 ml 5.5 N HCl was added and the mixture was heated at 100°C for 3 hrs. The HCl was removed by blowing a stream of air over the hydrolysed sample overnight. The sugars were reduced with 1 ml NaBH<sub>4</sub> (100 mg in 10 ml 1 M aqueous NH<sub>3</sub>) for two hours at room temperature. Glacial acetic acid was added dropwise until frothing stopped (to neutralize excess NaBH<sub>4</sub>).

Methanol (1 ml) was added and an air stream passed over the solution to blow off methyl borate. The addition of methanol was repeated two or three times. This procedure took 2-3 hrs at 45°C and gave a final dry sample.

1 ml of acetic anhydride in pyridine (1:1, v/v) was added and the reaction mixture was incubated for one hour at 100°C (for 12 hrs at room temperature). 1 ml CH<sub>2</sub>Cl<sub>2</sub> and 1 ml of distilled water were then added and the sample swirled. The CH<sub>2</sub>Cl<sub>2</sub> layer (bottom) containing the acetylated carbohydrates was collected. The CH<sub>2</sub>Cl<sub>2</sub> extraction was repeated



twice and the combined  $\text{CH}_2\text{Cl}_2$  extracts were dried under an air stream. The dried extract was then redissolved in 0.5 ml  $\text{CH}_2\text{Cl}_2$  and applied to the gas chromatograph.

Samples were applied to a Varian Aerograph gas chromatograph using a OV275 steel column 2 meters long with a 2 mm i.d. at  $210^\circ\text{C}$  and a flow rate of  $30 \text{ ml N}_2 \text{ min}^{-1}$ . Alternatively samples were also applied to a Pye 104 chromatograph with an SP2340 glass column 2.4 meters long with an i.d. of 3 mm at  $230^\circ\text{C}$  and a gas flow rate of  $20 \text{ ml N}_2 \text{ min}^{-1}$ .

#### 2.4.5: Pyridine hemochromogen derivatives of cytochromes

Pyridine hemochromogens were prepared and determined by the method of Weston and Knowles (1973). Alpha band wavelengths and extinction coefficients as listed by Smith (1978) were used to identify and quantify cytochromes. Characteristics of the pyridine hemochromogens of the main cytochrome types are listed in Table 2.4.5.1.

#### 2.5: Determination of Electron Transport Activities

##### 2.5.1: Determination of dehydrogenase activities

D-lactate, L-lactate, NADH and succinate dehydrogenase activities were measured in continuous spectrophotometric assays using DICPIP or  $\text{Fe}(\text{CN})_6$  as electron acceptors. PMS accelerates the reduction of DICPIP and is thought to act by coupling the dehydrogenase and DICPIP directly, thus bypassing components of the electron transport chain subsequent

Table 2.4.5.1: Absorption Characteristics of Alkaline Pyridine Hemochromogens

Type	Alpha Band	$E_{mM}$	Ether solubility
a	580-590 nm	22.8	soluble
b	556-558	34.4	soluble
c	549-551	29.1	insoluble
d	600-620	-	soluble

to the dehydrogenase. However, the mode of action of PMS is probably more complex than this (see section 3.1.2 and 3.2). Oxygen was also found to compete with  $\text{Fe}(\text{CN})_6$  and DICPIP as an electron acceptor in some assays (see section 3.1.2).

The assay mixtures were prepared in 1 cm cuvettes in final volumes of 3.1 ml. All reagents were prepared in Tris/EDTA (0.1 M and 1 mM, respectively) buffer (pH 7.4). Cuvettes containing buffer, membranes and either DICPIP or ferricyanide at final concentrations of  $5.2 \times 10^{-5}$  M or  $9.5 \times 10^{-4}$  M, respectively, were allowed to equilibrate to  $30^\circ\text{C}$  in the spectrophotometer for 5-10 min. The assay was initiated by the addition of 0.1 ml 50 mM D- or L-lactate or succinate or 0.1 ml 30 mM NADH. Reduction of DICPIP or ferricyanide was followed at 600 nm or 405 nm, respectively, in a Pye-Unicam SP1800 spectrophotometer. Extinction coefficients for DICPIP and ferricyanide at these wavelengths are  $19.1$  and  $1.05 \text{ mM}^{-1} \text{ cm}^{-1}$ , respectively. PMS, if included, was added to a final concentration of 0.1 to 0.5 mM.

If assays were to be carried out anaerobically, all the reagents were prepared using Tris/EDTA buffer which had been boiled and equilibrated to  $30^\circ\text{C}$  under oxygen-free  $\text{N}_2$  and stored in the refrigerator in butyl rubber-stoppered bottles. Each cuvette was prepared under oxygen-free  $\text{N}_2$ . Anaerobic conditions were further assured by incubating cuvettes in the presence of reducing substrate (D-lactate, L-lactate, succinate or NADH) for a few minutes before starting the reaction by the addition of oxidant (ferricyanide

or DICPIP) which had been prepared anaerobically.

#### 2.5.2: Fumarate oxidoreductase activity

Fumarate oxidoreductase activity was determined by measuring the decrease in absorbance due to fumarate at 270 nm at 30°C. Spectrosil grade cuvettes of 1 cm light path with circular ground glass openings were gassed under O<sub>2</sub>-free N<sub>2</sub> and made to 2.4 ml with anaerobic 0.1 M Tris (pH 7.4), membrane suspension and reducing substrate (D-lactate, L-lactate to a final concentration of 2 mM in 2.5 ml). The cuvettes were closed with butyl rubber septa and allowed to preincubate at 30°C for 5-10 min to allow membrane oxidases to remove any traces of oxygen present before the assay was started by addition of 0.1 ml 50 mM anaerobic sodium fumarate. Fumarase activity was tested for by an appropriate control (buffer, membrane suspension plus fumarate).

NADH-fumarate oxidoreductase cannot be measured at 270 nm due to the high absorbance of NADH and NAD at this wavelength. NADH-fumarate oxidoreductase was therefore measured by following the decrease in absorbance due to NADH at 340 nm. This same assay mixture without fumarate can be used to test the quality of the anaerobic technique by following the decrease in absorbance at 340 nm of cuvettes prepared anaerobically with buffer plus membranes which have been equilibrated to 30°C. Any oxygen present will result in oxidation of NADH due to NADH-oxygen oxidoreductase. The reaction is started by addition of NADH to about 0.16 mM

final concentration. The rate at which absorbance at 340 nm decreases prior to addition of fumarate provides a measure of NADH-oxygen oxidoreductase and the total change in absorbance provides a measure of the amount of oxygen present in the supposedly anaerobic assay mixture. A decrease of absorbance greater than 0.1 at 340 nm due to the presence of oxygen was regarded as unacceptable for subsequently measuring NADH-fumarate oxidoreductase. After the absorbance at 340 nm in the absence of fumarate ceased to decrease, 0.1 ml of 50 mM anaerobic sodium fumarate was added and the rate of decrease of absorbance at 340 nm was measured.

An end-point assay for measuring fumarate utilization was developed to assay larger numbers of samples used in determining inhibition curves. A reaction mixture of 0.8 ml containing 0.1 M Tris (pH 7.4) and membrane suspension was prepared in centrifuge tubes (10 ml polypropylene) which had been flushed with oxygen free  $N_2$  and capped with rubber serum caps. D- or L-lactate (0.1 ml of a 60 mM anaerobic solution) was added and the tubes were incubated for 5-10 min at 30°C to allow consumption of residual traces of oxygen by oxidases present in the membranes. The reaction was started by addition of 0.1 ml 60 mM anaerobic sodium fumarate and the reaction was stopped after 10 min by addition of 5 ml spectral grade absolute ethanol. Two other protein precipitants tried in this assay were trichloroacetic acid and barium sulfate, but both altered the absorption spectrum of fumarate and so were not used. The samples containing ethanol were shaken vigorously and

allowed to stand on the bench for 5-10 min before centrifuging in a bench centrifuge for 5 min. The absorbance of the ethanolic solution was read against a blank composed of all reagents except fumarate. Absorbance at  $t = 0$  was determined by adding fumarate to an ethanol-precipitated sample and reading at 270 nm. Fumarase activity was checked by measuring the decrease in fumarate in the absence of a reducing substrate. NADH-fumarate oxidoreductase cannot be measured by this assay due to high absorbance of NADH and NAD at 270 nm. NADH-fumarate oxidoreductase must be measured at 340 nm in a continuous assay as described above.

Fumarate reductase activity as measured above involves a complex electron transport system composed of dehydrogenases quinones and cytochromes. As a more direct measure of the activity of the fumarate reductase system itself, reduced benzyl viologen was used as electron donor (Unden, *et al*, 1980). Reaction mixtures were prepared anaerobically in 2.5 ml volumes in 1 cm light path cuvettes containing about 0.14 mM benzyl viologen and membranes in 0.1 M Tris buffer (pH 7.4). Cuvettes were warmed to 30°C in an SP 1800 spectrophotometer and the benzyl viologen was reduced by adding 1-5  $\mu$ l 2% (w/v)  $\text{KBH}_4$  ( $\text{KBH}_4$  was added 1  $\mu$ l at a time until the benzyl viologen appeared fully reduced; cuvettes requiring more than 5  $\mu$ l  $\text{KBH}_4$  solution to become reduced were not used). The reaction was started by the addition of fumarate to a final concentration of 2 mM and the change in absorbance at 560 nm was followed. The millimolar extinction coefficient of reduced benzyl viologen is 7.5  $\text{mM}^{-1} \text{cm}^{-1}$  at 560 nm.

### 2.5.3: Oxygen oxidoreductase activity

Oxygen consumption was followed using a Clark oxygen electrode (Model 5301 chamber with a Model 53 amplifier, Yellow Springs Instrument Co). Assay mixtures of 3.0 ml total volume were prepared by equilibrating 2.9 ml 0.1 M Tris buffer (pH 7.4), which contained membranes in air-saturated buffer for a few minutes in the water jacketed chamber at 30°C and then starting the assay by the addition of 0.1 ml 60 mM D- or L-lactate or succinate or 30 mM NADH. The amount of oxygen initially present was assumed to be 0.235  $\mu\text{mol ml}^{-1}$  and the instrument was calibrated prior to use by reduction with a few grains of sodium dithionite.

## 2.6: Spectrophotometric Examination of Cytochromes

### 2.6.1: Low temperature spectrophotometry

Low temperature difference spectra were obtained using a Shimadzu (MPS) 5000 double beam spectrophotometer with low temperature attachments. Membrane suspensions in 0.1 M Tris buffer (pH 7.4) with or without 50% glycerol, were placed in 2 mm light path low temperature cells and frozen in liquid air. Liquid air was used in place of liquid nitrogen because it was readily available in the department. Comparison of several spectra obtained from samples frozen in liquid air with those of duplicate samples frozen in liquid nitrogen revealed no detectable differences.

Samples containing 50% glycerol form a transparent glass when originally frozen. If these samples are then

warmed slowly to  $-70$  to  $-50^{\circ}\text{C}$ , they take on a thick milky appearance as the glycerol and water separate and the water forms microcrystals. The microcrystalline state is retained when the samples are recooled in liquid air.

Samples frozen to liquid air temperatures give an approximately tenfold amplification of the b peak absorbance relative to that at room temperature if the solvent is wholly aqueous, and approximately 20-fold if the samples are made microcrystalline in 50% glycerol as described above. The  $\alpha$ -peak absorption wavelength of the b cytochromes observed at liquid air temperature is shifted to about 5 nm less than that observed at room temperature.

If sufficient care was taken to keep the protein concentrations in sample and reference cuvettes equal, definite  $\alpha$ ,  $\beta$  and Soret peaks could be seen in spectra of membranes prepared in aqueous solvent. However if 50% glycerol was the solvent, the shape of the peaks in the Soret region was not very reproducible. This was probably due to the increased light path in the presence of glycerol and the increased scattering of light at lower wavelengths, both of which would tend to amplify even very slight differences between preparations in the sample and reference cuvettes. The  $\alpha$ -peak spectra were highly reproducible both with and without glycerol in the solvent.



## 2.6.2: Redox titrations

### 2.6.2.1: Room temperature redox titrations

Redox titrations at room temperature were performed in a double beam spectrophotometer (Cary 219) using a specially constructed cuvette accommodating the electrode, a magnetic stirring bar, titrant and gas ports. The redox potential was measured with a pH-mV meter (PHM62, Radiometer, Copenhagen). A platinum electrode was immersed in the cuvette and a calomel electrode was used as a reference connected to the cuvette by a KCl salt bridge (2 M KCl in 1% agar). The accuracy of the voltmeter was tested by use of a quinhydrone electrode. The cuvette was stirred by means of a magnetic stirrer housed in the sample compartment. Reductant (5% sodium dithionite, pH 7.0) or oxidant (2% potassium ferricyanide, pH 7.0) were added in microliter quantities to the sample cuvette by capillary tubing connected to micrometer syringes (Agla, Burroughs-Wellcome Co., London). Oxygen-free  $N_2$  was passed continuously through the sample cuvette during the redox titration. After each addition of reductant or oxidant, 5-10 min were allowed for the potential to stabilize before a difference spectrum was recorded. The reference cuvette contained an equal concentration of mediators and membranes which had been thoroughly mixed with air to keep the cytochromes in an oxidized state. The reaction mixture contained about  $3 \text{ mg ml}^{-1}$  membrane protein in 0.1 M Tris buffer (pH 7.0). It was necessary to include redox mediators acting over the range of +200 mV to -100 mV. These are listed in Table 2.6.2.1.

Table 2.6.2.1: Redox mediators

Mediator	$E_{m7}$ (mV)	Concentration (M)
EDTA, $\text{FeCl}_3$	+117	$10^{-2}$ , $3 \times 10^{-4}$ (resp.)
PMS	+80	$5 \times 10^{-4}$
Juglone	+30	$5 \times 10^{-5}$
Duroquinone	+5	$5 \times 10^{-5}$
Resazurin	-46	$3 \times 10^{-6}$
2-OH-1,4-napthoquinone	-152	$10^{-5}$

### 2.6.2.2: Low temperature redox titrations

Membranes poised at known redox potentials were frozen and the absorption spectra recorded at low temperatures as described above (section 2.6.1). This was done in order to resolve  $\alpha$ -peaks of the b type cytochromes with absorption maxima differing by 3-5 nm and to distinguish between the  $\alpha$ -peaks of b type cytochromes which had  $E_{m7}$  values insufficiently separated (less than 50 mV) to be distinguishable by room temperature redox titrations.

A 15 ml suspension of membranes (0.3 to 0.6 mg membrane protein ml<sup>-1</sup>) from which samples were to be removed and frozen at specific redox potentials was placed in a magnetically stirred, water jacketted chamber (constructed according to the design of Dutton, 1978) maintained at 25°C with a circulating water bath. A platinum electrode and a calomel electrode were immersed directly in the sample and the redox potential was followed using a pH-mV meter. Reductant (5% sodium dithionite, pH 7.0) or oxidant (2% potassium ferricyanide, pH 7.0) were added via capillary tubing in amounts controlled by micrometer syringes. Oxygen-free N<sub>2</sub> was continuously passed over the sample and allowed to escape via the sampling tube. Prior to sampling, the external end of the sampling tube was placed inside the low temperature spectrophotometer cell to flush the cell free of air. Sampling was performed by forcing the sampling tube into the redox-equilibrated membrane suspension. The subsequent rise in internal pressure forced the sample up the sampling tube into the low temperature cuvette without exposure to air. The cuvette was immediately frozen in

liquid air. Redox mediators were the same as those listed in Table 2.6.2.1.

In several titrations carried out by the above method, the membranes were suspended in 50% aqueous glycerol in order to reduce the amount of membrane required to perform a titration. However, the inclusion of glycerol caused problems due to the increased viscosity. This meant that stirring had to be very vigorous to obtain a homogeneous solution and sufficient time had to be allowed between additions of reductant or oxidant to allow complete equilibration between the cytochromes, the media and the electrodes. Furthermore, the use of glycerol gave less reproducible Soret peaks as explained earlier. More satisfactory redox titrations were obtained using more concentrated membrane samples without glycerol.

Experimental procedures for obtaining steady-state and substrate reduced spectra and for studying the effects of inhibitors on the oxidation and reduction of cytochromes are described in the appropriate section of the results (3.2.1.1.).

## 2.7: Sources of Chemicals

The chemicals used in experiments performed for this thesis and their sources are listed below.

Potassium ferricyanide ( $K_3FE(CN)_6$ ) UCB, Brussels

Resazurin

L. Light & Co.,  
Ltd. Colnbrook,  
England.

Sodium succinate	BDH
Yeast Extract	GIBCO

The following chemicals were all purchased from Sigma Chemical Company.

Benzyl viologen (1,1'-dibenzyl-4,4'-bipyridinium chloride)

Casein, enzymatic hydrolysate

Coomassie brilliant blue G

Deoxyribonuclease, grade II

2,6-dichlorophenol-indophenol

Duroquinone (tetramethyl-p-benzoquinone)

Fumarate, sodium salt

Juglone (5-OH-1,4-napthoquinone)

D-lactate, lithium salt

D,L-lactate, sodium, 60% syrup

L-lactate, lithium salt

Lawsone (2-OH-1,4-napthoquinone)

$\beta$ -nicotinamide adenine dinucleotide

Phenazine methosulfate

Tris (hydroxymethyl) aminoethane

Reagents not listed above but mentioned in the text were of the highest purity available.

3. RESULTS

### 3.1: Characterization of Membrane Particles

In this section, some of the properties of the membrane fraction prepared as described in section 2.2 are presented. The membrane preparation was characterized by use of electron microscopy, density gradient centrifugation, analysis for electron transport components and assay of specific activities of electron transport processes under various conditions.

#### 3.1.1: Investigation of the membrane fraction by electron microscopy and density gradient centrifugation

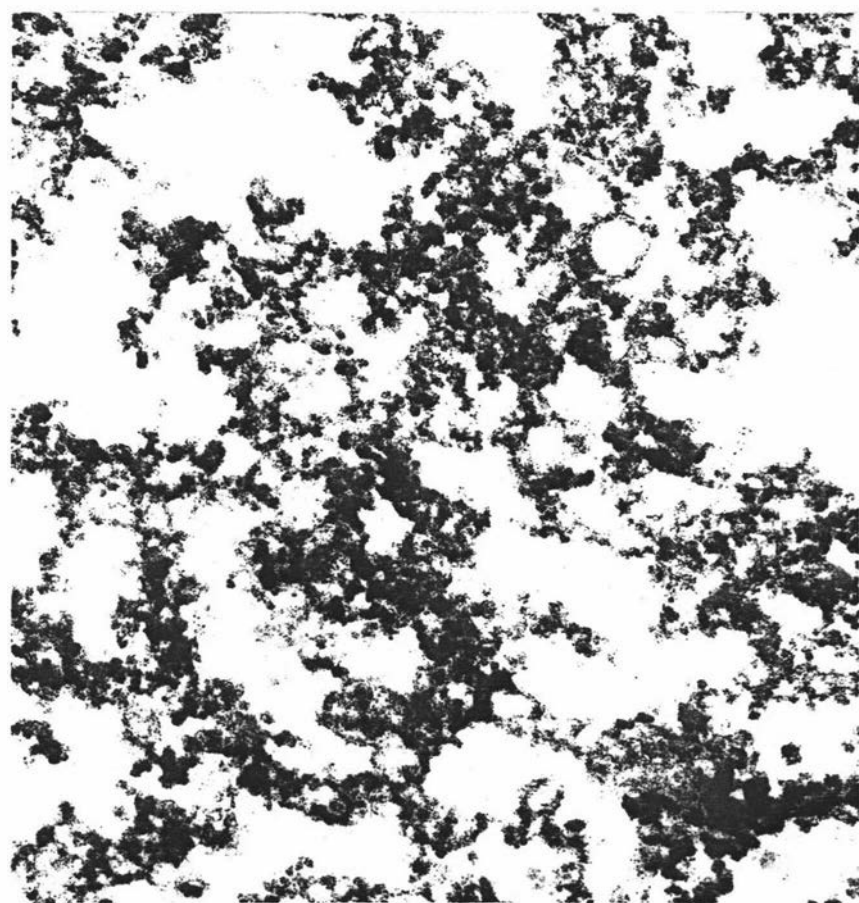
The procedure originally adopted for preparation of membranes, which employed buffer containing 10 mM  $\text{MgCl}_2$ , gave a very dense preparation which sedimented through 60% sucrose within 30 min at 160,000 g. Membranes prepared in this manner and examined in thin sections by transmission electron microscopy showed a mass of small particles with a diameter of about 13 nm (Plate 3.1.1.1) in which there was very little evidence of membrane structure.

It was subsequently realized that the particulate nature of the membrane fraction was due to clumping of ribosomes in the presence of high  $\text{Mg}^{++}$  concentrations. When  $\text{Mg}^{++}$  was omitted from the preparation buffer, the membrane fraction was less dense and this permitted the inclusion of a centrifugation over sucrose layers (20%/40%/60%, as described in 2.2) in the isolation procedure. This served the purpose of removing further soluble or cytoplasmic material above the 20%/40% interface as well as separating whole cells and cell walls, which formed a pellet. The membranes formed

Plate 3.1.1.1: ELECTRON MICROGRAPH OF MEMBRANES  
PREPARED IN THE PRESENCE OF  $Mg^{++}$

Membranes prepared with buffer, containing  $10^{-2}$   
M  $Mg^{++}$  as described in Methods (2.2) were prepared  
for thin section electron microscopy as described  
previously (2.3). Magnification X74,000.





a layer at the 40%/60% sucrose interface.

To test whether the omission of  $Mg^{++}$  had a significant effect on the specific activities of electron transport processes, a cell extract obtained by cell breakage in the Hughes press was divided into two aliquots. Membranes were prepared by differential centrifugation from one aliquot using buffer containing 10 mM  $MgCl_2$  and from the other aliquot using buffer lacking  $MgCl_2$ . A comparison of certain electron transport activities (Table 3.1.1.1) showed that there was little difference between the two aliquots, so that results obtained with membranes prepared with or without  $MgCl_2$  are probably comparable.

Electron microscopy of whole cells using negative staining (Plates 3.1.1.2, A & B) gave some indication that mesosomes might occur in *P. shermanii*. Thin sections of apparently whole cells present in membrane preparations also frequently showed extensive internal membrane structure (Plate 3.1.1.3, C, ii). This raised the possibility of particular specialization of the cellular membrane in certain regions. Variations in specific activities of electron transport reactions might also be associated with variations in the amount of cell wall attached to membranes, the ratio of inside-out to right-side-out vesicles, and contamination with unbroken cells.

The possibility of membrane particle heterogeneity was investigated by density equilibrium separation of membranes prepared in the absence of  $Mg^{++}$ . Membranes collected from between 40% and 60% sucrose layers were dialysed against 0.1 M Tris buffer (pH 7.4, at 5°C) for 8 hrs and then applied

Table 3.1.1.1: Comparison of recovery of electron transport activities in membranes prepared with and without  $Mg^{++}$  in the isolation buffer

	Total Protein	D-lac-DICPIP oxidoreductase	D-lac-oxygen oxidoreductase	NADH-oxygen oxidoreductase
+ $Mg^{++}$	2.37	17.6	20.4	35.4
- $Mg^{++}$	3.47	16.2	16.9	25.7

The values presented in this table are the recoveries of total protein or activity in the purified membrane fraction as a percentage of the total protein or activity in the original broken cell suspension.

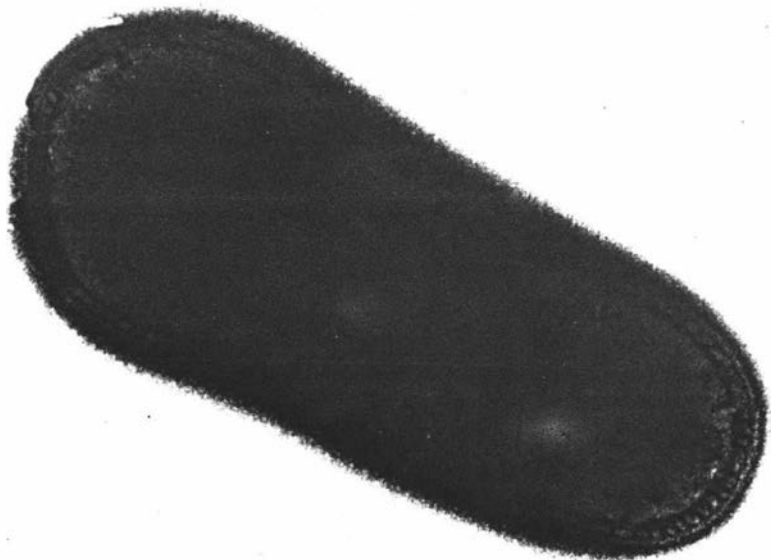
Plate 3.1.1.2: ELECTRON MICROGRAPHS OF NEGATIVELY  
STAINED CELLS OF *P. shermanii*.

Cells collected at the end of log growth and kept as a pellet were prepared for electron microscopy as follows:

- A. 0.25% PTA, spray (as described in section 2.3).  
Magnification, X55,000.
- B. The sample was prepared as in the drop wash method (section 2.3) except that 1% ammonium molybdate was used instead of 2% PTA.  
Magnification, X55,000.

A

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B

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to a 30 ml 40% to 60% linear sucrose gradient and centrifuged overnight (14 hrs) at 100,000 g at 5°C. Fractions of 1.2 ml were collected by forcing 65% sucrose in from the bottom of the tube. These fractions were analysed for protein, specific activities of D-lactate-, L-lactate- and NADH-DICPIP oxidoreductases, D-lactate- and NADH-oxygen oxidoreductases and specific gravity. Data from this are presented in Figure 3.1.1.1 and Table 3.1.1.2.

Membrane material was concentrated in three major zones appearing as three peaks in Figure 3.1.1.1. However, the specific activities of all the electron transport processes measured were very similar in all fractions, apart from the four most dense fractions, which may have included some unbroken cells, and the first four fractions (Table 3.1.1.2). The specific activities of D-lactate-DICPIP oxidoreductase in fractions 2, 3 and 4 were significantly higher than the mean activity of all samples. The same is true for L-lactate-DICPIP oxidoreductase in fraction 2. These high activities are probably due to a form of D-lactate and L-lactate dehydrogenase which has dissociated from the membrane, since the specific gravities of these fractions lie below the linear portion of the density gradient.

The shaded portions of Figure 3.1.1.1 represent pooled fractions which were dialysed to remove sucrose and examined by transmission electron microscopy (Plates 3.1.1.5, A, B & C). The size distribution of these particles was estimated by measuring thirty vesicle diameters from each of 3 or 6 photographic prints. Results of these measurements are tabulated in Table 3.1.1.3.

Figure 3.1.1.1: D-lactate-DICPIP oxidoreductase and total protein in fractions from density gradient centrifugation of membranes prepared from *P. shermanii*.

Data shown graphically in this figure are tabulated in Table 3.1.1.2. Shaded portions represent the pooled fractions which were examined by electron microscopy (Plate 3.1.1.2). Total D-lactate-DICPIP oxidoreductase ( $\mu\text{mol DICPIP reduced min}^{-1}$ ) ●; protein concentration ( $\mu\text{g ml}^{-1}$ ) ▲; specific gravity, ■.

Figure 3.1.1.1

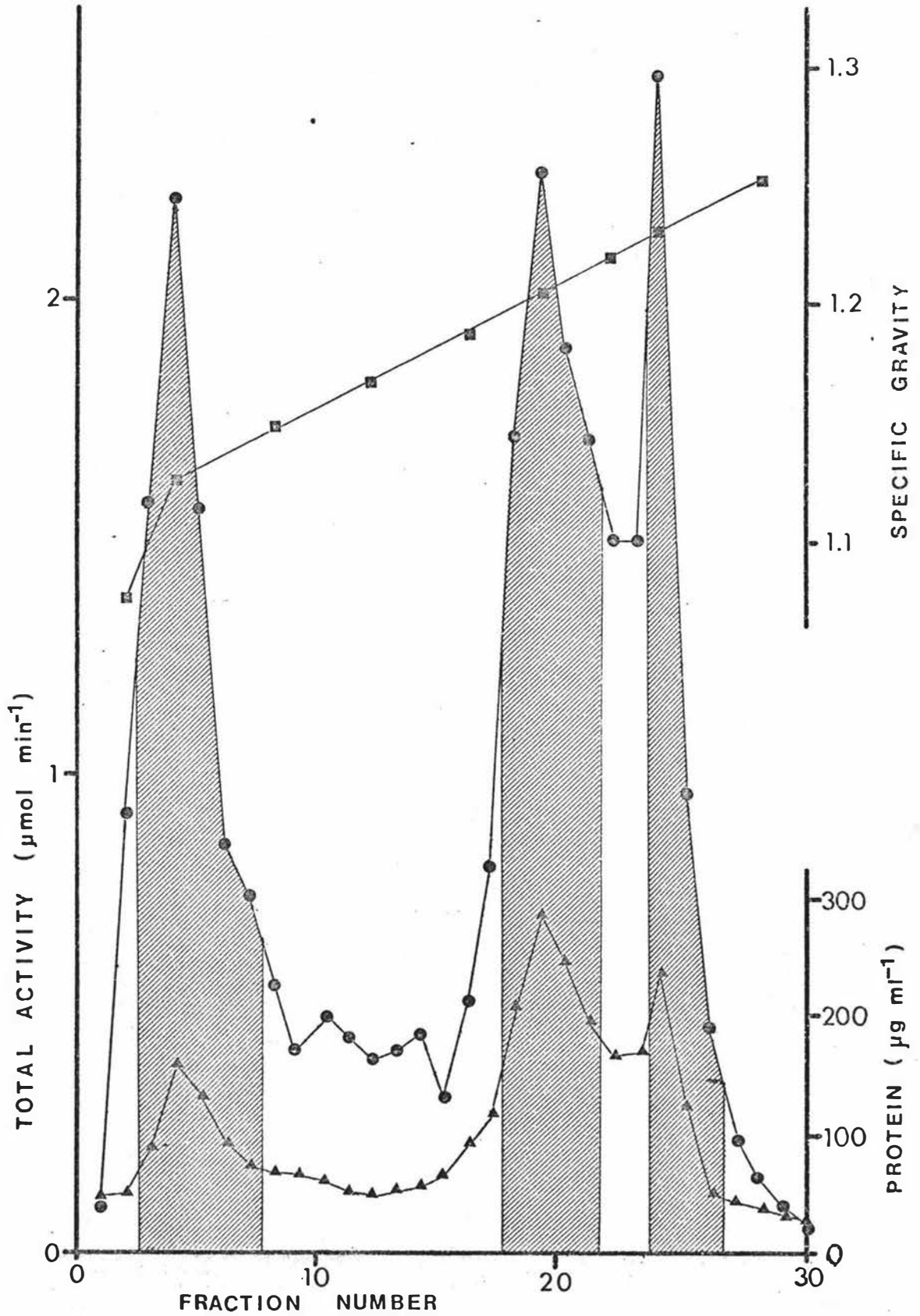




Table 3.1.1.2: Sucrose Density Gradient Fractionation of *P. shermanii* membranes: Protein, Specific Activities and Specific gravity of Fractions.

#	Protein ( $\mu\text{g ml}^{-1}$ )	D-lac DICPIP	L-lac DICPIP	NADH DICPIP	D-lac Oxygen	NADH Oxygen	S.D.
1	45	1.62	0.133	0.413	0	0	-
2	50	15.2	2.46	0.566	0	0.235	1.137
3	90	14.5	1.80	0.762	1.159	0.273	-
4	160	11.4	1.15	1.304	1.216	0.500	1.161
5	130	9.87	1.06	1.22	1.102	0.576	-
6	90	7.80	0.911	1.108	0.791	0.567	-
7	70	8.78	0.792	1.03	1.33	0.713	-
8	65	7.10	0.884	0.980	-	0.672	1.172
9	65	6.49	0.818	0.923	-	-	-
10	60	6.83	0.824	0.811	-	0.538	-
11	52	7.20	0.835	1.0	-	-	-
12	50	6.71	0.714	1.01	1.03	0.654	1.181
13	52	6.62	0.692	1.05	-	-	-
14	55	6.83	0.722	1.008	-	0.614	-
15	65	5.98	0.624	0.659	-	-	-
16	92	4.66	0.515	0.764	0.391	0.515	1.191
17	118	5.71	0.642	1.131	0.772	-	-
18	202	7.07	0.642	1.18	0.691	0.572	-
19	280	6.71	0.599	1.15	0.706	0.631	1.200
20	246	6.38	0.65	1.318	0.779	0.725	-
21	196	7.17	0.634	1.312	0.641	0.895	-
22	166	7.44	0.659	1.299	0.925	0.756	1.207
23	166	7.40	0.598	1.205	1.042	0.801	-
24	237	8.61	0.627	1.32	0.892	0.758	1.213
25	128	6.20	0.437	1.105	0.683	0.798	-
26.	58	6.87	0.325	1.113	0.975	-	-
27	47	4.13	0.116	0.754	0.750	0.0495	-
28	40	3.30	0.093	0.543	-	-	1.223
29	35	2.16	0.106	0.253	-	-	-
30	32	1.41	0.116	0.286	-	-	-
$\bar{x}$		6.94	0.797	1.028	0.882	0.592	
s.d.		3.02	0.445	0.226	0.230	0.202	

Specific activities are expressed as umoles DICPIP or oxygen reduced per min per mg protein.

Table 3.1.1.3: Particle sizes from three different density gradient fractions

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Specific Gravity ( $\text{g/cm}^3$ )	1.160	1.200	1.212
Diameter (nm)	32.4 <sup>(a)</sup>	103 <sup>(b)</sup>	98.7 <sup>(b)</sup>
Standard deviation (nm)	0.67	13.3	14.1
Range (nm)	12-61	22-410	22-480

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(a) average of 90 determinations

(b) average of 180 determinations

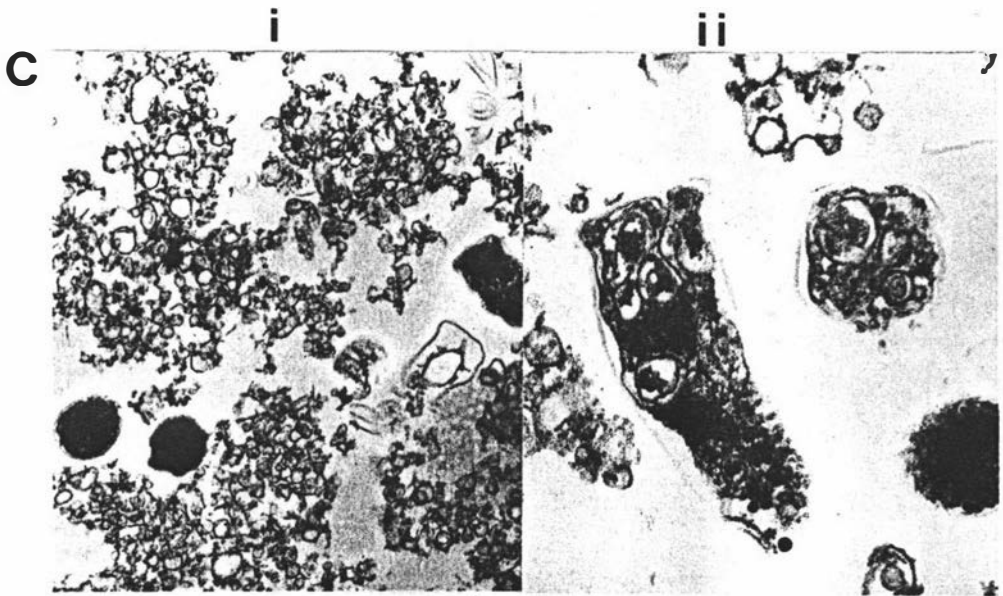
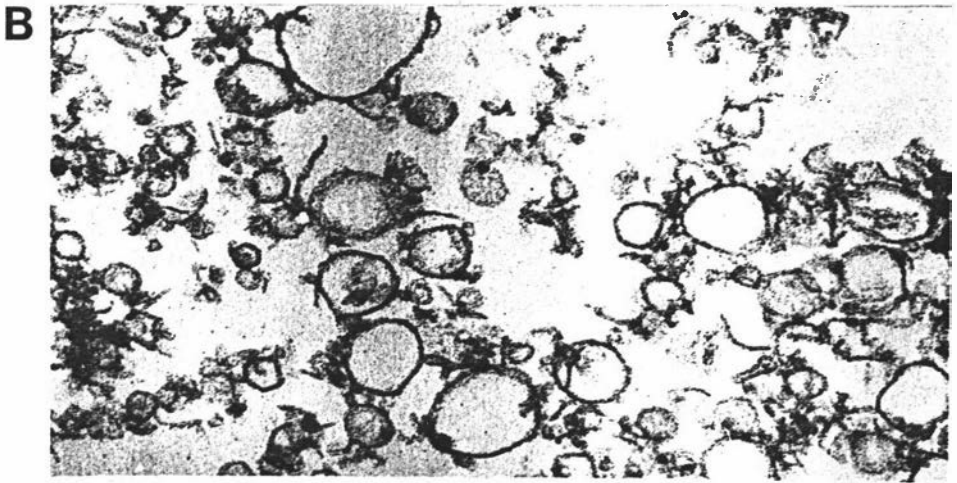
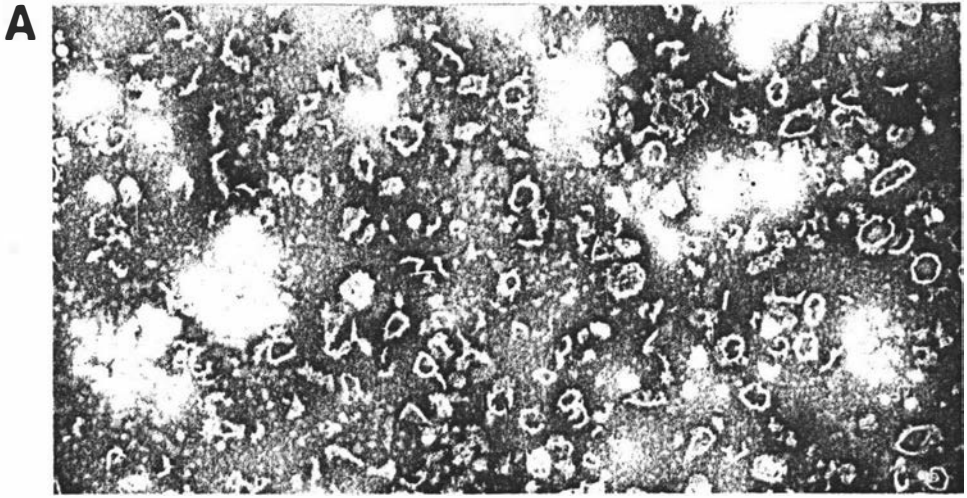
The small particles (specific gravity  $1.160 \text{ g cm}^{-3}$ ) correspond approximately in size (30-40 nm diameter) to the 'mesosome' size observed in electron microscopy of thin sections of whole cells (Plate 3.1.1.2). However, the 'mesosome-like' features observed by electron microscopy may well be artifacts created by the procedures for preparing cells for electron microscopy which causes bonding between DNA and the cellular membrane (Parks *et al*, 1981). Since there is no significant difference in specific activity of any of the electron transport processes assayed in the light particle fraction (specific gravity of  $1.160 \text{ g cm}^{-3}$ ) from that in the heavier fraction (specific gravity of  $1.200 \text{ g cm}^{-3}$ ), there is no evidence of functional specialization of these membranes.

The two more dense pooled fractions (specific gravity of  $1.200$  and  $1.212 \text{ g cm}^{-3}$ ) also did not differ significantly in specific activities. They did differ in that the heavier fraction appeared to contain more cell wall material than did the s.g.  $1.200$  membranes as well as some whole cells.

The whole cells present in the s.g.  $1.212$  fraction (Plate 3.1.1.3, C) formed a small proportion of the fraction. Some of these cells show considerable membrane structure in the cytoplasmic space, whereas others show very little. This probably represents variation in degree to which the cells were broken, the more damaged cells showing greater internal membrane structure.

Plate 3.1.1.3: ELECTRON MICROGRAPHS OF POOLED FRACTIONS  
FROM DENSITY GRADIENT CENTRIFUGATION.

- A. Negative stain (0.25% PTA, as described in 2.3) of 'light' membrane fraction (s.g., 1.150). X82,000.
- B. Thin section electron micrographs of 'middle' membrane fraction (s.g., 1.200). X46,000.
- C. Thin section electron micrograph of 'heavy' membrane fraction (s.g., 1.212).
  - (i) X17,500
  - (ii) X46,000



### 3.1.2: Electron transport activities of *P. shermanii* membrane preparations

Membranes prepared as described in the section 2.2 were able to couple the oxidation of D-lactate, L-lactate NADH and succinate with the reduction of oxygen and oxidation of the first three of these donors with the reduction of fumarate. The electron transport activities dependent on D-lactate, NADH and succinate as donors were fairly stable, although it was noticed that NADH coupled activities decreased slightly with time. L-lactate-coupled activities were much less stable, decreasing from levels similar to those for D-lactate if assayed the day of preparation to 20% or less if assayed the following day. Therefore, L-lactate-coupled reactions were normally assayed on the day of preparation or as soon as possible thereafter, whereas other activities could be measured during the four days subsequent to preparation without substantial loss of activity when stored at 4°C. Membrane fractions were rarely kept longer than this.

#### 3.1.2.1: Specific activities of oxygen and fumarate oxidoreductases

Specific activities of electron transport processes coupled to oxygen or fumarate reduction (the latter in an oxygen-free assay) are shown in Table 3.1.2.1. The membranes for these assays were prepared in the presence of 10 mM Mg<sup>++</sup> and were assayed on the day of preparation. Specific activities determined with membranes which had been prepared using a sucrose gradient step and assayed after storage at

4°C for 1-4 days were lower, normally 25-50% of these values. However, the ratios of activity of oxygen to fumarate consumption, of oxygen consumption in the presence and absence of PMS, and of D-lactate to NADH to succinate dependent activities remained fairly constant.

If the rates of oxygen consumption are expressed as  $\mu\text{g}$  atoms of oxygen  $\text{min}^{-1} \text{mg}^{-1}$ , the rates of reduction of oxygen and fumarate are roughly equal for each substrate. PMS accelerated oxygen consumption 2.5 fold with D-lactate, 6.0 fold with L-lactate and 19.2 fold with succinate. Succinate-oxygen oxidoreductase activity was very much lower than that with other donors (2.5% of D-lactate-oxygen oxidoreductase). However, PMS stimulated it by a much greater factor, suggesting that the low rate of electron transfer from succinate to oxygen is not limited by a low dehydrogenase activity.

Table 3.1.2.1: Specific Activities of Oxygen and Fumarate Oxidoreductase in Membranes of *P. shermanii*.

Acceptor	Reductant			
	D-lac	L-lac	NADH	Succ
Oxygen	7.67	2.03	5.8	0.192
Oxygen + PMS	19.7	12.2	-	3.69
Fumarate	4.02	1.54	3.21	-

Specific activities are in  $\mu\text{moles}$  fumarate or  $\mu\text{moles}$  oxygen  $\text{min}^{-1} \text{mg}^{-1} \text{protein}^{-1}$ . PMS was present, when indicated, at  $10^{-4}$  M. Membranes were prepared in the presence of 10 mM  $\text{Mg}^{++}$  and assayed on the day preparation. Fumarate

reductase activities were determined in an anaerobic assay. as described in section 2.3.7. Oxygen consumption was followed using an oxygen electrode as described in section 2.3.8.

3.1.2.2: Specific activities of the  $\text{Fe}(\text{CN})_6$  and DICPIP oxidoreductases

Specific activities of reductases measured with  $\text{Fe}(\text{CN})_6$  or DICPIP as electron acceptors are shown in Table 3.1.2.2 & 3.1.2.3 respectively. Activities were compared in the presence and absence of PMS and also in the presence and absence of oxygen to ascertain the extent to which oxygen competes with the dyes for electron flow.

Table 3.1.2.2: Specific activities of  $\text{Fe}(\text{CN})_6$  Reductases

$\text{O}_2$ (a)	PMS (b)	D-lac	L-lac	NADH	Succ
+	-	30.8	2.02	24.7	6.22
+	+	94.1	3.10	-	13.2
-	-	33.2	4.33	28.0	6.71
-	+	91.0	7.44	-	13.2

Values presented are specific activities of  $\text{Fe}(\text{CN})_6$  reduction in  $\mu\text{moles per min per mg protein}$ .

(a) Assay performed using reagents equilibrated with atmospheric oxygen (+) or oxygen-free, under nitrogen (-).

(b) PMS present, when indicated, at 0.1 mM.



Table 3.1.2.3: Specific Activities of DICPIP Reductases

O <sub>2</sub> (a)	PMS (b)	D-lac	L-lac	NADH	Succ
+	-	43.4	1.55	3.44	1.45
+	+	67.2	1.25	-	7.88
-	-	39.5	7.64	8.67	1.83
-	+	71.9	8.34	-	9.41

Figures are specific activity of DICPIP reduction in micro moles per min. per mg protein. (a) and (b) are the same as in Fig. 3.1.2.2.

The ratios of activity measured in the presence of oxygen to that in its absence are given in Table 3.1.2.4. It can be seen that oxygen competes strongly with Fe(CN)<sub>6</sub> or DICPIP in some assays but not at all in others. In particular, L-lactate-DICPIP oxidoreductase and, to a lesser extent, L-lactate-Fe(CN)<sub>6</sub> oxidoreductase activities were inhibited by the presence of oxygen. NADH-DICPIP oxidoreductase activity was also quite strongly inhibited, while NADH-Fe(CN)<sub>6</sub> oxidoreductase activity was not markedly affected. The presence of PMS had little effect on the ratios.

The ratios of activities in the presence and absence of PMS are shown in Table 3.1.2.5. While PMS stimulated the oxidoreductase activities in all cases except for L-lactate-DICPIP oxidoreductase, the extent of stimulation differed

Table 3.1.2.4: Effects of oxygen on  $\text{Fe}(\text{CN})_6$  and DICPIP Reductases.

Oxidant	PMS	D-lac	L-lac	NADH	Succ
$\text{Fe}(\text{CN})_6$	-	1.08	2.14	1.13	1.08
$\text{Fe}(\text{CN})_6$	+	0.97	2.40	-	1.00
DICPIP	-	0.91	4.93	2.52	1.26
DICPIP	+	1.07	5.38	-	1.19

The values presented in this table are the ratios obtained by dividing the specific activity determined in the absence of oxygen by that determined in its presence, using data presented in Tables 3.1.2.2 and 3.1.2.3.

Table 3.1.2.5: Effect of PMS on  $\text{Fe}(\text{CN})_6$  and DICPIP Reductases.

Oxidant	$\text{O}_2$	D-lac	L-lac	Succ
$\text{Fe}(\text{CN})_6$	+	3.06	1.53	2.12
$\text{Fe}(\text{CN})_6$	-	2.74	1.72	1.97
DICPIP	+	1.55	0.086	5.43
DICPIP	-	1.82	1.09	5.14

The values presented in this table are the ratios obtained by dividing the specific activity determined in the presence of PMS by that determined in its absence, using data presented in Tables 3.1.1.5 and 3.1.1.6.

markedly for the different systems. PMS stimulated  $\text{Fe}(\text{CN})_6$  reductase much more than DICPIP reductase with D-lactate as donor, but had the opposite effect with succinate. The acceleration of reductase activities by PMS was unaffected by oxygen. It is also of interest to note that PMS stimulated activity of the reductases by a lesser factor than that for PMS stimulation of oxygen consumption (see Table 3.1.1.4).

A final comparison of interest is that while the specific activities of the DICPIP reductase and the  $\text{Fe}(\text{CN})_6$  reductase (in the absence of PMS) are approximately comparable for D- and L-lactate, the  $\text{Fe}(\text{CN})_6$  reductase activity is markedly higher than DICPIP activity for NADH and succinate.

From these studies, it is apparent that DICPIP and  $\text{Fe}(\text{CN})_6$  interact with different components of the electron transport chain. These results will be discussed later in this thesis.

#### Determination of Apparent $K_m$ Values for Substrates

Michaelis constants for D-lactate, L-lactate and NADH were determined using a number of different assays in order to ensure that a sufficiently high concentration of substrate was used to achieve saturation of electron transport activities in routine assays. Data from linear regressions of Lineweaver-Burk plots ( $1/v$  vs  $1/S$ ) are shown in Table 3.1.2.6. Because the activities being measured are in all cases multi-step processes in which the rate limiting step may not be the same with different electron acceptors,

Table 3.1.2.6: Apparent  $K_m$  values for electron donors of some oxidoreductase systems

donor	acceptor	$K_m$ (mM)	r	n
D-lac	oxygen	0.15	0.99	9
D-lac	DICPIP	0.364	0.99	8
L-lac	oxygen	0.027	0.98	8
L-lac	DICPIP	0.105	0.98	8
NADH	oxygen	0.72	0.99	8

Linear regressions of data ( $1/v$  vs  $1/S$ ) were calculated using the internal program on a Sharp 5100 calculator.  $r$  is the correlation coefficient from a regression of  $n$  pairs of data.

interpretation of the significance of the  $K_m$  values is complex and will not be attempted. The determinations were made only to estimate saturating substrate concentrations for these reactions.

The apparent  $K_m$  values for succinate- $\text{Fe}(\text{CN})_6$  and succinate-DICPIP oxidoreductases were also determined. Plots of  $1/v$  vs  $1/S$  are concave downward (Figures 3.1.2.1 A & B). This suggests that there are two binding sites for succinate for both oxidoreductase systems with different  $K_m$  values (Cleland, 1970) or that negative cooperativity exists between binding sites for succinate on the same enzyme (Fromm, 1975). Linear regressions fitted to the points on the assumption that the former interpretation is correct give approximate  $K_a$  values for the two succinate binding sites as shown in Table 3.1.2.7.

Table 3.1.2.7: Apparent succinate binding constants for succinate-Fe(CN)<sub>6</sub> and succinate-DICPIP oxidoreductase systems.

	$K_{a1}$ (mM)	$K_{a2}$ (mM)	$r^2$ (a)
Fe(CN) <sub>6</sub>	0.157	2.17	0.996
DICPIP	0.0502	0.742	0.975

(a)  $r^2$  is the multiple correlation coefficient (Seber, 1977) of the data shown in Figure 3.1.2.1 fitted to the equation of Cleland, (1970).

The difference between the constants determined using DICPIP and Fe(CN)<sub>6</sub> probably depends on the sites at which these acceptors interact with the complex system coupling oxidation-reduction reactions with the normal substrates. The DICPIP reducing system apparently becomes saturated at lower concentrations of succinate than does that reducing Fe(CN)<sub>6</sub>. This possibly indicates that the rate limiting steps in electron transport from succinate to the two acceptors are different.

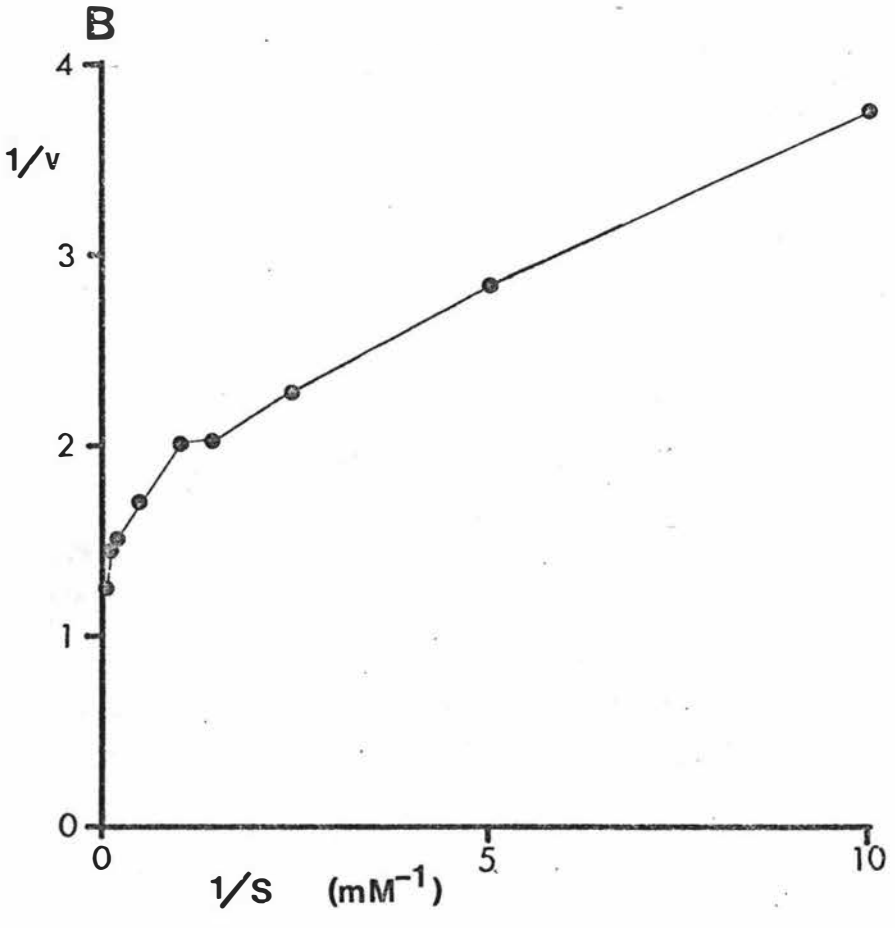
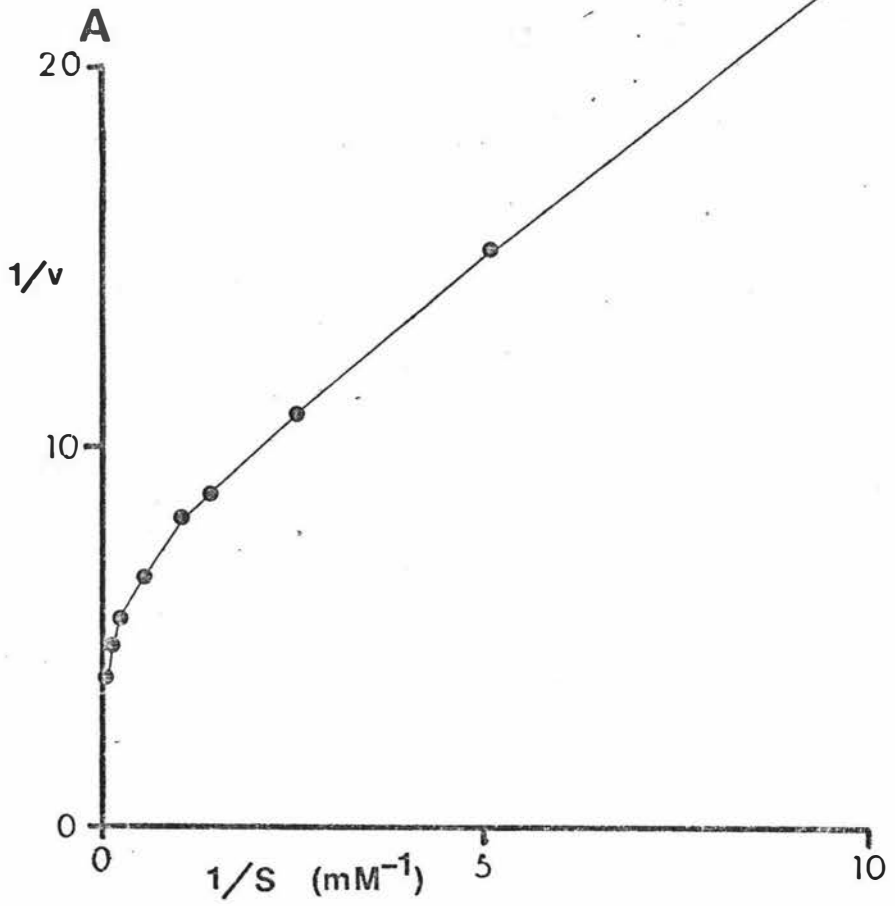
Succinate-coupled reactions were routinely assayed using 2 mM succinate. This concentration effectively saturates the higher affinity site, but not the lower affinity site. Since these values were determined at a late stage in the work described in this thesis, this aspect of succinate-coupled activities could not be further investigated.

Figure 3.1.2.1: Lineweaver-Burk Plots of succinate-Fe(CN)<sub>6</sub> and succinate-DICPIP oxidoreductases

Data plotted are the inverse rate of reaction versus the inverse of succinate concentration (mM). The reaction rate of succinate-Fe(CN)<sub>6</sub> oxidoreductase was expressed as the change in absorption at 405 nm per minute and that of succinate-DICPIP oxidoreductase as the percent maximum rate measured, both in the presence of 0.1 mM PMS.  $V_{\max}$  was estimated as 4.99 and 2.50  $\mu\text{M min}^{-1} \text{mg}^{-1}$  for succinate-Fe(CN)<sub>6</sub> and succinate-DICPIP oxidoreductases, respectively.

- A. Succinate-Fe(CN)<sub>6</sub> oxidoreductase
- B. Succinate-DICPIP oxidoreductase

Figure 3.1.2.1



### 3.1.3: Protein, carbohydrate and lipid content of membranes

Before it was realized that  $Mg^{++}$  was causing the high density of membrane fractions, it was thought that this high density might be due to a peculiar composition of the membrane. Determination of the major chemical constituents was therefore attempted to examine the extent to which membranes may be contaminated with 'non-membranous' cellular materials and as part of the general membrane characterization.

A membrane fraction was analysed for protein content by five different methods (Table 3.1.3.1). The Coomassie blue binding assay gave the lowest value. However, due to ease of use, it was adopted as the routine method for estimating the protein content of membrane suspensions throughout this work.

In the same membrane preparation used above, phosphate, analysed by the method of Jaenicke (1974), which includes a perchloric acid digest, was present at 13  $\mu g$  per mg dry weight. Occasionally, however, a black membrane preparation resulted. One of these, prepared in the absence of  $Mg^{++}$  and finally collected from between 40% and 60% sucrose layers, was analysed for phosphate. Phosphate was present in this membrane fraction at 1.67 mg per mg protein. Cells of propionibacteria grown as described in this study contain a large polyphosphate granule which is the probable source of phosphate observed in the black membranes. Black membranes appeared infrequently and did not appear to differ from other membrane preparations in any of the electron transport activities assayed.



Table 3.1.3.1: Protein Analysis of Membranes

Method	mg protein per mg dry weight
Phenol hypochlorite	0.36
Kjeldahl	0.49
Coomassie blue	0.28 <sup>(a)</sup>
biuret	0.47
Lowry	0.38

Membranes prepared by differential centrifugation in the presence of 10 mM MgCl<sub>2</sub> were dialysed against distilled water twice for 10 hrs before analysis. Values presented are the average of duplicates. Each assay was standardized against bovine serum albumin.

(a) This value was duplicated on a separate membrane preparation.

In the membrane preparation used for the determinations presented in Table 3.1.3.1, carbohydrate was present at 0.20 mg per mg dry weight. This unusually high carbohydrate content prompted the determination of carbohydrate in a number of separate membrane preparations (Table 3.1.3.2). Carbohydrate was present at an average concentration of 2.13 mg per mg protein and lipid was present at an average of 3.27 mg per mg protein, giving a membrane composition of 16% protein, 51% lipid and 33% carbohydrate. The major deviation from normal membrane composition (Law & Synder, 1972) is the presence of a high content of carbohydrate.

Table 3.1.3.2: Carbohydrate and Lipid Content of Membranes

Protein <sup>(a)</sup> (mg/ml)	Carbohydrate <sup>(b)</sup> (mg/ml)	Lipid <sup>(c)</sup> (mg/ml)	Carbohydrate in lipid fraction (mg/ml)
9.0	16.0	-	-
4.4	10.5	14.2	0.32
0.45	1.16	-	-
0.98	1.72	3.25	0.0545

(a) Protein was assayed with the Coomassie blue binding method.

(b) Carbohydrate was assayed in duplicate by the phenol sulfuric acid method using a standard composed of glucose, mannose and inositol in the ratio 6.4 to 3.2 to 1.0, respectively (see below).

(c) Lipid was measured by extraction in duplicate with chloroform-methanol (2:1 (v/v)) and weighing the dried extract.

An attempt to determine the source of carbohydrate was made by identification of its monosaccharide composition. This was done by acid hydrolysis of a membrane fraction, acetylation of the hydrolysed monosaccharides and separation by gas liquid chromatography as described in Section 2.4.4. A chromatogram obtained using an SP2340 column is shown in Figure 3.1.3.1.

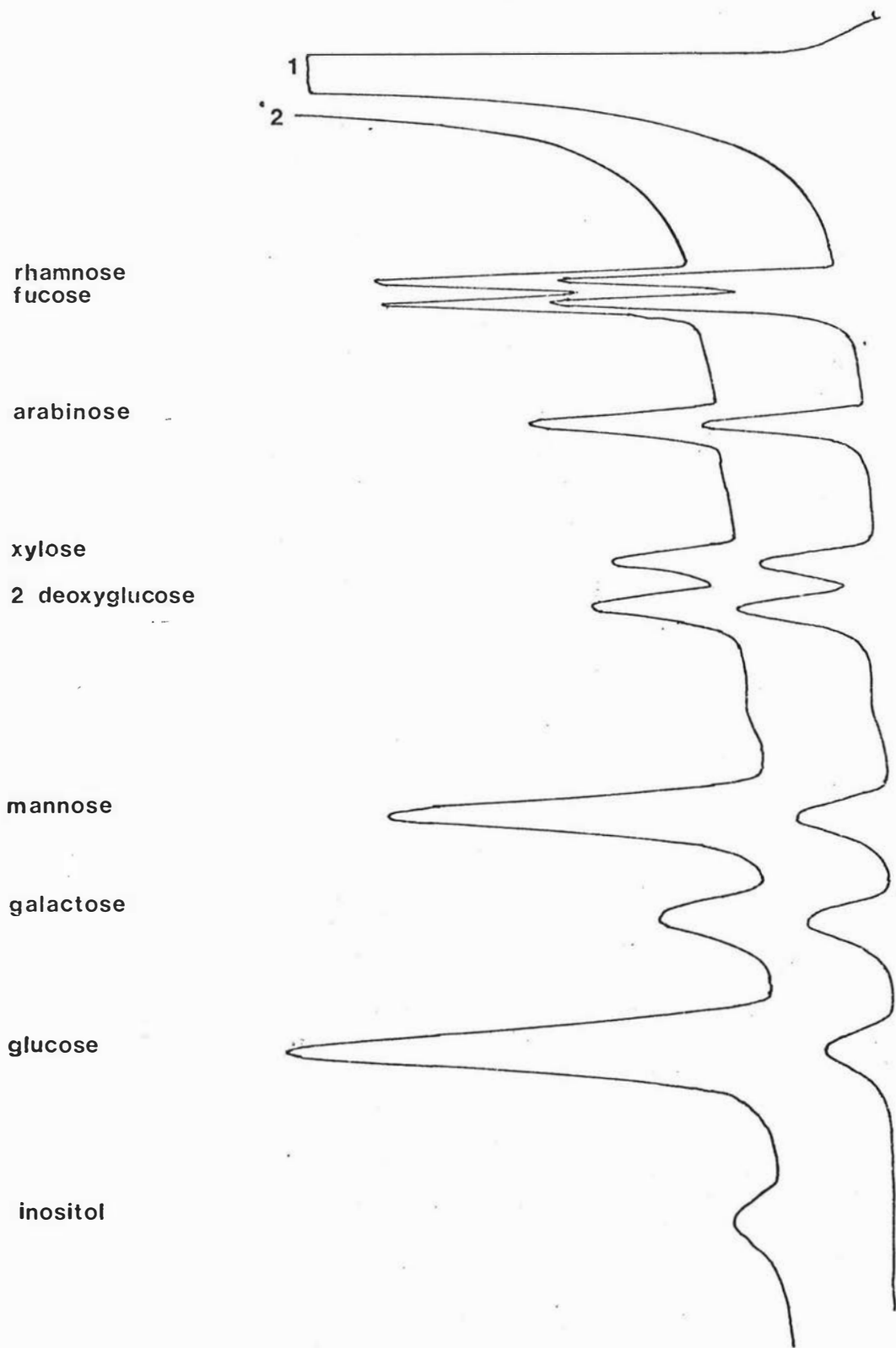
All monosaccharides in the sample co-chromatographed with sugars in the standard, except inositol, which was identified by co-chromatography with another standard. The ratios of monosaccharides were determined by comparison of peak heights to be 6.4 glucose : 3.2 mannose : 1 inositol.

Figure 3.1.3.1: Gas Liquid Chromatography Tracing of  
Acetylated Monosaccharides.

Samples of acetylated sugars were prepared as described (2.3.4). Tracings obtained using the Sp2340 column are shown.

1. 4  $\mu$ l standard containing equimolar proportions of all sugars.
2. 4  $\mu$ l standard plus 1  $\mu$ l sample.

**Figure 3.1.3.1**



Traces of galactose and arabinose were also present.

An acylated inositol mannoside is known to be a major constituent of membrane lipids in *P. shermanii* (Shaw and Dinglinger, 1969; Prottey and Ballou, 1968), but assuming all the inositol is present as a mannoside, there remains a considerable amount of glucose and mannose to account for. The probable source of this carbohydrate is capsular polysaccharide which has adhered to the membranes during preparation. Glucose and mannose are known to be constituents of capsular polysaccharides in propionibacteria (Skogen *et al*, 1974).

#### 3.1.4: Quinone Content of Membranes

Both Schwartz (1973) and Sone (1974) have isolated the major quinone in *P. shermanii* and *P. arabinosum* and identified it as (II, III)-tetrahydromenaquinone-9 by use of chromatography, UV absorption spectrometry and mass spectrometry. Concentrations reported were 4-7 nanomoles per mg protein (Sone, *ibid*) or 18.3 manomoles per mg protein (Schwartz, *ibid*). Absorption maxima in iso-octane were at 243, 248, 260 and 269 nm. Schwartz noted a fluorescent spot running in the position of ubiquinone on a thin layer chromatogram of his chloroform-methanol extract, but stated that it comprised less than 10% of the total quinone.

Quinones were extracted from freeze-dried membranes with eight successive 5 ml portions of sodium-dried n-pentane by grinding in a Duall tissue grinder. The combined n-pentane extracts were vacuum evaporated to dryness, resuspended in n-pentane and scanned over the UV

region against an n-pentane blank in a double beam spectrophotometer. Figure 3.1.4.1 shows an ultraviolet absorption spectrum of such an extract. This spectrum corresponds to that reported by both Sone (1974) and Schwartz (1973) except for the high absorbance below 240 nm. Using a millimolar extinction coefficient of menaquinone at 249 nm of 18.0 in iso-octane and assuming that the sample in Figure 3.1.4.1 is pure, the menaquinone content of membranes is calculated as 16.1 nanomoles per mg protein. This agrees well with the value of 18.3 nanomoles per mg protein quoted by Schwartz and Sporckenbach (1975).

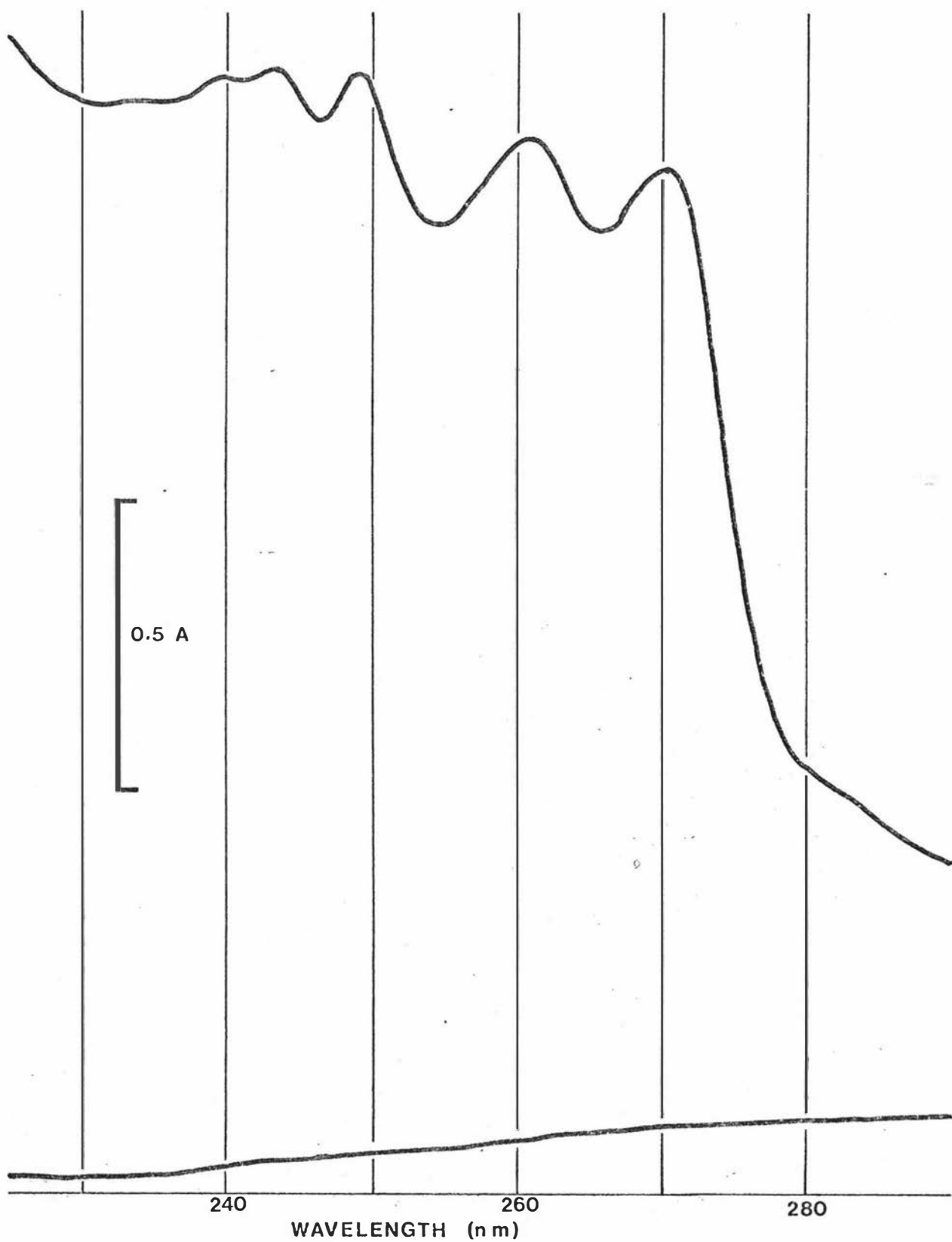
#### 3.1.5.1: Cytochrome content of *P. shermanii* membranes

The cytochrome content of propionibacteria has been described by Sone (1972), Schwartz and Sporckenbach (1975) and deVries *et al* (1972). Sone reported only a cytochrome b peak in dithionite reduced minus oxidized spectra of membranes of *P. arabinosum*. However, this published spectrum did not extend above 600 nm, so he would not have detected cytochrome d. Schwartz and Sporckenbach observed, in low temperature spectra of membranes from *P. shermanii*, the presence of cytochromes b, a, d and traces of c (based on a small shoulder at 548 nm). DeVries *et al* observed cytochromes b, a and d in *P. shermanii*, *P. freudenreichii*, *P. rubrum* and *P. pentosaceum*. The b peak was described as complex by Schwartz and Sporckenbach and was thought to contain an o type cytochrome on the basis of carbon monoxide difference spectra.

Figure 3.1.4.1: Ultraviolet Absorption Spectrum of the  
Quinone Extracted from Membranes of  
*P. shermanii*.

The sample used for measuring the ultraviolet absorption spectrum shown was prepared from 0.206 g dry weight of dried membranes which were extracted 5 times with sodium-dried n-pentane. The extract was rotary vacuum evaporated to dryness, redissolved in 10 ml n-pentane and scanned against a blank of n-pentane. The majority of absorption appears to be due to menaquinone, but there is some other ultraviolet absorbing material present which appears to increase in absorbance as the wavelength decreases. The lower line is a scan with n-pentane in the sample and reference cuvettes.

Figure 3.1.4.1





Reduced minus oxidized difference spectra of membranes obtained using dithionite, D-lactate, NADH or succinate as reductants are shown in Figure 3.1.5.1. These spectra are similar to those of Schwartz and Sporkenbach (1975), showing a shift in the absorption peak to shorter wavelengths in low temperature spectra as compared with room temperature spectra, and also in the complexity of the b peak. The low temperature spectra show that the b peak is composed of at least two b type cytochromes, and there is a slight shoulder at 548 nm in low temperature spectra (most clearly evident in the succinate reduced spectrum) which may be due to a c type cytochrome.\*

D-lactate and NADH reduce most of the cytochrome b reducible by dithionite, but succinate reduces a smaller fraction of the cytochrome b. The spectrum, Succ(LT) (Figure 3.1.5.1), indicates that succinate reduces cytochromes d, c and the b type cytochrome absorbing at about 562 nm, but has not fully reduced the b type cytochrome absorbing at 553-4 nm. This indicates that the b-type cytochrome absorbing at about 562 nm, cytochrome d and the putative cytochrome c have redox potentials above +33 mV, the potential of the succinate/fumarate redox couple (Thauer, Jungermann and Decker, 1977), whereas the b-cytochrome absorbing at 553-4 nm has a redox potential below +33 mV.

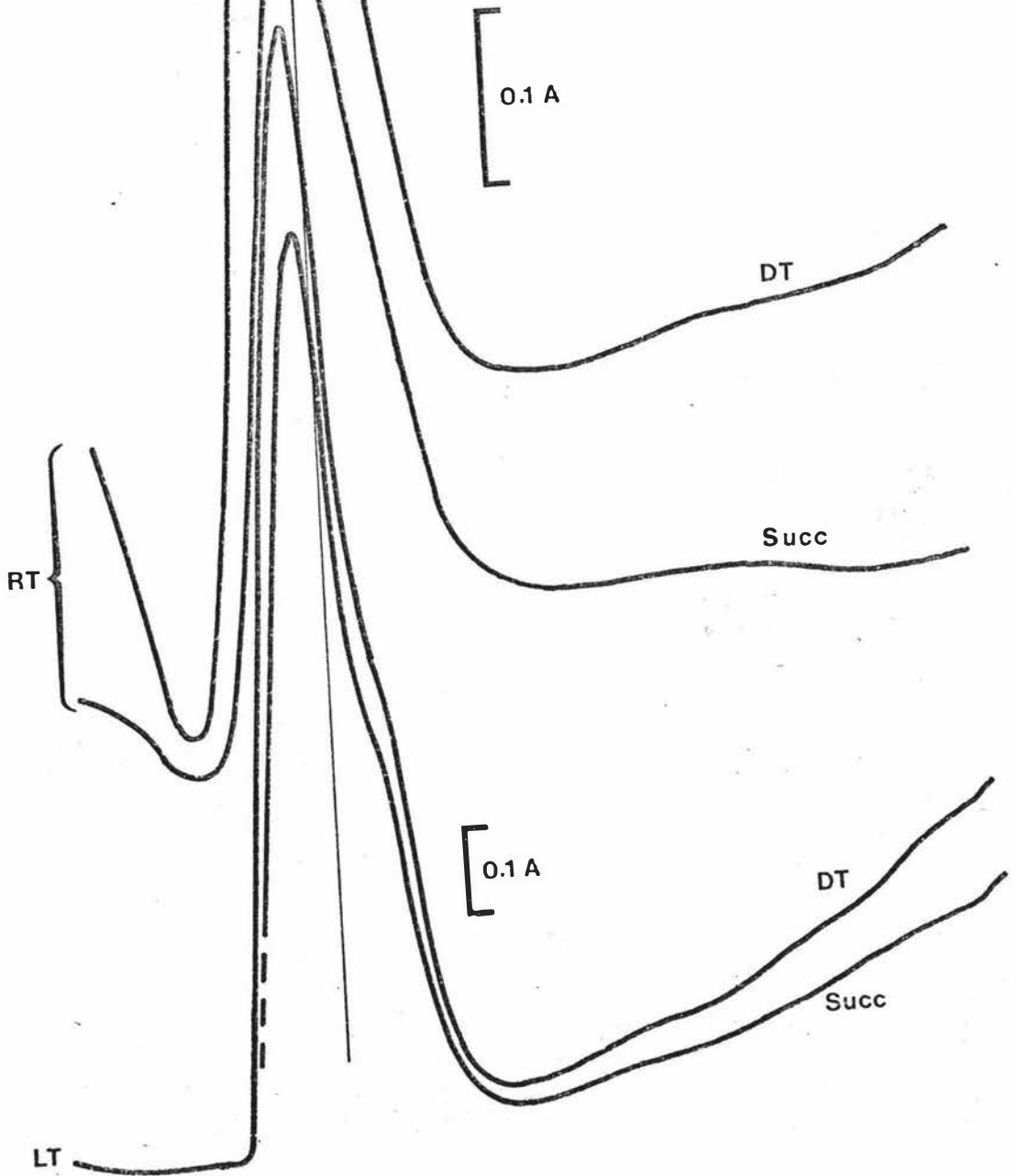
\* Note that throughout this thesis, the wavelengths specified for cytochromes are those obtained from low temperature spectra, except where otherwise specified.

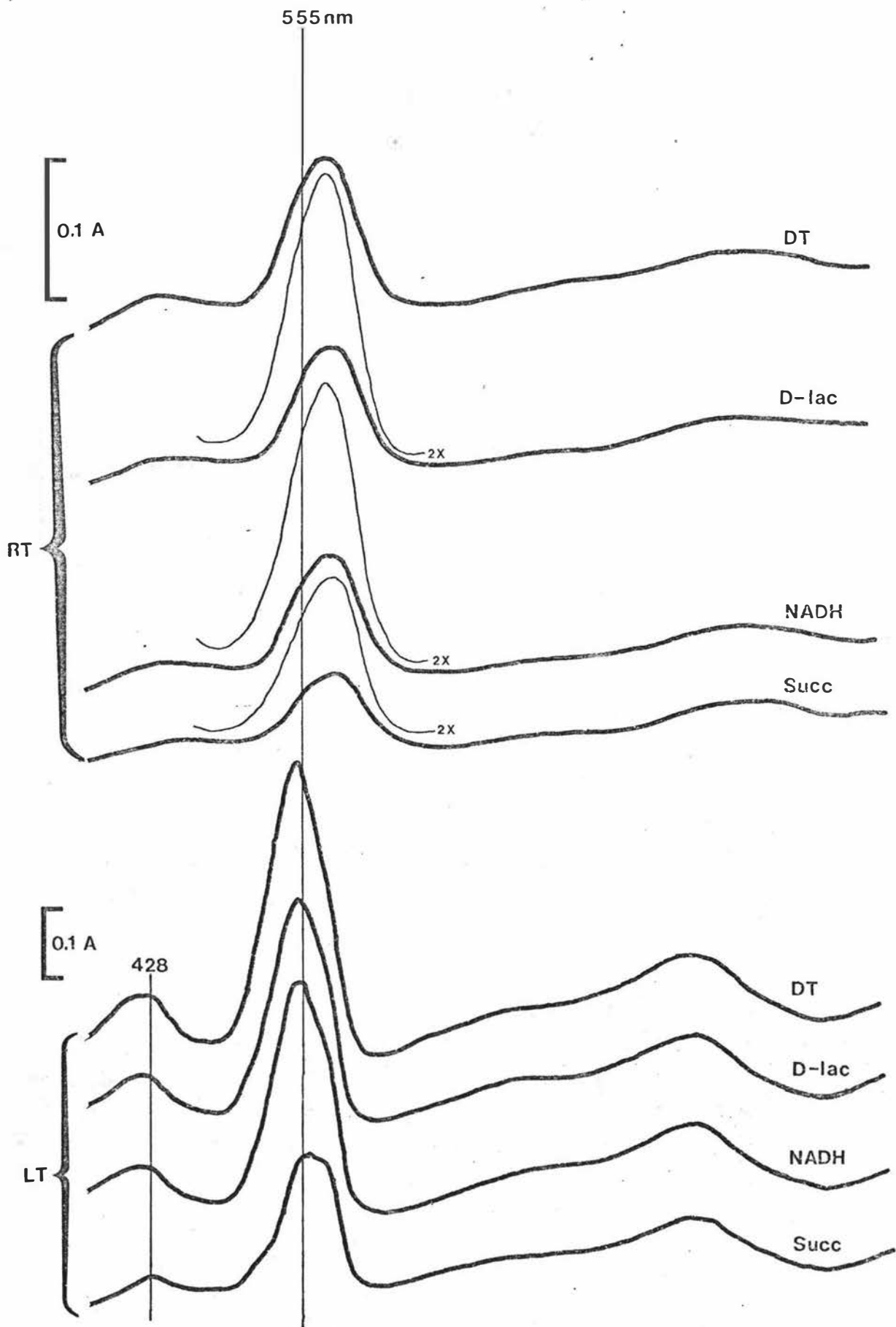
Figure 3.1.5.1: Room Temperature and Low Temperature Spectra of Dithionite and Substrate Reduced Membranes.

Membranes prepared as described were reduced with D-lactate, NADH, succinate or sodium dithionite and scanned at room temperature (RT) or at low temperature (LT) in the Shimadzu MPS 5000. The reference cuvette contained membrane suspension oxidized by addition of a small volume of  $H_2O_2$  solution. Membranes were present at a protein concentration of  $0.62 \text{ mg ml}^{-1}$ .

430nm

Figure 3.1.5.1





Castor and Chance (1959) demonstrated the presence of a cytochrome with an alpha-peak in the region of the b-type cytochromes which bound CO and acted as a terminal oxidase, which has been designated as cytochrome o. They utilized difference spectrophotometry in which the sample was reduced in the presence of CO while the reference was reduced by the same agent in the absence of CO. This gives a spectrum in which the alpha-peak of cytochrome o appears as a trough with low peaks on each side which are due to CO-bound cytochrome o. Difference spectra of *P. shermanii* membranes reduced in the presence of CO by dithionite, D-lactate, NADH and succinate minus membranes reduced by these same substrates in the absence of CO are shown in Figure 3.1.5.2. Assuming the interpretation given by Schwartz (1975) is correct, (i.e., that the trough minimum in difference spectra of S + CO/S reflects the peak absorbance of reduced cytochrome o), the cytochrome o of *P. shermanii* corresponds to a b-type cytochrome which has an alpha peak at 555-557 nm. However the  $\lambda_{\min}$  of the CO-induced trough may be influenced by the absorbance peaks due to CO-bound cytochrome.

Pyridine hemochromogen derivatives of the cytochromes were prepared as described in section 2.4.5. The absorption spectrum of these hemochromogens is shown in Figure 3.1.5.3. Alpha peaks for b-type heme and d-type heme are present at 556 nm and 649 nm, respectively. Using the extinction coefficient of heme b at 556 nm (Table 2.4.4.1), a concentration of 0.665 nmoles heme b per mg protein is estimated.

Figure 3.1.5.2: Carbon monoxide difference spectra of membranes.

Membranes (final concentration of 0.7 mg protein per ml) were placed in butyl rubber stoppered vials and one of each of four pairs was injected with 5 ml of carbon monoxide gas. After 15 min, reductant was added and 30 min was allowed for the sample to reduce. Sample reduced in the presence of CO was placed in the sample beam cuvette while that reduced in the absence of CO was placed in the reference beam cuvette. DT refers to dithionite.

Figure 3.1.5.2

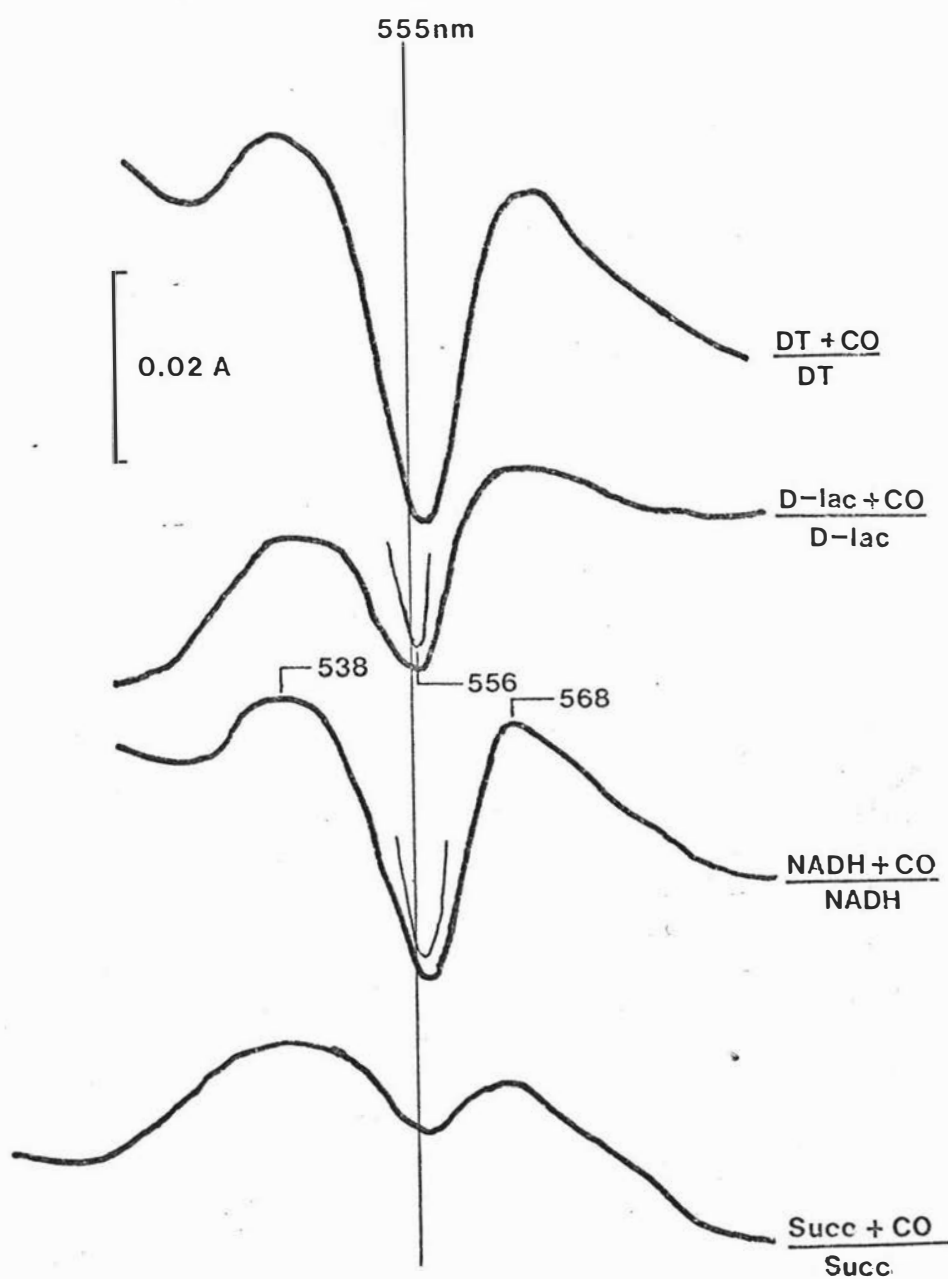
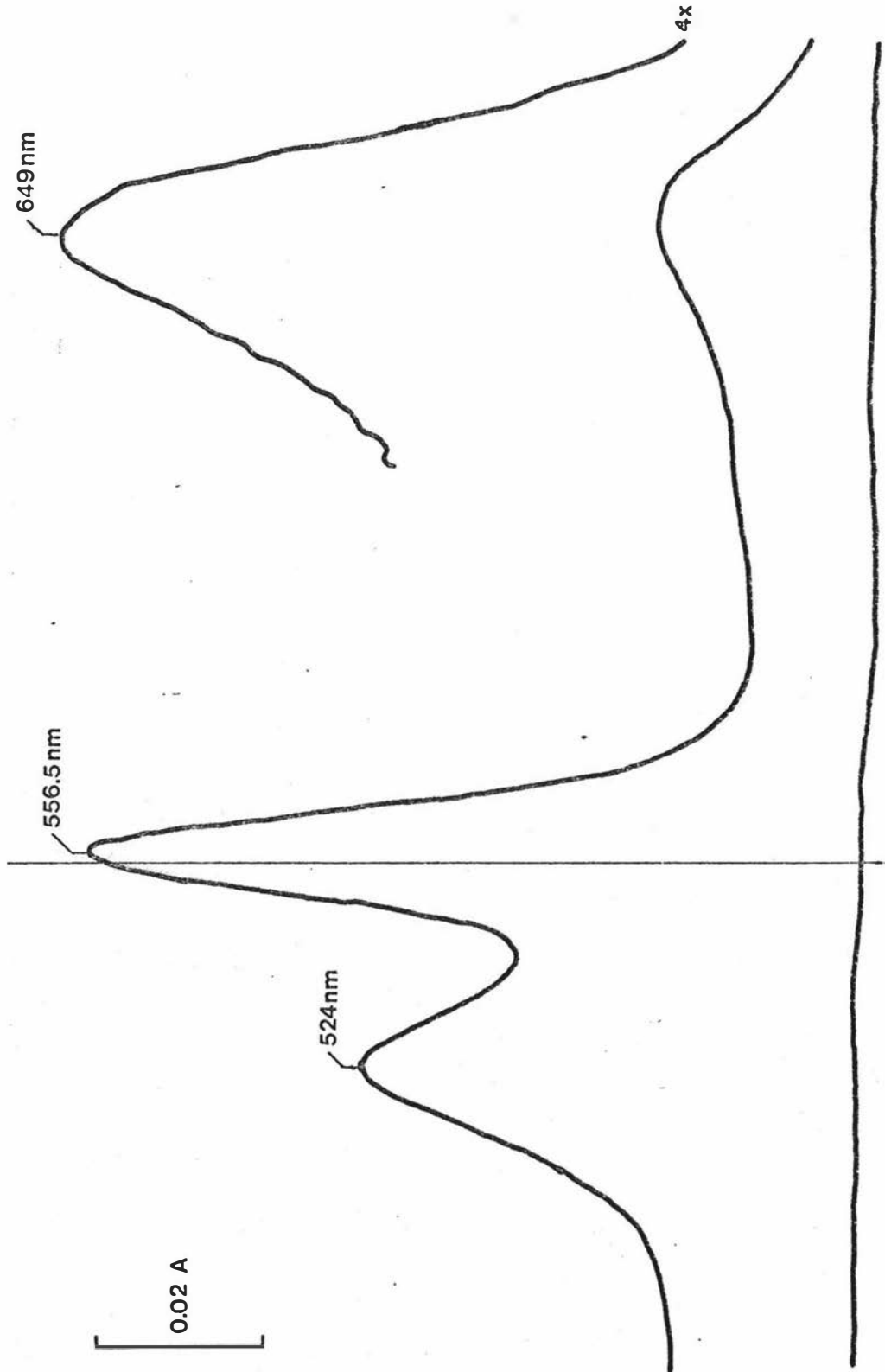


Figure 3.1.5.3: Spectra of Pyridine Hemochromogens

The visible absorption spectrum of pyridine hemochromogens prepared from membranes of *P. shermanii* (7.0 mg protein per ml) as described in 2.4.5. is shown. Heme d is present as indicated by a peak at 640 nm and heme b is present as indicated by a peak at 556 nm.



Figure 3.1.5.3



This compares with the value<sup>obtained</sup> by Schwartz and Sporkenbach (1975) of 1.8 nanomoles per mg protein. There was no cytochrome c hemochromogen observed in the acid acetone insoluble fraction, but this may be due to the low quantity of this cytochrome.

#### 3.1.5.2: Room temperature redox titrations

It was evident from the low temperature spectra that the b peak was composed of two or more cytochrome b components, which probably differed in midpoint redox potential. If the midpoint redox potentials of cytochrome components differ by 50 mV or more, a redox titration will distinguish between them. Redox titrations of membrane suspensions were therefore performed as described in section 2.6.2.1.

Representative difference spectra of the  $\alpha$ -peaks of cytochrome(s) b at known redox potentials are shown in Figure 3.1.5.4. From a whole series of these scans (between 20 and 25 for each titration) the differences in absorbance at the wavelengths pairs 565 nm - 575 nm\* and 559 nm - 575 nm were determined. The absorbance differences were plotted as percent maximum absorbance change versus redox potential ( $E_h$ ). Two such plots are shown in Figure 3.1.5.5, A and B.

The curve formed by plotting the absorbance difference between 559 nm and 575 nm shows two clearly separable components with approximate midpoints of +80 mV to +100 mV and -35 mV to -40 mV. The high redox potential fraction of

\* Note that wavelengths quoted in this section refer to room temperature spectra and will be different from wavelengths used in the other sections which refer to low temperature spectra.

Figure 3.1.5.4: Room Temperature Spectra of Redox Poised Membranes

Tracings shown are scans of the b peak performed in the Cary 219 at room temperature. The redox potential of the membranes in the sample beam are shown on the right hand side of each spectrum. The reference sample was a suspension of membranes at equal concentration which were fully oxidized by addition of a few granules of potassium ferricyanide. Membranes were present at a protein concentration of  $1.8 \text{ mg ml}^{-1}$ .

Figure 3.1.5.4

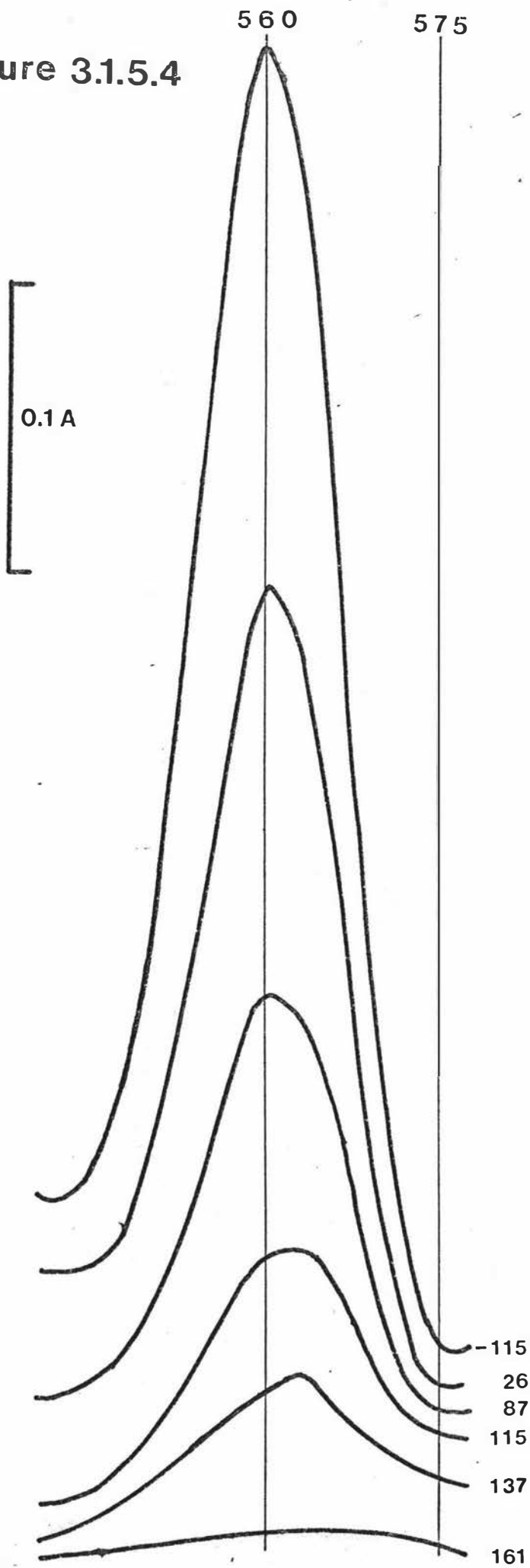
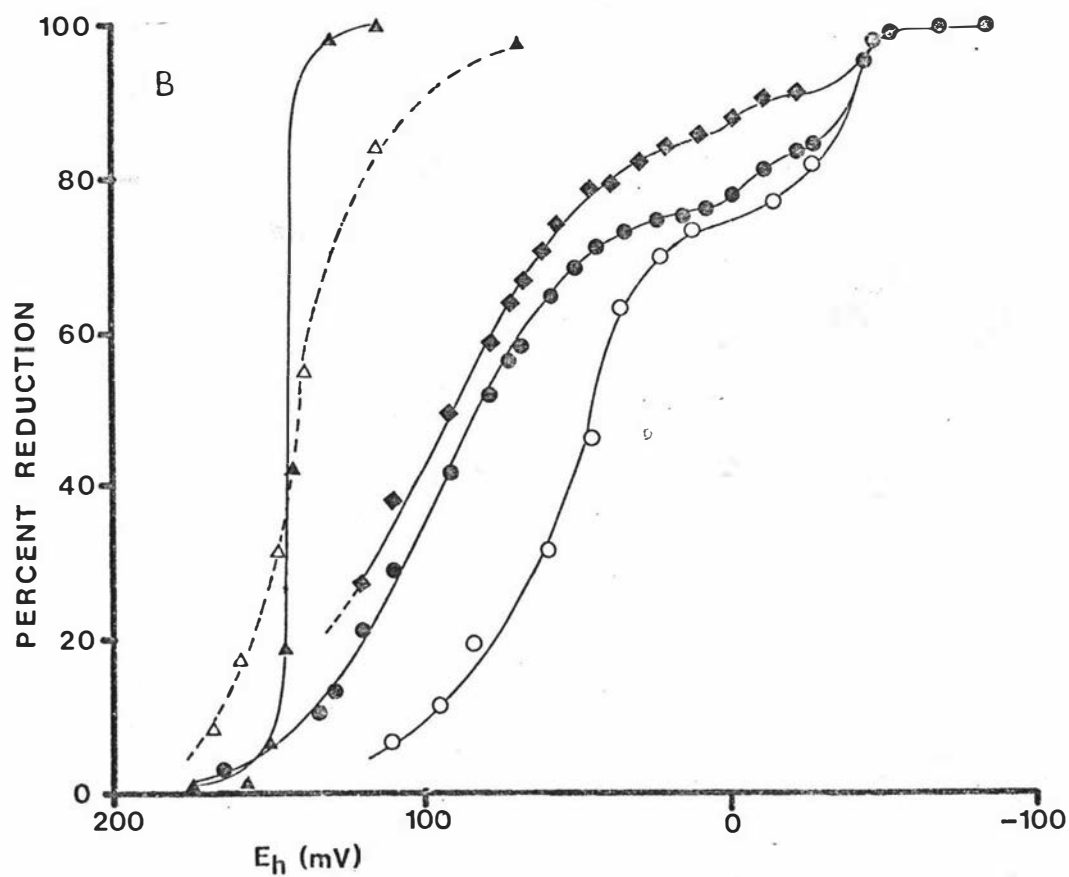
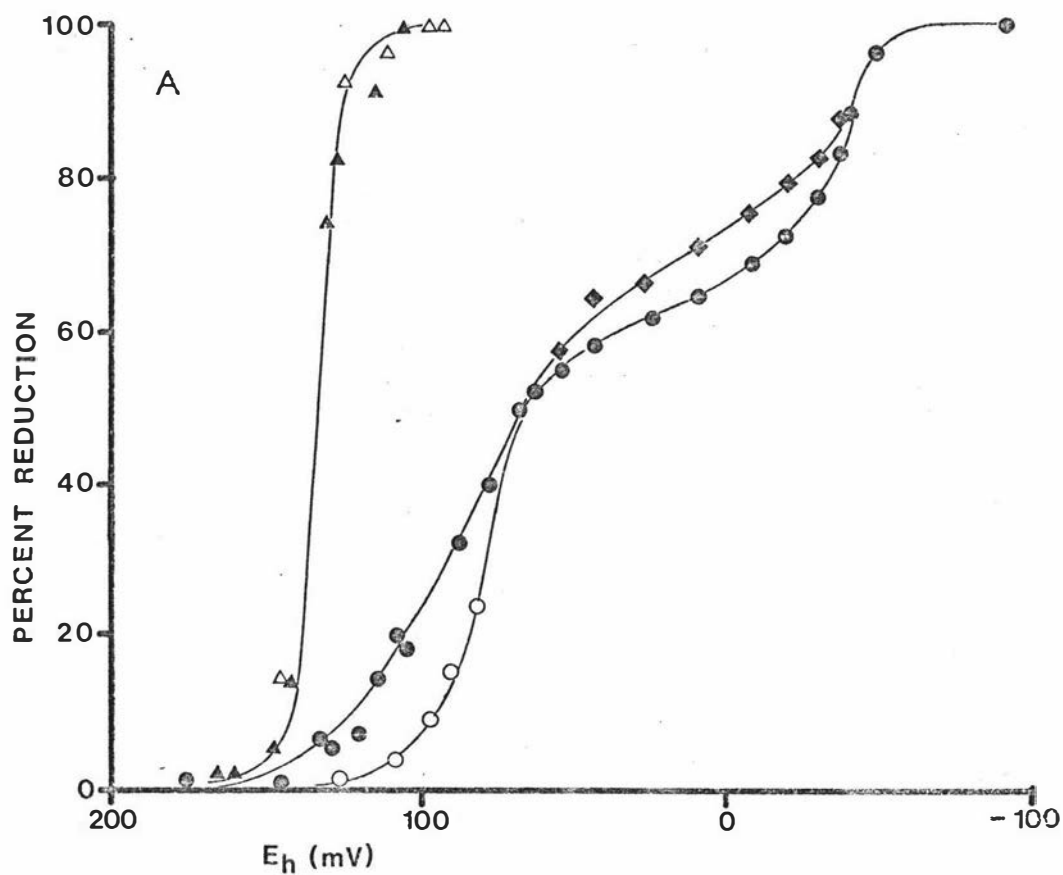


Figure 3.1.5.5: Room Temperature Redox Titrations of  
Cytochromes b and d

Absorbance differences at 559 nm - 575 nm (●, ○) at 565 nm - 575 nm (◆) for cytochrome b and the absorbance differences for cytochrome d (▲, △) as estimated by the difference<sub>at 630nm</sub> between the recorded spectrum and a straight line connecting points on the recorded spectrum at 660 nm and 605 nm. Solid symbols were determined from titrations with 5% sodium dithionite and open figures were determined from titrations with 2% potassium ferricyanide. The experiments were performed as described in 2.6.2.1.

In the dithionite titration of the cytochrome b peak in 3.1.5.5, A, the values for absorbance difference at 565 nm - 575 nm (◆) over the redox range + 50 mV to -180 mV are so close to the values for 559 nm - 575 nm that the points have been omitted from the figure for clarity.

Figure 3.1.5.5



cytochrome b appears to compose 60-70% of the total cytochrome b, while the low potential fraction appears to compose 30-40% of the total cytochrome b.

The high redox potential fraction accounts for a higher proportion of the total titratable cytochrome b peak when the wavelength pair of 565 nm - 575 nm is used. This can be attributed to the contribution of a cytochrome b absorbing in the region 562-565 nm which is reduced at higher (about 100 mV) redox potentials and the decreased contribution of a component (or components) with a maximum absorption wavelength less than 559 nm which is reduced at lower potentials. This is more clearly seen in Figure 3.1.5.4, where the absorption peak shifts to a shorter wavelength as the redox potential is lowered.

From data obtained using the wavelengths 565 nm and 575 nm, there is some indication of another step between these two major groups. This appears as a small step in Figure 3.1.5.5, B at approximately -10 mV (solid squares). While this step appeared in the majority of room temperature redox titrations, it can account for no more than 5% of the b cytochrome.

The midpoints of the two main steps are similar in the two curves shown (Figure 3.1.5.5, A & B), however, with several other redox titrations, some variation in midpoint redox potential values was found. Nine different redox titrations were performed which were similar in shape and separation of midpoint values but differing somewhat in the actual values estimated for the midpoints (Table 3.1.5.1). Back titrations of membranes reduced with dithionite were performed using ferricyanide as oxidant in

two instances and these both showed marked hysteresis (Figure 3.1.5.5). Hysteresis between reducing and oxidizing titrations has been attributed to non-establishment of equilibrium between the platinum electrode and the cytochrome components, which may be due to inadequate concentrations of mediators.

The variability in midpoint values estimated from reductive titrations and the observation of hysteresis with reverse titrations means that accurate midpoint potentials cannot be assigned. However, it is clear from these titrations that there are at least two major cytochrome b components with redox potentials of the order of -25 mV and +90 mV (Table 3.1.5.1) and possibly a very minor component with a midpoint potential in between these, present in membranes of *P. shermanii*.

### 3.1.5.3: Low temperature spectra of redox-poised membranes

The greater resolution of low temperature spectrophotometry allows finer distinction between cytochromes with closely adjacent absorbance maxima than is possible using room temperature spectrophotometry. Low temperature spectrophotometry also permits the investigation of effects of incremental changes in the redox potential by determining the difference between frozen samples containing membranes poised at different redox potentials. The resulting difference spectrum shows the amount of cytochrome reduced between the known potentials, eliminating any reduction which has occurred at potentials higher than that of the reference sample.



Membrane suspensions in 50% glycerol were poised at known redox potentials, frozen in liquid air and difference spectra measured in a Shimadzu MPS spectrophotometer, as described in sections 2.6.1 and 2.6.2.2. In each experiment, samples were taken at as many as ten different redox potentials. The first sample was normally fully oxidized ( $E_h = +180$  mV or greater) and the redox potential was progressively lowered until ten samples covering the desired range of redox potentials had been collected.

Nineteen separate redox titrations were performed by this method. Some of these encompassed the full range of redox potentials shown by the room temperature titrations to include all of the cytochromes, i.e., an  $E_h$  range of +200 mV to -100 mV. Some experiments covered narrower redox ranges in order to distinguish between b-type cytochromes with midpoint potentials differing by less than 50 mV.

A set of spectra covering the full range of redox potentials (from +304 mV to -121 mV) is shown in Figure 3.1.5.6. In these spectra, the reference sample was fully oxidized. Cytochrome d was fully reduced at +147 mV and there appeared to be no further increase in peak height at 630 nm as the redox potential was lowered below this value. The Soret peak for cytochrome d appears to be at about 436 nm. This contrasts with the statement by Schwartz and Sporkenbach (1975) that a shoulder in their spectra at 419 nm is due to cytochrome d. The position of the cytochrome d Soret absorption appearing as a shoulder at about 440 nm in the spectra shown in Figure 3.1.5.6, B corresponds reasonably well with that reported for cytochrome d in

*E. coli* (442 nm (Pudek and Bragg, 1974)).

In the cytochrome b region there are clearly two components in the peak in the scan 147 mV/304 mV of Figure 3.1.5.6 with alpha peak absorbance maxima at 562-3 nm and about 556-7 nm. As the redox potential of the sample was lowered (114 mV/304 mV), the 556-7 nm component became more reduced, indicating that it has a lower redox midpoint potential than the component with peak absorbance at 562-3 nm. As the redox potential of the membrane suspension in the sample cuvette was decreased, the b peak size increased, but additional separate components cannot be identified in these spectra.

More detail can be seen when the difference spectra between each increment of redox potential are examined (Figure 3.1.5.6,B). These show that between +147 mV and +88 mV the  $b_{562-3}$  and  $b_{556-7}$  components become progressively reduced with the  $b_{562-3}$  component being more completely reduced at +114 mV than the  $b_{556-7}$  component. Both these components appeared to be fully reduced at +88 mV, since there is no difference between +80 mV and +88 mV.

Between +24 mV and +80 mV, a small asymmetric peak appeared which may possibly be due to a further component or components since the adjacent spectra (+80 mV minus +88 mV and -3 mV minus +24 mV) show no b peak. However, the observed peaks are small and could be due to minor changes of the redox potential when the samples were collected. This is the probable explanation of the negative peak observed in the spectrum 24/57 (Figure 3.1.5.6,B). Between -3 mV and -121 mV, two further components with approximate absorbance peaks at 556-7 nm and 553-4 nm become reduced.

The b type cytochromes can thus be divided into two major groups, a high potential pair,  $b_{562-3}$  and  $b_{556-7}^{HP}$ , with midpoint potential lying between +88 mV and +304 mV, and a low potential pair,  $b_{553-4}$  and  $b_{556-7}^{LP}$ , with midpoint potentials lying between -3 mV and -121 mV (Figure 3.1.5.6,C). These two groups correspond to the two major steps observed in room temperature redox titrations. Two minor components with midpoint potentials between these two may be present, but the evidence for these is less convincing.

The  $\beta$  and Soret peaks of the b type cytochromes have peaks at 529 nm and 426 nm, respectively, except for  $b_{553-4}$  for which the peaks are about 5 nm less for both. It is assumed that the shoulder at 421 nm is the Soret peak of the component designated  $b_{553-4}$ , since both peaks appear over the same redox potential range. This Soret peak wavelength is close to that of c type cytochromes (417 to 419 nm) but the pyridine hemochromogen evidence suggests that there is insufficient cytochrome c to account for the absorbance at 421 and 553-4 nm, so this component is regarded as a b type cytochrome. The minor cytochromes, i.e., the ' $c_{548}$ ' and the ' $a_{595}$ ' detected as small shoulders on the low wavelength side of the b and d peaks respectively, are not clearly resolved in the redox titrations.

Another redox titration is shown in Figure 3.1.5.7. Although the redox potentials appear slightly higher, a similar result to the above is obtained. Cytochrome  $b_{562-3}$  becomes reduced first, followed closely by  $b_{556-7}^{HP}$ . The other two cytochromes,  $b_{556-7}^{LP}$  and  $b_{553-4}$ , are reduced at lower potentials, between -126 mV and +78 mV. Between the

Figure 3.1.5.6: Low Temperature Spectra of Redox-Poised Membranes

Membranes were prepared as described in 2.6.2.2. Spectra are identified as  $E_h$  (Sample)/ $E_h$  (Reference), where  $E_h$  is in mV. Membranes were present at a protein concentration of  $1.5 \text{ mg ml}^{-1}$ .

- A. Difference spectra of redox poised membranes versus oxidized membranes.
- B. Difference spectra of redox poised membranes versus membranes poised at the nearest higher potential.
- C. Difference spectra of redox-poised membranes showing the major redox groups of cytochromes.

FIGURE 3.1.5.6

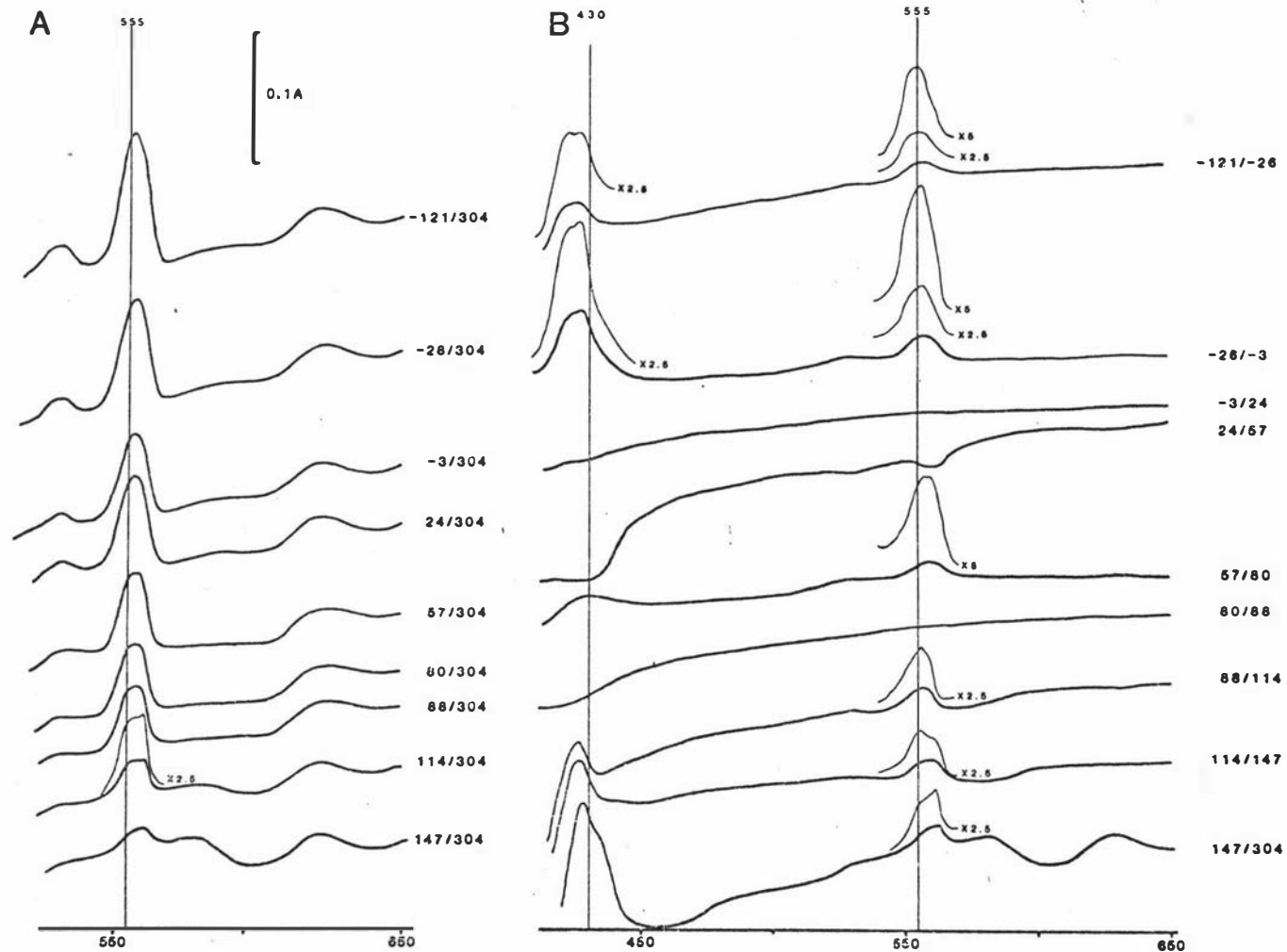


Figure 3.1.5.6,C

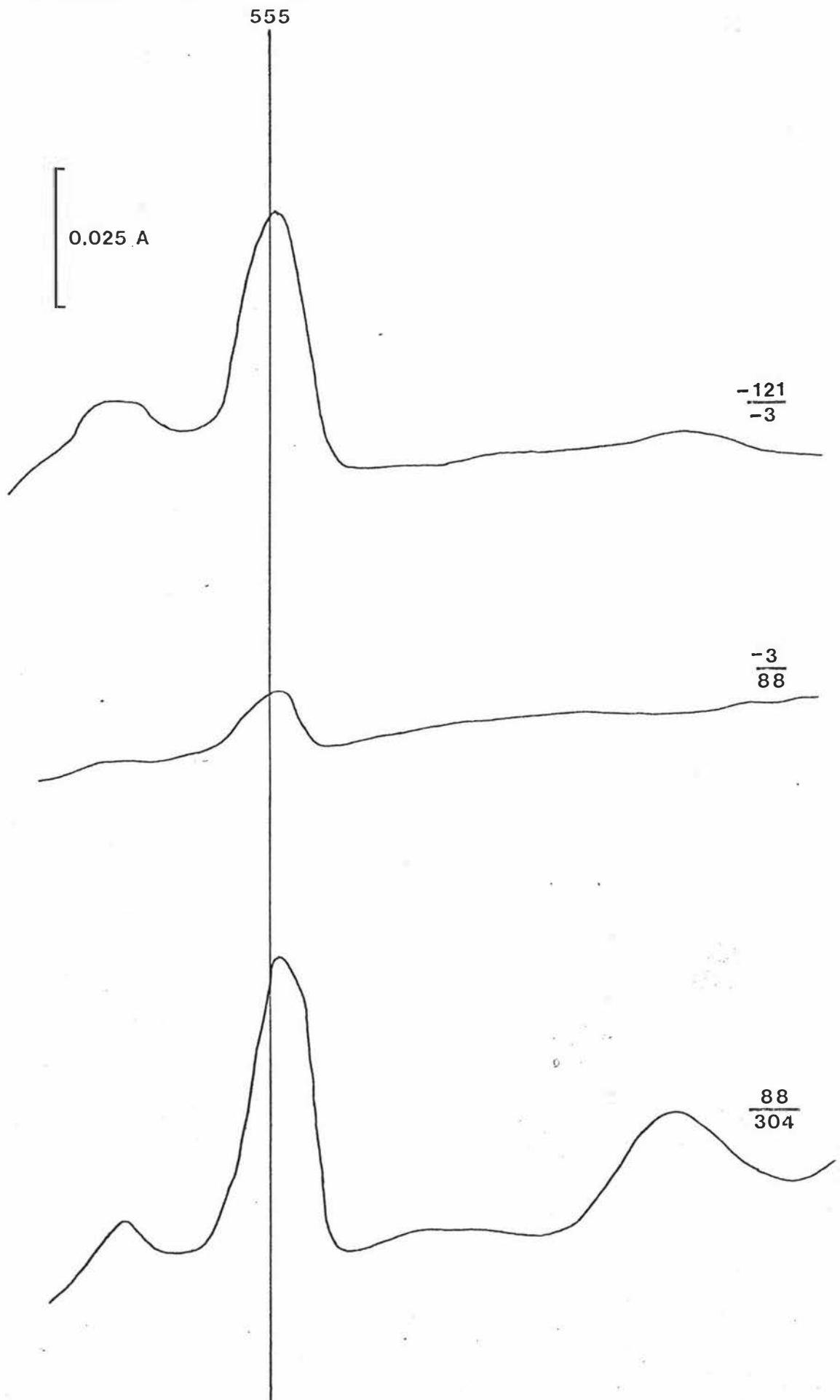


Figure 3.1.5.7: Low Temperature Spectra of Redox-  
Poised Membranes

Spectra were prepared as described in 2.6.2.2. Spectra are designated as  $E_h$  (mV, sample cuvette/ $E_h$  (mV, reference cuvette). Membranes were present at a protein concentration of  $6.0 \text{ mg ml}^{-1}$ . The solvent was wholly aqueous.

- A. Spectra of membranes poised at various  $E_h$  versus oxidized membranes ( $E_h = 244 \text{ mV}$ ).
- B. Spectra of membranes reduced between increments of redox potential.

FIGURE 3.1.5.7

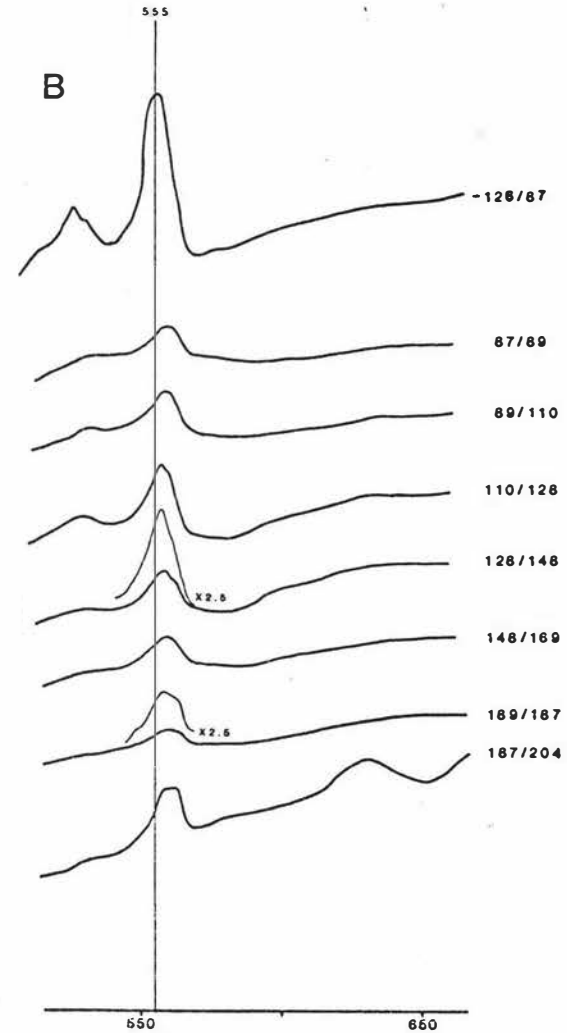
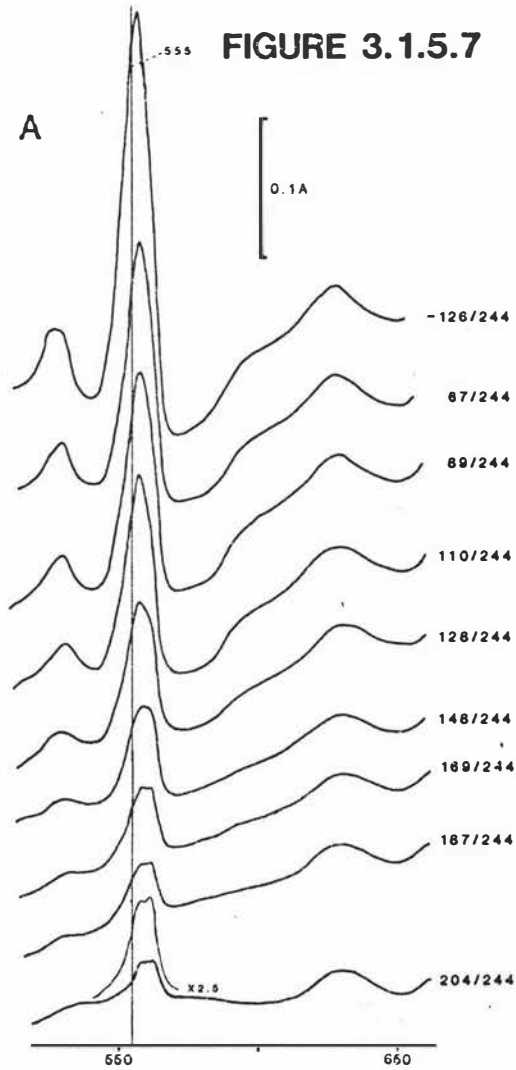


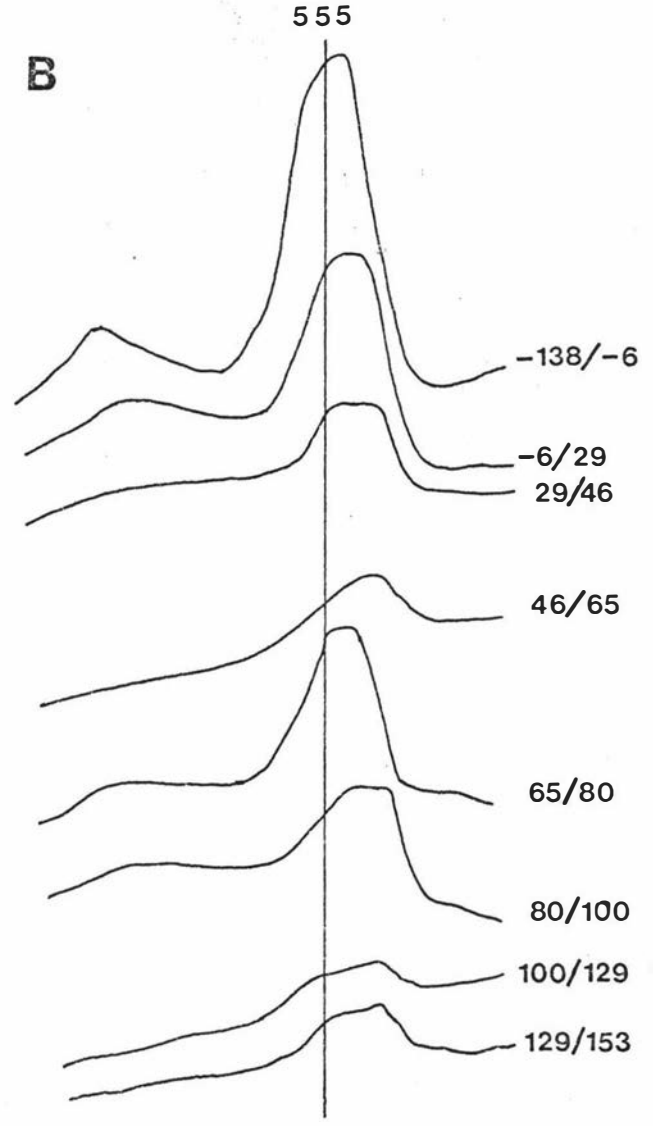
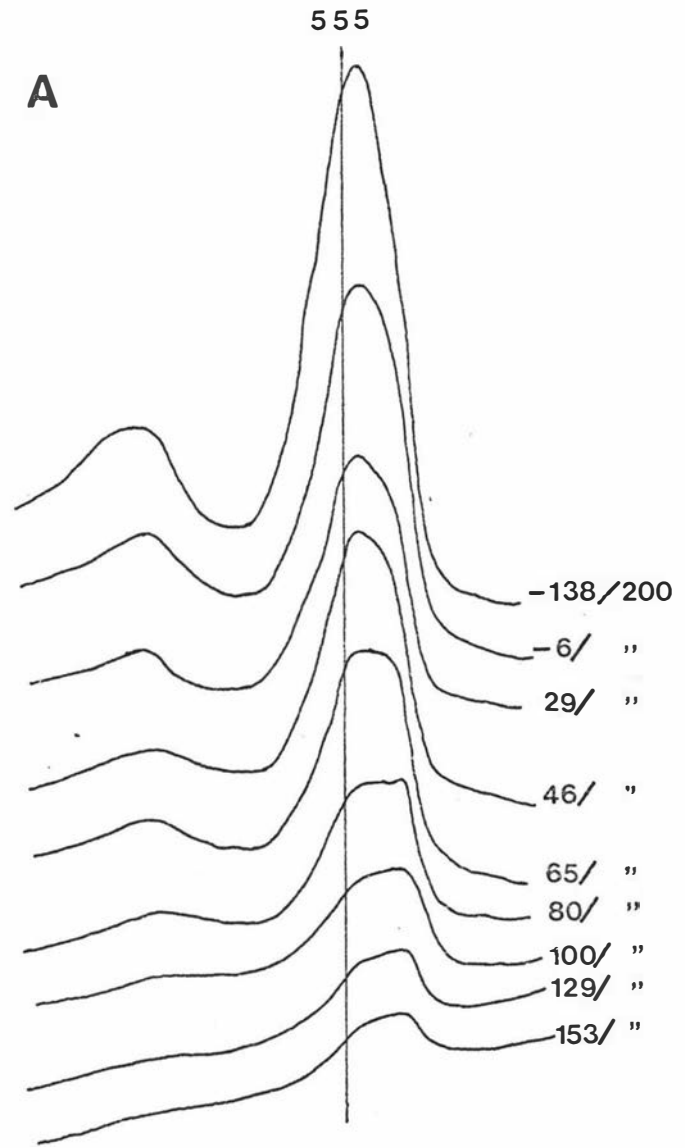


Figure 3.1.5.8: Low Temperature Spectra of Redox-Poised  
Membranes

Spectra were prepared as described in 2.6.2.2. Spectra are designated as  $E_h$  (mV, sample cuvette)/ $E_h$  (mV, reference cuvette). The membranes were suspended in buffer without glycerol.

- A. Spectra of membranes poised at various  $E_h$  versus oxidized membranes ( $E_h = 200$  mV).
- B. Spectra of membranes reduced between increments of redox potential.

Figure 3.1.5.8



two high redox potential cytochromes and the two low potential cytochromes there is a small b peak with alpha peak absorption between 557 nm and 560 nm (spectra 67/89 and 89/119, Figure 3.1.5.7,B), which may indicate the presence of an additional minor b component. Cytochrome d is fully reduced by 187 mV, indicating a higher midpoint potential than that indicated by room temperature redox titrations (+150 mV).

A further experiment covering the full range of potentials is shown in Figure 3.1.5.8. Here the midpoint potentials estimated more closely approximated the average values (Table 3.1.5.1), with two b type cytochromes ( $b_{562-3}$  and  $b_{556-7}^{HP}$ ) showing midpoints near +100 mV and two b type cytochromes ( $b_{556-7}^{LP}$  and  $b_{553-4}$ ) showing midpoints slightly below 0 mV. The spectra 46/65 and 29/46 give a slight indication that there may be small amounts of b cytochrome with intermediate redox potentials (about +40 mV) and alpha peaks between 557 and 560 nm.

Titrations over the high potential cytochrome region ( $E_h$  greater than +60 mV) showed that the two cytochromes in this region differ in redox midpoint by 20 to 30 mV with the  $b_{562-3}$  component showing the higher midpoint. The pair of cytochromes at lower redox potentials also titrated separately, but showed a much narrower separation of midpoints.

In summary, redox titrations at room temperature and low temperature scans of redox poised membranes have indicated the presence of four major b cytochrome in membranes of *P. shermanii*. The characteristics of these

cytochromes b and of cytochrome d are shown in Table 3.1.5.1. The redox titrations did not provide any clear cut evidence for c or a type cytochromes, since these peaks are too small to be detected in the spectra of redox poised membranes.

Table 3.1.5.1: Absorption Maxima and Approximate Midpoint Potentials of Cytochromes of *P. shermanii*.

Cytochrome	Absorption Maximum (nm)			Midpoint Potential (mV)
	alpha	beta	Soret	
d	630	-	436	+150
b <sub>562-3</sub>	562-3	529	429	+120(+ <u>21</u> ) (a)
b <sup>HP</sup> <sub>556-7</sub>	526-7	529	429	+88(+ <u>22</u> )
b <sup>LP</sup> <sub>556-7</sub>	556-7	529	429	-23(+ <u>16</u> )
b <sub>553-4</sub>	553-4	525	421	-23(+ <u>16</u> )

(a) Figures presented are the average of 8 or 9 determinations in the case of the b cytochromes. Bracketted figures are the 95% confidence limits. (Alder and Roessler, 1964).

### 3.1.6: Summary of conclusions from membrane characterization Studies

The membrane preparation used in this study was composed primarily of membrane vesicles but contained some contamination by cells and cell walls, as well as a large amount of carbohydrate, probably extracellular polysaccharide. There

was no evidence of specialization (at least with respect to electron transport activities) observed in membranes of different densities.

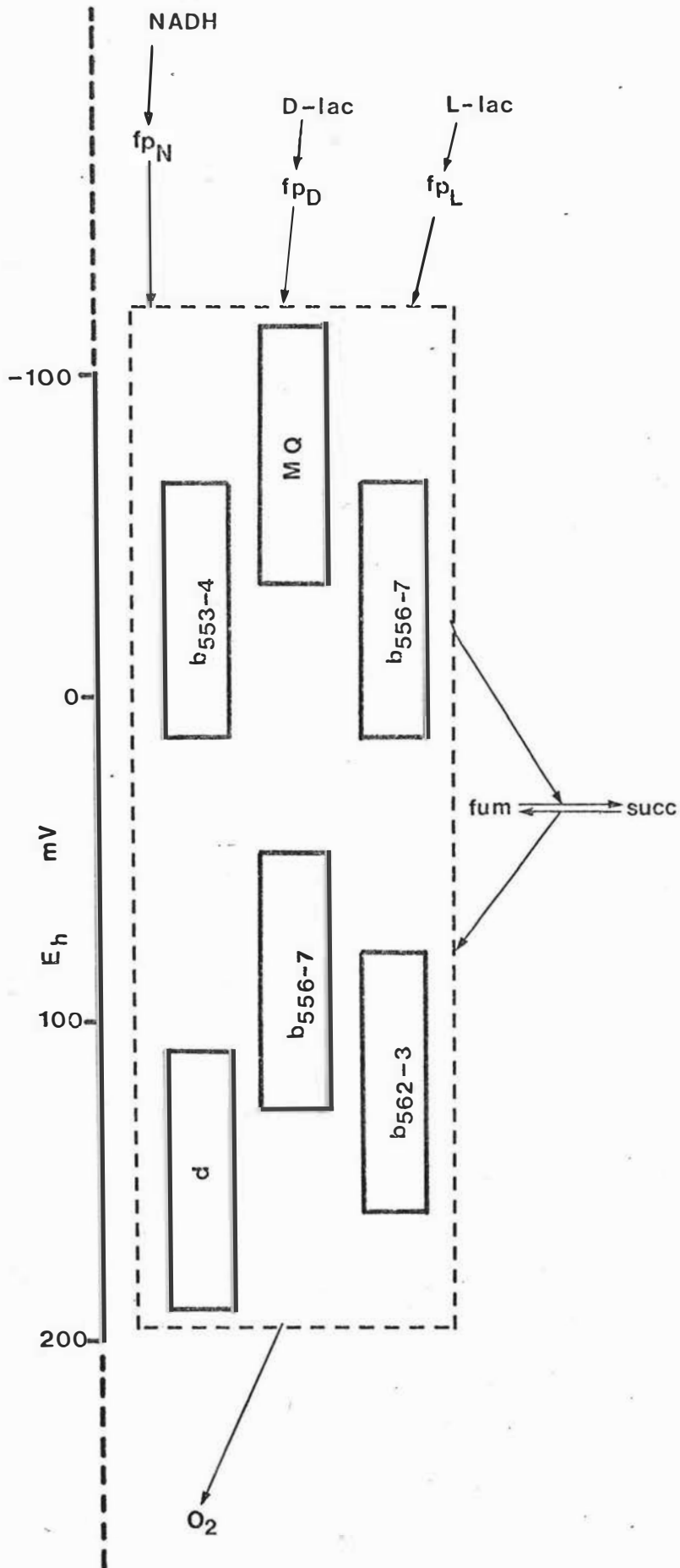
Membranes contain an electron transport system coupling oxidation of D- and L-lactate and NADH to fumarate reduction and the oxidation of these three substrates and succinate to reduction of oxygen. The specific activities of oxygen and fumarate oxidoreductase systems were of the same order. D-lactate, NADH and succinate coupled activities were relatively stable, but L-lactate coupled activities were unstable.

The artificial electron acceptors,  $\text{Fe}(\text{CN})_6$  and DICPIP, also interact with the electron transport system of these membranes but the sites of interaction differ depending on both the acceptor and the donor used. Oxygen competes strongly with the artificial acceptor in the case of L-lactate-DICPIP, L-lactate- $\text{Fe}(\text{CN})_6$  and NADH-DICPIP oxidoreductases and slightly in the case of NADH- $\text{Fe}(\text{CN})_6$  and succinate-DICPIP oxidoreductases. PMS stimulated activities with DICPIP and  $\text{Fe}(\text{CN})_6$  but did not alter competition by oxygen.

The membranes contain menaquinone, four cytochromes b and a cytochrome d. Two of the b cytochromes have redox potentials low enough to permit them to function in an electron transport system to fumarate.

These results are summarized in Figure 3.1.6.1.

Figure 3.1.6.1



### 3.2: Effects of Inhibitors on Electron Transport Activities and Reduction Level of the Cytochromes in Membranes of *P. shermanii*.

#### 3.2.1: Introduction to principles and procedures used

As described in Chapter 1, one of the principal aims of this study was to investigate the role of the cytochrome components in the electron transport system of *P. shermanii*. The main method of approach employed for this purpose was firstly to determine the effects of inhibitors or inhibitory treatments on the specific activities of membrane-bound electron transport processes, and secondly to determine the effect of these same inhibitors on the reduction levels of the cytochrome components.

Before presenting the results of the studies with inhibitors a description of the procedure used for studying the effect of inhibitors on the reduction level of cytochromes and of the cytochrome pattern in various states in uninhibited particles will be presented in the two following sections.

#### 3.2.1.1: General description of experimental procedure

The reduction level of cytochromes is most conveniently measured using a dual wavelength spectrophotometer. Since such an instrument was not available while work for this thesis was being undertaken, an alternative method was adopted. This consisted of freezing membranes in various redox states and examining the frozen membranes by means of low temperature difference spectrophotometry. The general

procedure adopted in all the experiments with inhibitors is described and discussed below, since the advantages and limitations of this approach, and the assumptions involved must be kept in mind when interpreting the results of these experiments.

A typical time-course following the reduction level of a hypothetical b-type cytochrome in a membrane suspension using a dual wavelength spectrophotometer is shown in Figure 3.2.1.1. Addition of a reducing substrate (S) to an air-equilibrated membrane suspension causes a rapid partial reduction to a level which depends on the rates at which the cytochrome is reduced by S (via the dehydrogenases, etc.) and oxidized by oxygen (via oxidases, etc.). This condition is the aerobic ( $O_2$ ) steady state and it continues until all the oxygen has been consumed. After the oxygen has been consumed, the reduction level of the cytochrome increases rapidly to the maximum level for that particular substrate. This latter level is referred to as the substrate-reduced state.

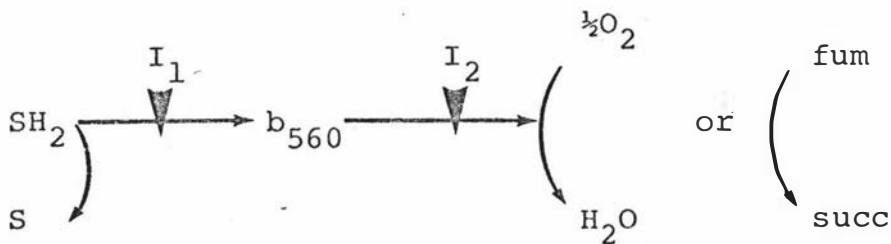
If anaerobic fumarate is added to substrate-reduced membranes, the reduction level decreases to a steady-state which is dependent on the rate of reduction by substrate and the rate of oxidation by fumarate (Figure 3.2.1.1,B). This state is referred to as the anaerobic steady-state.

If, instead of fumarate, a solution of  $H_2O_2$  is added, the reduction level decreases to the aerobic ( $H_2O_2$ ) steady-state (Figure 3.2.1.1,A).  $H_2O_2$  was used as a convenient method of rapid oxygen addition since membrane particles have sufficient catalase activity to release oxygen rapidly from  $H_2O_2$ .



Figure 3.2.1.1: Dual Wavelength Spectrophotometer Tracing of Inhibited and Uninhibited Cytochrome b Reduction

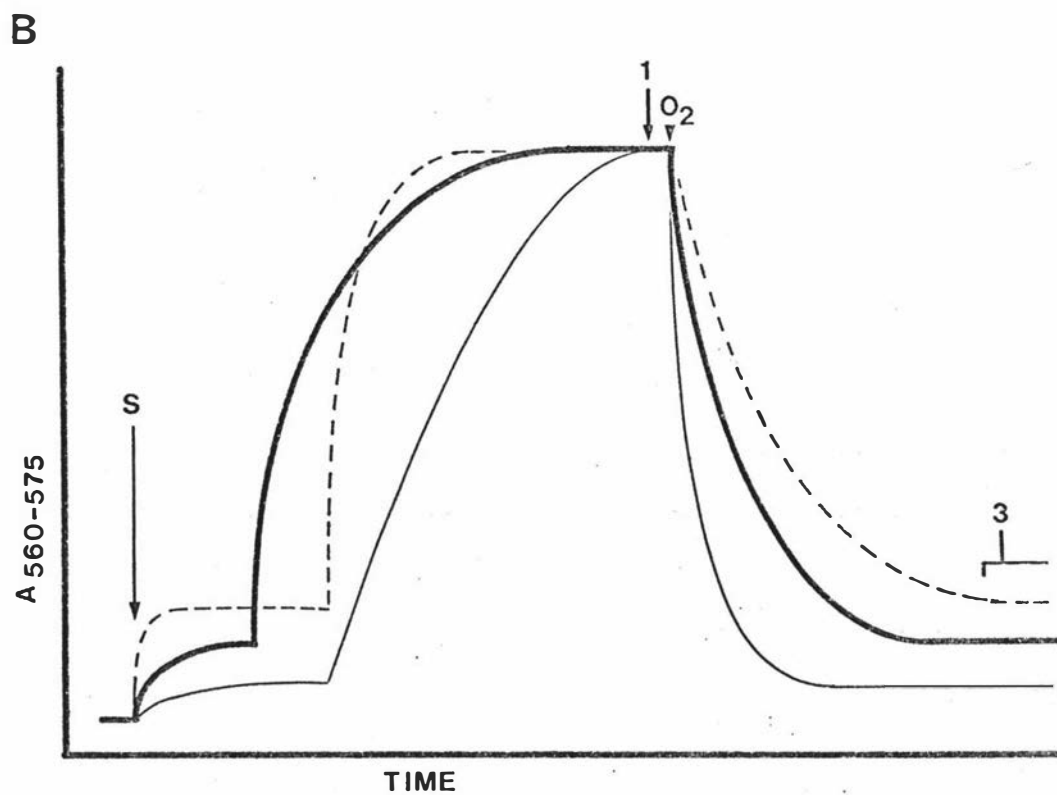
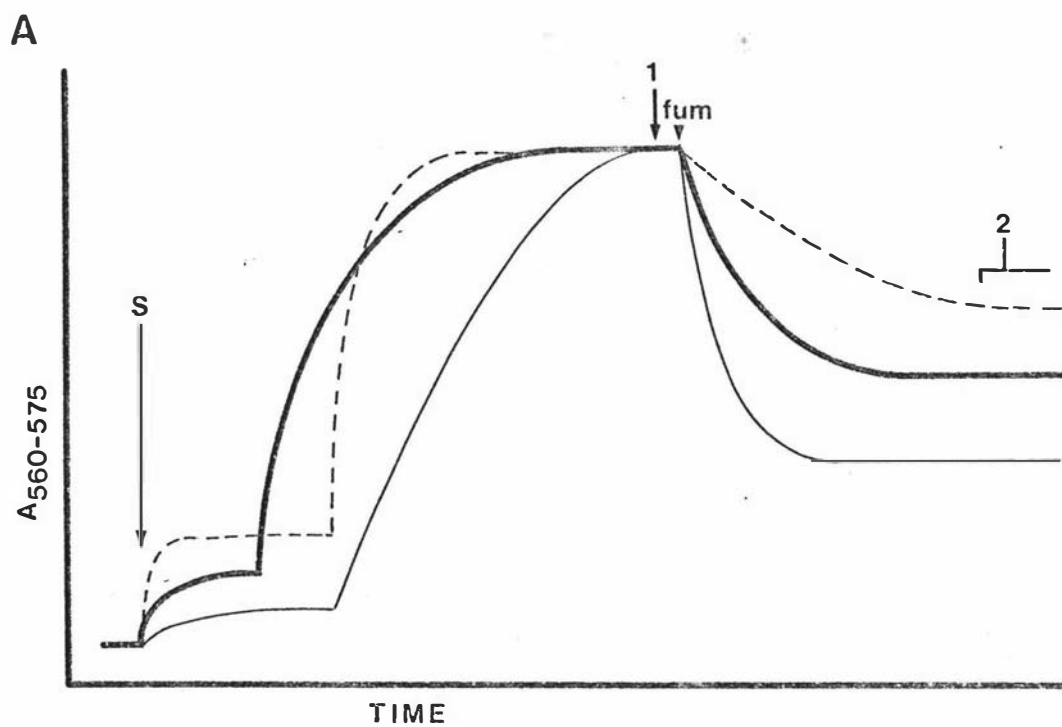
This figure presents a hypothetical dual wavelength spectrophotometer tracing of a cytochrome b which is an intermediate in the coupled oxidation-reduction reaction depicted in scheme 1.



Tracings are shown which may be expected to result, starting with a fully oxidized membrane suspension, where reduction is initiated by a reducing substrate  $\text{SH}_2$ . The three conditions are (a) no inhibitors present (—), (b) inhibitor ( $\text{I}_1$ ) inhibits reduction of cytochrome (—) and (c) inhibitor ( $\text{I}_2$ ) inhibits oxidation of cytochrome b (---). The three states examined by low temperature spectrophotometry are substrate reduced (1), anaerobic steady-state (2) and aerobic steady-state (3).

- A. Membranes reduced by  $\text{SH}_2$  and then placed in the anaerobic steady-state with fumarate.
- B. Membranes reduced by  $\text{SH}_2$  and then placed in the aerobic steady-state with oxygen (using  $\text{H}_2\text{O}_2$  as a source of  $\text{O}_2$ ).

Figure 3.2.1.1



The presence of inhibitors will alter the time course of cytochrome reduction outlined above, affecting both the rate at which the cytochrome approaches the steady-states and the substrate-reduced state, and the proportion of cytochrome reduced when in the steady-state. The change, relative to the uninhibited condition, will depend on the site of action of the inhibitor, i.e., on whether it decreases the rate of reduction or the rate of oxidation of the cytochrome.

If the inhibitor acts by decreasing the rate of reduction, it will prolong the initial aerobic steady-state as well as slow the approach to the substrate-reduced state. The level of reduction of the cytochrome will be less than that in an uninhibited control in both the aerobic and anaerobic steady-states.

If the inhibitor affects the rate of oxidation of the cytochrome, it will also prolong the initial aerobic steady-state, but will not alter the rate at which the substrate-reduced state is reached once the oxygen is removed. In this case, the steady-state level of reduction will be greater than that of the uninhibited state.

This line of reasoning forms the basis of the design of experiments described in the following sections in which the effects of inhibitors on the reduction level of cytochromes under three conditions were examined. The three conditions were the substrate-reduced state, the aerobic ( $H_2O_2$ ) steady-state and the anaerobic (fum) steady-state.

The first condition was used to determine whether the inhibitor inhibited reduction of the cytochromes. This was

done by allowing a membrane suspension in the oxidized state to equilibrate with inhibitor for 10 min at 30°C and then adding substrate to reduce the cytochrome components. The oxidized membrane suspension contained only the small amount of oxygen present in 0.1 ml of air-equilibrated concentrated membrane suspension since the other 0.9 ml of reagents (buffer plus reducing and oxidizing substrates, except H<sub>2</sub>O<sub>2</sub>) was prepared anaerobically. The time necessary to reduce the cytochromes in uninhibited membranes was determined by following the reduction of the b peak in similarly prepared anaerobic cuvettes at 30°C. In the absence of a dual wavelength spectrophotometer, this was done by manually alternating the wavelength between 560 nm and 580 nm in an SP1800 double-beam spectrophotometer. With D-lactate and NADH as reductants, it was found that the time required to reach the substrate-reduced state was only a few seconds. Samples for examination of the low temperature spectra were therefore collected one minute after addition of substrate, the minimum time to allow for mixing, transfer to low temperature cells and freezing. With L-lactate and succinate, longer periods were required and samples were collected three minutes after adding reductant. In samples treated with inhibitor, the duration of the aerobic steady-state and the time required for the cytochrome to reach the substrate reduced state may be considerably extended, so that the point on the reduction curve at which the sample was taken was not known. Thus an unambiguous location of the site of action of the inhibitor, i.e., whether it is on the reducing substrate or acceptor side of the cytochrome or not, cannot

always be obtained by this procedure by itself. Due to the very short duration of the initial steady-state in the largely anaerobic reaction mixture it is usually obvious when the inhibitor is blocking reduction. The effect of the inhibitor on the steady state reduction level gives a much more unambiguous indication of the site of inhibitor action.

Steady-state samples were prepared by first fully reducing membrane suspensions with substrate (10 min with D-lactate and NADH, and 20 to 30 min with L-lactate and succinate), then exposing one of a pair of samples to anaerobically prepared inhibitor for 10 min. An  $H_2O_2$  solution or anaerobically prepared fumarate was then added via a syringe and after 1 min of gentle mixing, the samples were removed to low temperature cells and frozen. Comparison of the height and shape of the  $\alpha$ -peak of the cytochrome spectra in the inhibited and uninhibited steady-state samples provides information on the site of the inhibitor.

Careful precautions were taken when preparing these samples to keep the membrane concentration equal under all conditions examined in a single experiment, thus making it possible to obtain difference spectra between uninhibited and inhibited steady-states and the substrate-reduced state. This possibility constitutes a major advantage over dual wavelength experiments where only a single state can be examined at a time and therefore differences between inhibited and uninhibited states cannot be directly assessed. Low temperature spectrophotometry also provides greater resolution than that of room temperature spectrophotometry, making it possible to distinguish between some of the

different b type cytochromes present in *P. shermanii* membranes.

After surveying a range of known inhibitors of electron transport processes, four inhibitors - dicumarol, HOQNO, p-chloromercuribenzoate and cyanide - were selected for a more intensive study of their effect on the rate of various electron transport processes and on the oxidation and reduction of the cytochrome components. Other inhibitors tested were antimycin A, rotenone, o-phenanthroline, oxamate and carbon monoxide. Antimycin A and rotenone produced only very small inhibition of electron transport, even at high concentration. o-Phenanthroline inhibited all electron transport processes equally strongly and therefore was not particularly useful for investigating the involvement of cytochromes in the different pathways. Oxamate, as expected, produced strong competitive inhibition of D- and L-lactate dependent activities, while leaving NADH dependent activities unaffected. Again this inhibitor was not particularly useful for investigating the regions of the electron transport pathway which were of particular interest.

The effect of CO on oxygen consumption was examined briefly. The result of this is recorded in Section 3.2.8.

HOQNO was added from stock solutions made up in DMSO (dimethyl sulfoxide). An equivalent volume of DMSO lacking inhibitor was always added to the controls in all experiments. At the levels added, the DMSO did not appear to produce any inhibition of electron transport. Dicumarol, cyanide and pCMB were added from aqueous solutions adjusted to pH 8. Cyanide solutions were stored in rubber stoppered vials after adjusting the pH to 8.

In selecting a suitable concentration for the studies of the effects of inhibitors on cytochrome oxidation and reduction a concentration found to give strong inhibition of electron transport activities was chosen in order to maximize differences between inhibited and uninhibited pathways. Since the protein concentration in the experiments on cytochrome oxidation and reduction was high and since it was considered desirable to maintain the ratio of inhibitor to membrane constant, the actual inhibitor concentrations used in the experiments are fairly high.

As well as the studies using these four inhibitors, UV irradiation was used as another means of inhibiting electron transport. The procedures used for this are fully described in section 3.2.2.

Before describing the effects of these inhibitors in detail, the spectra obtained from uninhibited membranes will first be presented and discussed.

#### 3.2.1.2: Spectra of membranes in substrate-reduced and steady-states

A selection of difference spectra prepared using membranes in the absence of inhibitors is shown in Figure 3.2.1.2. For each of the four reducing substrates used (NADH, D- and L-lactate and succinate) six difference spectra are shown:

1. The substrate-reduced membrane suspension in the sample beam and the  $H_2O_2$ -oxidized suspension in the reference beam. This is referred to as  $S/H_2O_2$ , where S is any one of the reducing substrates.

2. Anaerobic (fum) steady-state membranes in the sample beam and  $\text{H}_2\text{O}_2$ -oxidized membranes in the reference beam. This is referred to as S,fum/ $\text{H}_2\text{O}_2$ .
3. Aerobic ( $\text{H}_2\text{O}_2$ ) steady-state membranes in the sample beam and  $\text{H}_2\text{O}_2$ -oxidized membranes in the reference beam. This is referred to as S, $\text{H}_2\text{O}_2$ / $\text{H}_2\text{O}_2$ .
4. Substrate-reduced membranes in the reference beam and aerobic ( $\text{H}_2\text{O}_2$ ) steady-state membranes in the reference beam. This is referred to as S/S, $\text{H}_2\text{O}_2$ .
5. Substrate reduced membranes in the sample beam and anaerobic (fum) steady-state membranes in the reference beam. This is referred to as S/S,fum.
6. Anaerobic (fum) steady-state membranes in the sample beam and aerobic ( $\text{H}_2\text{O}_2$ ) steady-state membranes in the reference beam. This is referred to as S,fum/S, $\text{H}_2\text{O}_2$ .

Two features clearly emerge from comparison of the aerobic and anaerobic steady-states. One is that cytochrome d remains fully reduced in the anaerobic steady-state (S,fum/ $\text{H}_2\text{O}_2$ ) and is oxidized in the aerobic steady-state (S, $\text{H}_2\text{O}_2$ / $\text{H}_2\text{O}_2$ ). This indicates that cytochrome d is on an electron transport pathway to oxygen and not on the path to fumarate. This is also true for the small cytochrome a peak visible in these spectra. It should be noted that since the redox couple succinate/fumarate has a midpoint potential of +33 mV while cytochrome d has a midpoint potential of about +150 mV (Section 3.1.5.) Table 3.1.5.1), fumarate would not be expected to oxidize reduced cytochrome d.



Figure 3.2.1.2: Spectra of Membranes in Uninhibited  
Substrate Reduced and Steady-States.

Spectra of steady-state and substrate reduced membranes were prepared as described in methods and the text (Section 2.6.1, 3.2.1.1). Membrane was present at a final concentration of 0.83 mg protein ml<sup>-1</sup>. Fumarate, D-lactate, L-lactate and succinate were added, where indicated, at final concentration of 4.3 mM. NADH and H<sub>2</sub>O<sub>2</sub> were added, where indicated, at final concentrations of 2.14 mM and 0.0143%, respectively.

Spectra in this and later figures and in the text are referred to in a shorthand notation described in the following. When a particular reducing substrate (D- or L-lactate, NADH or succinate) is not specifically named, it is referred to as S. Aerobic and anaerobic steady-states are referred to as S, H<sub>2</sub>O<sub>2</sub> and S, fum, respectively. The membrane preparation in the sample beam of the double beam spectrophotometer is described first and the sample in the reference beam second with a slant (/) between the two. The order in which reagents were added is repeated by the order of this shorthand, so that S,CN, fum means the membranes were first reduced by S, then inhibitor (cyanide) was added, and finally fumarate was added, which gives the cyanide inhibited anaerobic (fum) steady-state.

- A. D-lactate
- B. L-lactate
- C. NADH
- D. Succinate

FIGURE 3.2.1.2

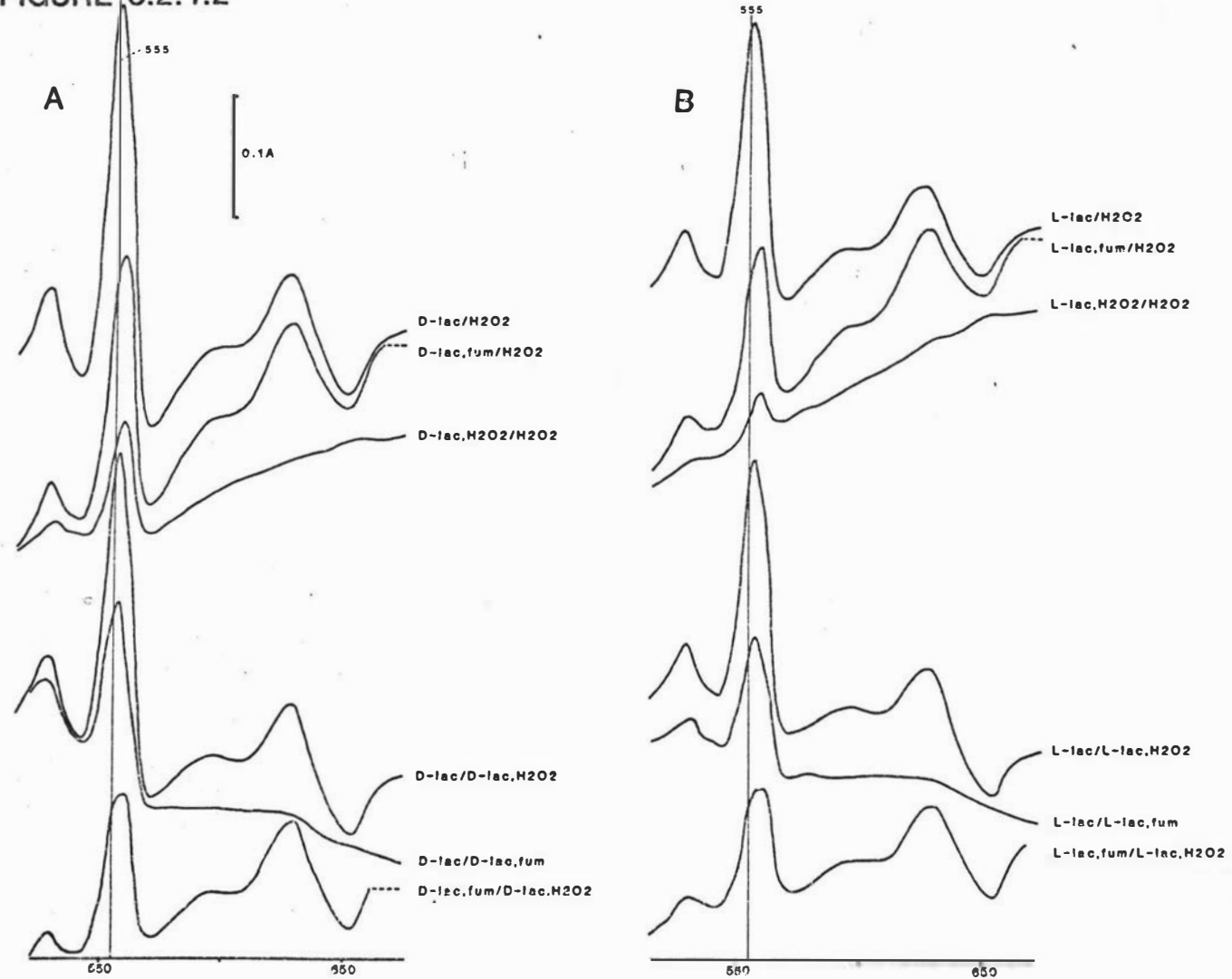
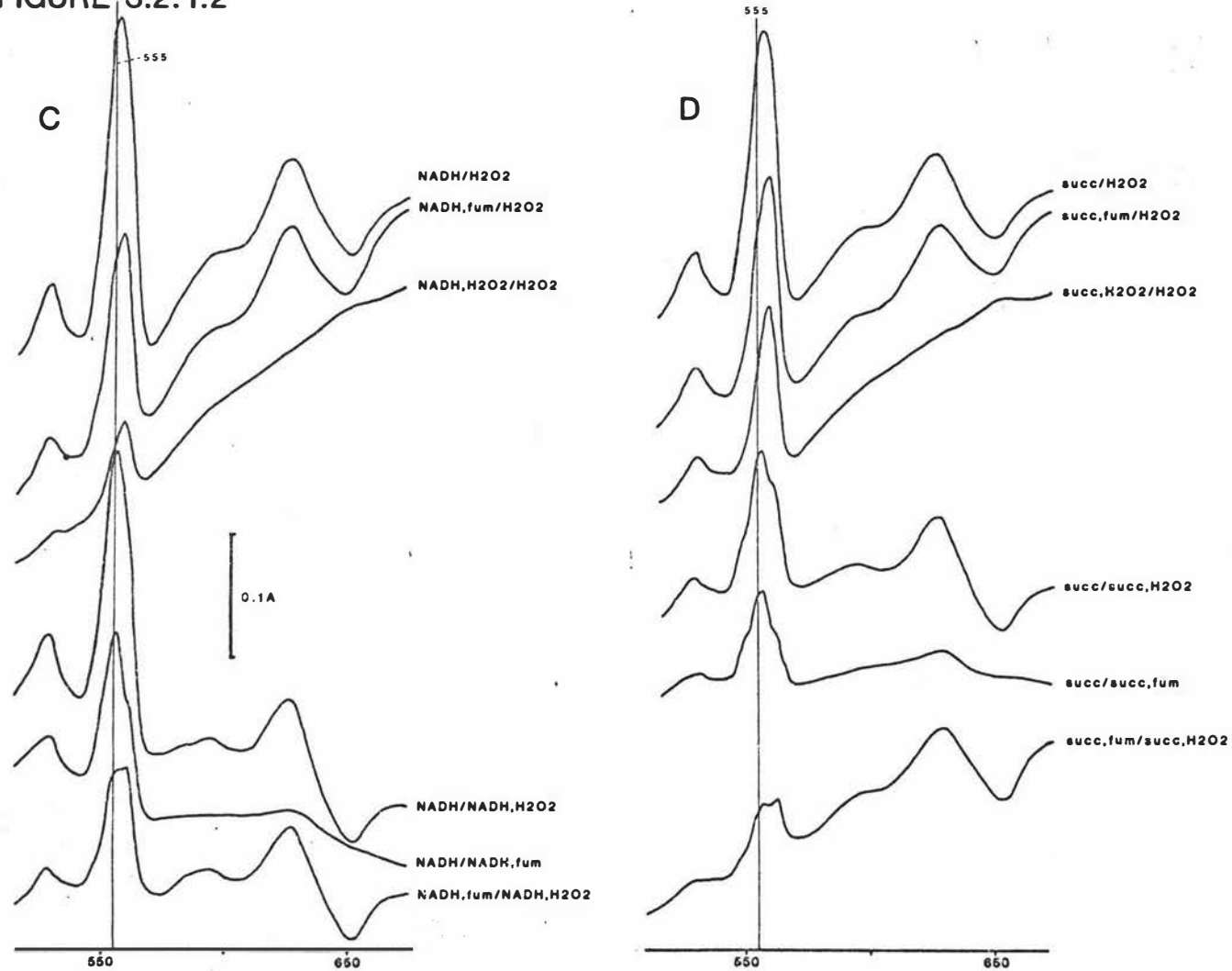


FIGURE 3.2.1.2



The second feature is that, with the exception of succinate, the reduction level of the cytochromes in the aerobic steady-state is much less than that of the cytochromes in the anaerobic steady-state. The cytochromes which remain reduced in the aerobic steady-state are primarily  $b_{562-3}$  and possibly some  $b_{556-7}$ . This conclusion is based on the position of the b peak at about 559 to 560 nm in spectra of membranes in the aerobic steady-state ( $S, H_2O_2/H_2O_2$ ), which indicates a very small contribution, if any, of  $b_{553-4}$  to this peak. However, the amount of  $b_{562-3}$  remaining reduced in the aerobic steady-state is only a fraction of the total  $b_{562-3}$  present. This residual fraction is even less in the case of  $b_{556-7}$  (both HP and LP).

With D-lactate in the aerobic steady-state, the  $\alpha$ -peak is considerably larger than that with L-lactate or NADH. This is consistent with the higher activity of D-lactate dehydrogenase (as estimated by the relative DICPIP oxidoreductase levels (Table 3.1.2.3)).

The aerobic steady-state with succinate shows a higher degree of reduction of the b cytochromes, especially  $b_{562-3}$ , than with any of the other reducing substrates tested. This difference has been found consistently in all steady-state spectra obtained in this study. This is surprising in view of the low dehydrogenase activities with succinate relative to those of D-lactate and NADH (Table 3.1.2.4). Also with succinate, the cytochromes in the aerobic and anaerobic steady states are reduced to very nearly the same extent, except for cytochrome d. In fact, the difference between aerobic and anaerobic steady state b peaks with succinate

was frequently even less than that shown in Figure 3.2.1.2,D (succ,fum/succ,  $H_2O_2$ ), where there is significant difference between the two states in the level of reduction of cytochromes  $b_{556-7}$  and  $b_{562-3}$ . The high level of reduction of cytochrome  $b_{562-3}$  in the aerobic steady-state with succinate is surprising since this cytochrome has the highest redox midpoint potential of all the b-type cytochromes (Table 3.1.5.1), a potential very similar to that of cytochrome d, which is fully oxidized in the aerobic steady state.

One interpretation of the higher degree of reduction of cytochrome  $b_{562-3}$  in the aerobic steady-state with succinate than with other reducing substrates is that this cytochrome is closely associated with a succinate dehydrogenase (which may or may not be the same enzyme as fumarate reductase), but is only slowly oxidized by oxygen. This is consistent with the very low succinate oxidase activity relative to that with other substrates (Table 3.1.2.1).

The difference spectra between anaerobic and aerobic steady-states (S,fum/S, $H_2O_2$ ) generally show a b peak apparently composed of approximately equal proportions of  $b_{562-3}$  and  $b_{556-7}$  and a lesser quantity of  $b_{553-4}$  (this particular difference spectrum should reveal those cytochromes which are more highly oxidized in the aerobic steady-state than in the anaerobic steady-state). This is consistent with the redox potential measurements (Table 3.1.5.1.), which show that  $b_{562-3}$  and  $b_{556-7}^{HP}$  have redox potentials considerably more positive than that of the succinate/fumarate couple. On the other hand the spectrum succ/succ,fum indicates that

fumarate does oxidize a  $b_{556-7}$  component. The low potential cytochrome  $b_{556-7}$  ( $b_{556-7}^{LP}$ ,  $E_{m7} = -23$  mV) has a midpoint potential which would permit a functional role in electron transport to fumarate.

Conclusions from the spectra described above are summarized below:

1. Cytochrome d is a terminal oxidase.
2. Cytochrome  $b_{556-7}^{LP}$  possibly has a functional role in electron transport to fumarate.
3. Cytochrome  $b_{562-3}$  possibly has a close relationship with succinate dehydrogenase.

### 3.2.2: Effects of UV irradiation and n-pentane extraction on electron transport

The possible role of quinones in electron transport in *P. shermanii* membranes was investigated by measuring the effect of quinone removal or inactivation on membrane-linked reactions. Quinones were removed by solvent extraction of freeze-dried membranes or inactivated by exposure of aqueous suspensions of membranes to UV light. The former method has been used by Kroger *et al* (1971) to determine the role of menaquinone and ubiquinone in *Proteus rettgeri* and by King and Drews (1973) to study the role of ubiquinone in *Rhodopseudomonas palustris*. Destruction of quinones by UV light was used by Brodie and Ballantine (1950) to study menaquinone function in *Mycobacterium phlei* and has subsequently been employed by deVries *et al* (1977) and by Sone (1974) to investigate the role of menaquinone in electron transport in propionibacteria.

The effects of UV irradiation are not, however, specific to quinones. Brodie (in Morton, 1965, p 362) reported that UV light also causes a slower destruction of FMN, although it has no effect on FAD (relative percentage inactivation quoted for quinone, FMN and FAD were 84%, 24% and 0%, respectively). Ultraviolet light also inactivates cytochrome d (Bragg, 1971).

#### 3.2.2.1: Effects of UV light on electron transport activities

Membranes were irradiated by placing an aqueous membrane suspension (2 mg protein ml<sup>-1</sup>) in a Petri dish supported on a bed of crushed ice 5 cm below an ultraviolet lamp (emission maximum 350 nm) and slowly rocking (12 rpm) during exposure. Samples were collected at 0, 5, 10, 20, 40 and 80 min and assayed for the various electron transport activities. The time courses of inactivation of oxygen and fumarate consumption, of Fe(CN)<sub>6</sub> and DICPIP reduction, and of the benzyl viologen-fumarate oxidoreductase were measured. Results of these experiments using D-lactate, L-lactate, NADH and succinate as reducing substrates are shown in Figure 3.2.2.1, A to E.

Of the various electron transport activities measured, D-lactate-fumarate and NADH-fumarate oxidoreductases were the most rapidly destroyed by UV light. Plots of log<sub>10</sub> % activity against time (Figure 3.2.2.1, E) indicate that the half-times for destruction of these two activities are 6 and 8 min for NADH and D-lactate, respectively. These semi-log plots are very nearly linear, suggesting that UV inactivation involves a single site.

Figure 3.2.2.1: UV inactivation of electron transport activities.

Values presented in these figures are the activities in membranes irradiated by UV light as described in the text expressed as a percentage of activities present in unirradiated membranes. The assay procedures are described in the method section (section 2.5). Oxygen + PMS,  $\odot$ ; oxygen, O; Fe (CN)<sub>6</sub>,  $\diamond$ ; DICPIP,  $\triangle$ ; fumarate, X.

- A. D-lactate-dependent activities
- B. L-lactate-dependent activities
- C. NADH-dependent activities and benzyl-viologen-fumarate oxidoreductase
- D. Succinate-dependent activities
- E. Semi-log plot of % residual activity versus time for NADH oxidase ( $\odot$ ); D-lactate oxidase ( $\triangle$ ); NADH-fumarate oxidoreductase, (O); D-lactate-fumarate oxidoreductase ( $\triangle$ ).



Figure 3.2.2.1

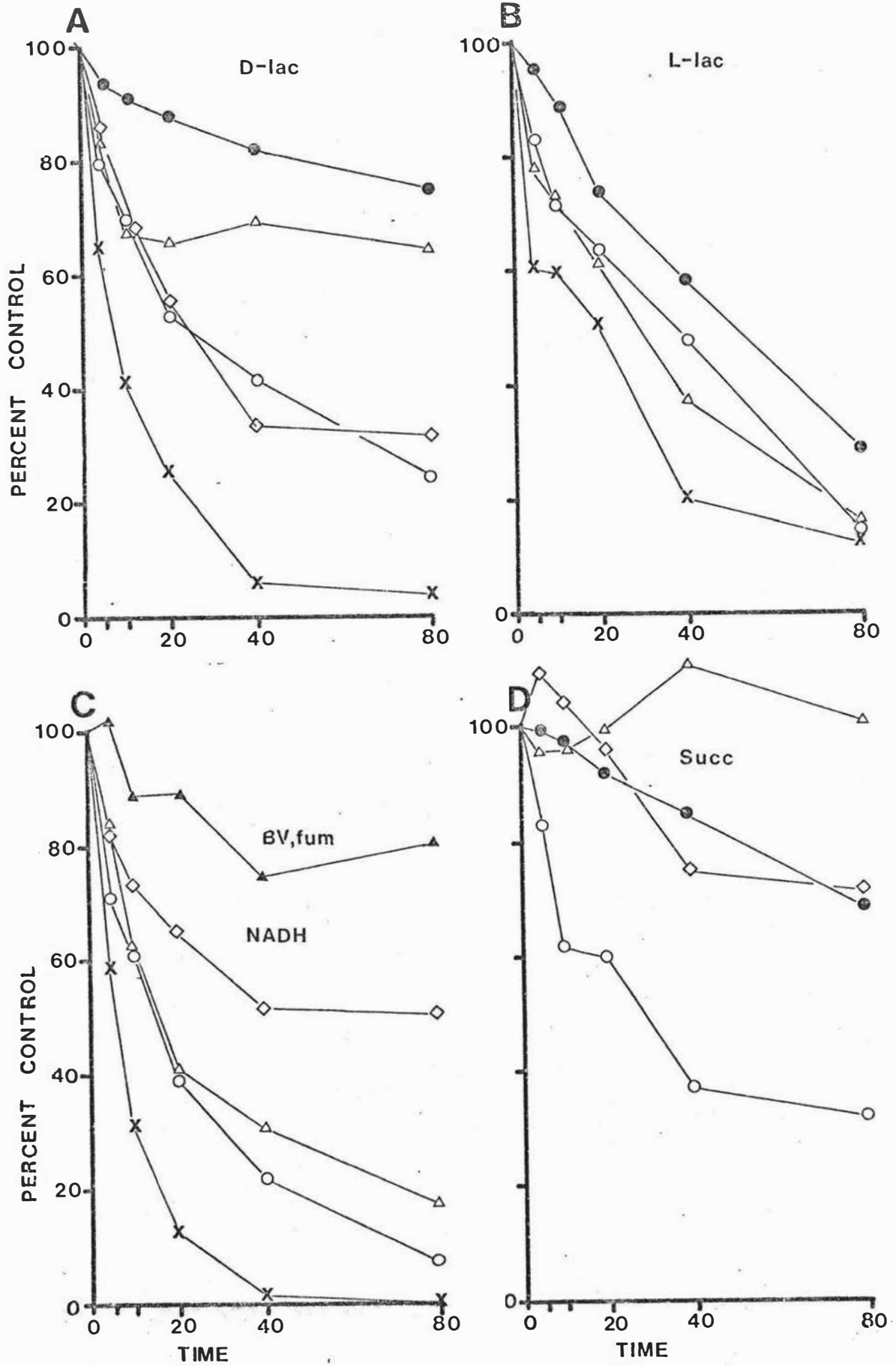
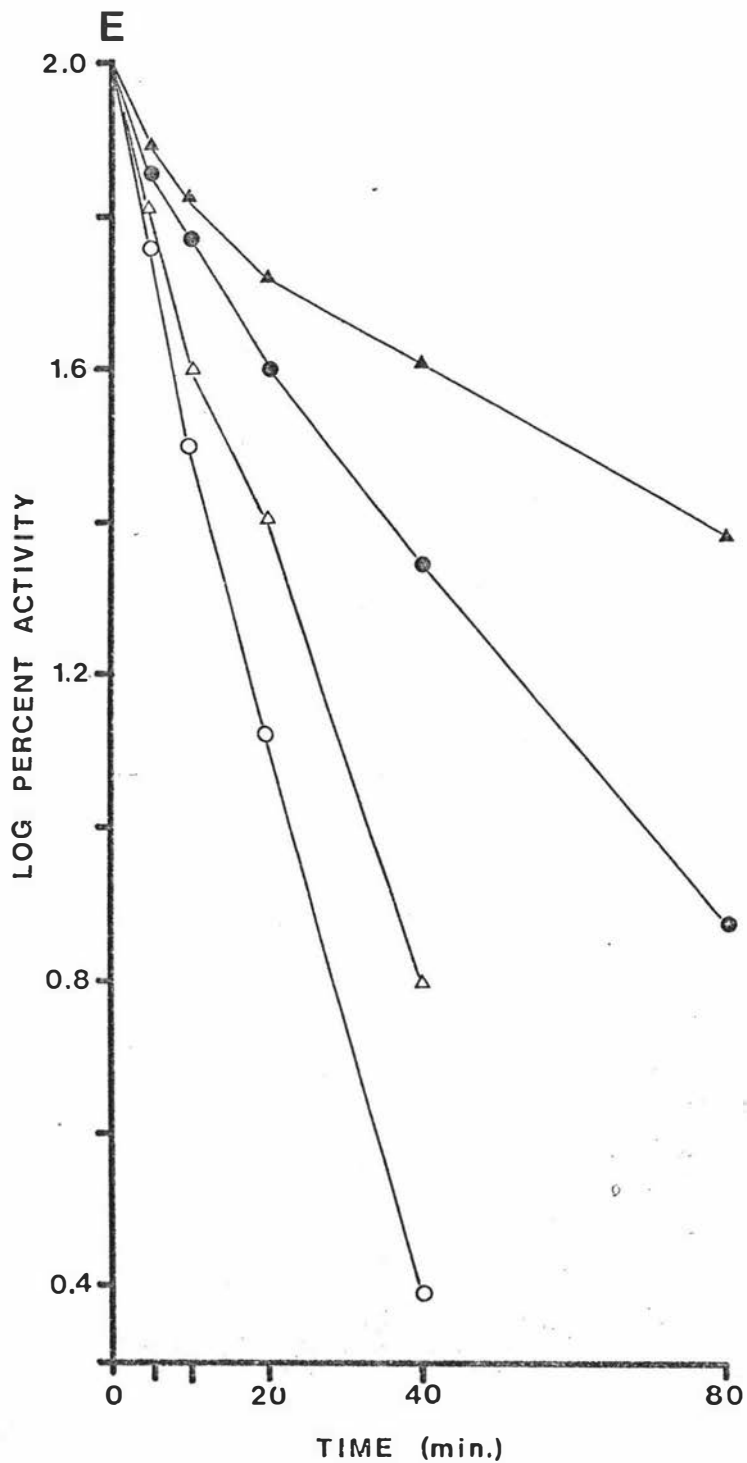


Figure 3.2.2.1



Electron transport to oxygen from D-lactate and NADH is less sensitive to UV than that to fumarate. The time of exposure required for 50% inactivation is 14 min and 23 min for NADH and D-lactate, respectively (Fig. 3.2.1.1,E). Semilog plots for the inactivation of D-lactate and NADH-oxygen oxidoreductases are non-linear, indicating that electron flow to oxygen occurs via at least two pathways with differing sensitivity to UV irradiation.

Oxygen consumption with D-lactate in the presence of PMS showed an initial small but rapid drop and then a slow decrease which approached linearity with time (Fig. 3.2.2.1,A). The rate of inactivation was very much slower than that in the absence of PMS which indicates that the main UV-sensitive site is largely by-passed when PMS is present.

D-lactate-DICPIP oxidoreductase activity was rapidly inactivated to about 60% of that at 0 min after 10 min exposure, but there was no further inactivation with continued exposure. On the other hand, NADH-DICPIP oxidoreductase showed progressive inactivation with increasing exposure time at a rate similar to that of NADH-oxygen oxidoreductase. The D-lactate- $\text{Fe}(\text{CN})_6$  oxidoreductase was inactivated to a much greater extent than the D-lactate DICPIP oxidoreductase and showed a rate of inactivation similar to that of D-lactate-oxygen oxidoreductase. In contrast to this, NADH- $\text{Fe}(\text{CN})_6$  oxidoreductase was less rapidly inactivated than NADH-DICPIP oxidoreductase. As noted previously (section 3.1.2),  $\text{Fe}(\text{CN})_6$  and DICPIP appear to interact at more than one site in the electron transport chain, and the extent or rate of interaction at the different sites is different for the different donors.

The data obtained using L-lactate are less reliable than those obtained with other donors since most of the original L-lactate-coupled activity had been lost from the membranes used in these experiments due to the time involved in preparation. The destruction by UV light of L-lactate dependent activities was similar to that with D-lactate in that fumarate reduction was the most sensitive reaction. However, L-lactate-DICPIP and L-lactate-oxygen (+PMS) oxidoreductases decayed under UV light at a rate similar to that of L-lactate and D-lactate-oxygen oxidoreductases. These results suggest that the L-lactate dehydrogenase system (unlike that for D-lactate) is destroyed by UV light and that it is inactivation at this site that determines the decay rate of oxygen consumption.

With succinate as reducing substrate, there was no inactivation of DICPIP reduction on exposure to UV light. The rate of reduction of oxygen in the presence of PMS and the rate of reduction of ferricyanide were also only very slowly inactivated. However, the rate of inactivation of succinate-oxygen oxidoreductase was very close to that of D-lactate-oxygen oxidoreductase.

#### 3.2.2.2: Effects of UV light on reduction and oxidation of cytochromes

The effects of UV light on reduction of cytochromes by D-lactate, NADH and succinate and on the level of aerobic and anaerobic steady-state cytochrome reduction were determined using low temperature spectrophotometry according to the procedure described in section 3.2.1. A membrane suspension

was divided into two aliquots, one of which was irradiated for 80 minutes as described above. One ml portions of irradiated and unirradiated membrane suspension were incubated with D-lactate, NADH or succinate (L-lactate was omitted because of the very low activity of L-lactate-dependent activities). Both irradiated and unirradiated samples were reduced for the same time intervals, then the samples were frozen and examined by low temperature spectrophotometry.

The effects of UV irradiation on reduction of the cytochromes by D-lactate, NADH and succinate are shown in Figure 3.2.2.2. Reduction of the b peak was inhibited by UV light in all three cases, although inhibition was small with succinate as electron donor. With NADH as reductant there was no reduced cytochrome d peak (630 nm) in the UV-treated sample (as compared with D-lactate and succinate) which indicates that those membranes were still in the aerobic steady state.

The reduced cytochrome d peak was also much smaller in the UV irradiated membranes reduced with D-lactate and succinate. This could be due to inhibition of cytochrome d reduction in UV irradiated membranes. However, if this were the case, there should be a higher content of oxidized cytochrome d, and consequently there should be a trough at 645 nm in the spectra D-lac/UV, D-lac and Succ/UV, succ, since oxidized cytochrome d has a peak at 645 nm. As can be seen from Figure 3.2.2.2,A, no such trough is evident. A more likely explanation is that the destruction of cytochrome d by UV light (Bragg, 1971) is responsible for the smaller d peak at 630 nm in the UV

Figure 3.2.2.2: Effects of UV Irradiation on Reduction  
Levels of Cytochromes

A membrane suspension exposed to UV light for 80 min as described in the text was compared with membranes which had not been exposed to ultraviolet light in the substrate reduced (A), anaerobic steady-state (B) and aerobic steady-state (C). Membranes were present at a protein concentration of  $0.42 \text{ mg ml}^{-1}$ . D-lactate, L-lactate, succinate and fumarate were present, where indicated, at 6 mM. NADH and  $\text{H}_2\text{O}_2$  were present, where indicated, at 3 mM and 0.1%, respectively.

NOTE: In the spectra shown in Figure 3.2.2.2., A, B & C (and in most subsequent spectra using inhibitors), the spectra shown on the left hand side of the page are the difference spectra between the substrate reduced or steady state samples, with and without inhibitor (sample cuvette) and a fully oxidized sample (reference cuvette). The spectra on the right hand side of the page are obtained by placing the uninhibited sample in the sample cuvette and the inhibited sample in the reference cuvette (e.g., D-lac/UV,D-lac). These spectra reveal more clearly differences in reduction level of the cytochromes in the inhibited and uninhibited samples.

- A. Substrate reduced membranes
- B. Anaerobic steady state
- C. Aerobic steady state.

FIGURE 3.2.2.2,A

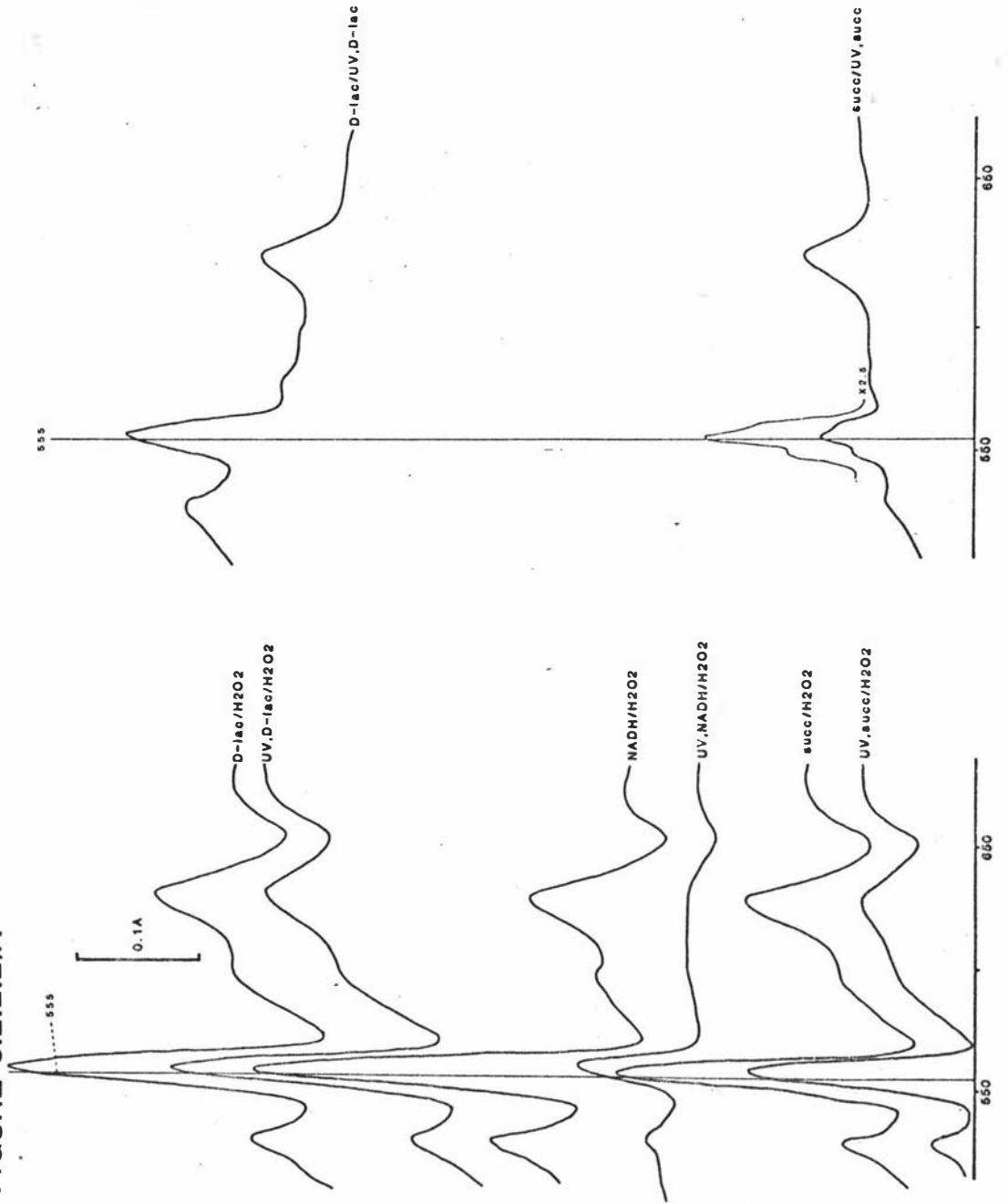


FIGURE 3.2.2.2.B

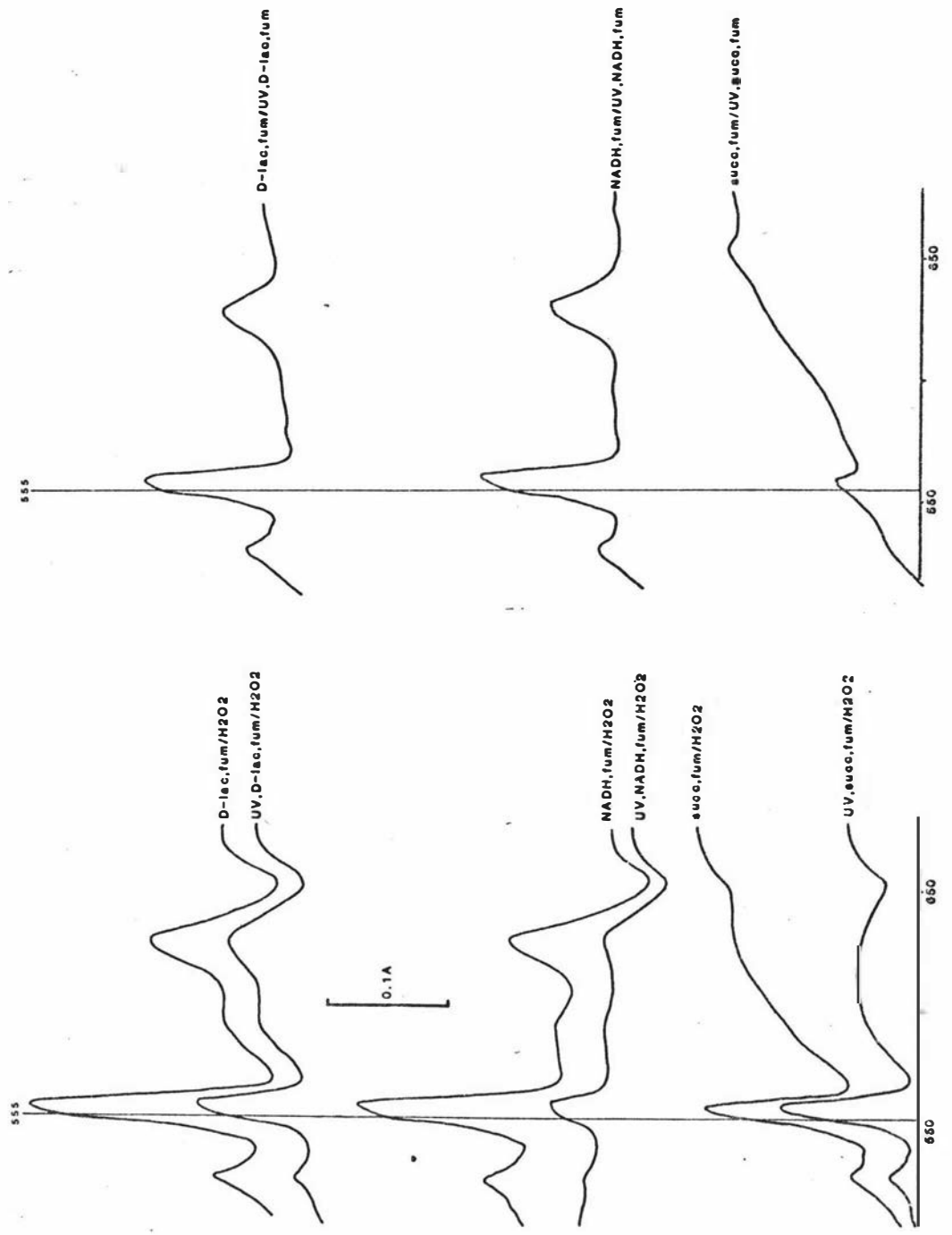
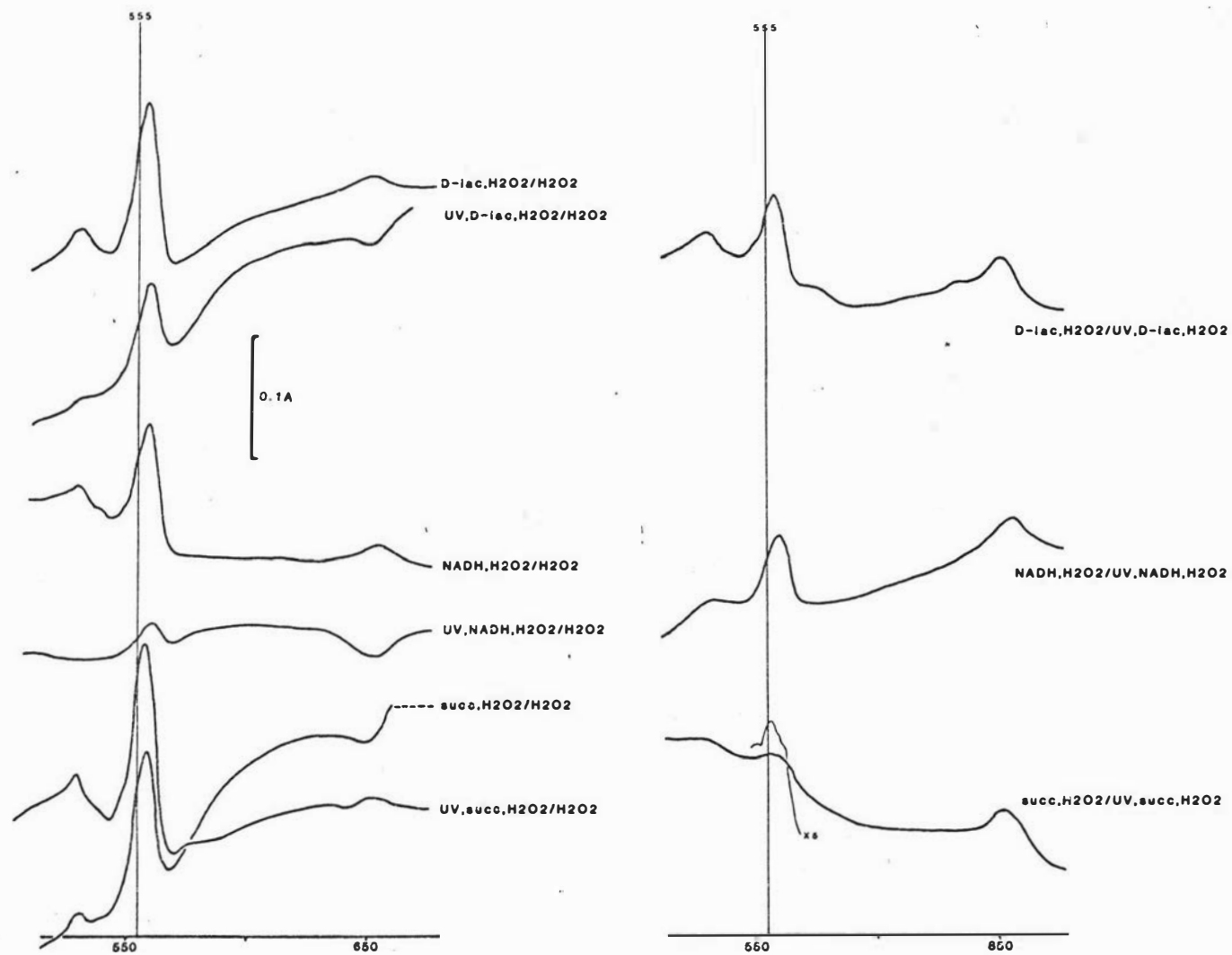




FIGURE 3.2.2.2,C



treated membrane spectra. Since, as will be discussed later, cytochrome d is probably the major terminal oxidase in *P. shermanii* membranes, this additional site of UV action would contribute to the non-linearity of the semi-log plots of UV inactivation of oxygen consumption (Fig. 3.2.2.1,E).

The aerobic and anaerobic steady-states of the cytochromes were also examined. Membranes were reduced by the various substrates (D-lactate, NADH and succinate) for 10 min (D-lactate or NADH) or for 30 min (succinate) at 30 C. These times were considered long enough to remove oxygen and to allow full reduction of the cytochromes in both irradiated and unirradiated membranes. Membranes in the steady-state were then prepared by adding either a solution of  $H_2O_2$  or of anaerobic fumarate, mixing gently for one minute, and freezing in low temperature cells in liquid air. Anaerobic and aerobic steady-state spectra of UV-irradiated and unirradiated membranes and the differences between them are shown in Figure 3.2.2.2.B and C.

Comparison of the aerobic steady-states of irradiated and untreated samples with D-lactate as reductant (D-lac,  $H_2O_2/H_2O_2$  and D-lac, UV,  $H_2O_2/H_2O_2$  (Figure 3.2.2.2,C)) shows a smaller b peak in the irradiated membranes, indicating that the major effect of UV light is on the reducing side of the b cytochromes. The difference spectrum between unirradiated and irradiated aerobic steady-states (D-lac,  $H_2O_2/D-lac, UV, H_2O_2$  (Fig. 3.2.2.2,C)) shows a complex b peak with contributions from at least two or more cytochromes b.

The anaerobic steady-state spectra with D-lactate also indicate that the major inhibition with UV light occurs on the reducing side of the b cytochromes. The difference between unirradiated and irradiated steady-state membranes (D-lac,fum/D-lac,UV,Fum (Figure 3.2.2.2,B)) shows a complex b peak indicating that fumarate oxidizes several of the b cytochromes, even when the majority of menaquinone is destroyed.

The same conclusions hold for NADH since the spectra observed when NADH was used as reducing substrate were nearly identical to those for D-lactate.

With succinate, UV irradiation had very little effect on the steady-state reduction levels of b type cytochromes as shown in Figure 3.2.2.2,C. This indicates that menaquinone does not have an essential role in the reduction of cytochrome b by succinate.

### 3.2.2.3: Effects of solvent extraction on electron transport activities

Membranes were extracted with n-pentane according to the method of Kroger (1978). Some variability was noted in the effect of n-pentane extraction on electron transport activity, which may have been due to the high humidity at the time these experiments were carried out. The membranes which had been lyophilized and dried *in vacuo* over phosphorus pentoxide may have taken up some water. It was emphasised by Kroger (ibid) that membranes and solvents should be thoroughly dry, especially during readdition of quinones to depleted membranes.

Sucrose had to be thoroughly dialysed away from membranes before n-pentane extraction, since, in the presence of sucrose, membranes bunched between the glass piston and the wall of the Duall homogenizer, preventing smooth mixing.

The effects of n-pentane extraction on oxygen reduction by membranes with the various substrates are shown in Table 3.2.2.2.

Table 3.2.2.2: Effects of n-pentane Extraction of Membranes On Rate of Oxygen Consumption

	Native	Extracted	Residue(%)
D-lac	2.43	0.578	23.8
L-lac	.312	0.088	28.2
NADH	2.30	0.760	33.0
Succ	0.302	0.179	59.3

Values presented are the average of duplicate assays of specific activity ( $\mu\text{M O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$ ).

In this experiment, the specific activity of freeze dried extracted membranes was compared with membranes which were kept in aqueous suspension. The rate of oxygen consumption was much less in the n-pentane extracted membranes. Part of this loss of activity may have been due to the effect of freeze drying rather than n-pentane extraction, so that the results are of limited value. However, they are presented here because they provide further confirmation that oxidation

of succinate is inhibited to a significantly lesser extent (about 40%) than that of D-lactate, L-lactate and NADH (about 70%).

The effect of n-pentane<sup>extraction</sup> on dehydrogenase activities was measured using DICPIP as electron acceptor in the presence and absence of PMS (Table 3.2.2.3).

Table 3.2.2.3: Effects Of n-pentane Extraction of Membranes on DICPIP reductase activity

	Unextracted	Extracted	Residue(%)
D-lac	4.34	4.08	94.0
" +PMS	8.18	7.22	88.3
NADH	4.46	3.94	88.3
Succ	0.712	0.826	116.0
" +PMS	3.36	3.91	116.4

Values presented are the average of duplicate assays of specific activity ( $\mu\text{M DICPIP min}^{-1} \text{mg protein}^{-1}$ ).

In this experiment, both extracted and unextracted membranes were freeze dried, so that changes in activity can be attributed to n-pentane extraction. All three dehydrogenase activities were relatively unaffected by n-pentane extraction. Extraction of membranes with n-pentane slightly accelerated succinate-DICPIP oxidoreductase in the presence or absence of PMS and this may be due to a partial removal of the competition between oxygen and DICPIP following n-pentane extraction.

The effect of n-pentane extraction on fumarate oxidoreductase is shown in Table 3.2.2.4. The relatively high activity of fumarase present in the particular membrane preparation used in this experiment permits only the qualitative conclusion that all three activities are inhibited by n-pentane extraction.

Table 3.2.2.4: The Effect Of n-pentane Extraction of Membranes On Fumarate Oxidoreductase Activity

	Unextracted membranes	Extracted membranes	Residual activity(%)
D-lac	0.418	0.0395	9.4
L-lac	0.103	0.0599	58.2
NADH	1.363	0.350	25.6

Specific activities are in units of  $\mu\text{mol fumarate min}^{-1} \text{ mg protein}^{-1}$  except with NADH which is  $\mu\text{mol NADH min}^{-1} \text{ mg protein}^{-1}$ . Fumarase activities were  $0.368 \mu\text{mol min}^{-1} \text{ mg}^{-1}$  in extracted membranes and  $0.736 \mu\text{mol min}^{-1} \text{ mg}^{-1}$  in unextracted membranes. Values shown are the average of duplicates.

Results of experiments in which a quinone extract (see Figure 3.1.4.1 for a UV scan of this extract) was added back to n-pentane extracted membranes is shown in Table 3.2.2.5. In no instance did readdition of the menaquinone extract give complete reactivation of membrane activities. The greatest reactivation occurred with D-lactate-fumarate oxidoreductase where about half the activity was recovered.

Table 3.2.2.5: The Effect Of Quinone Readdition to n-pentane Extracted Membranes.

Reductant	Oxidant	Unextracted	Extracted	Extracted +MQ
D-lac	oxygen	0.698	0.0483	0.0979
"	fumarate	0.564	0	0.259
"	DICPIP	6.53	3.11	3.77
"	Fe(CN) <sub>6</sub>	3.11	3.02	2.79
NADH	oxygen	1.57	0.0379	0.0608
"	fumarate	3.00	0.0198	0.0846
"	DICPIP	1.76	0.424	0.423
"	Fe(CN) <sub>6</sub>	2.78	5.49	7.62
benzyl viologen	fumarate	19.0	1.8	4.61

Values presented are the averages of duplicate assays in units of specific activity ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ). Values presented are corrected for fumarase (present in the membranes at  $0.0065 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ). Quinone readdition was at the level of  $1.3 \mu\text{mol mg}^{-1}$ .

In contrast to D-lactate-fumarate oxidoreductase reactivation, D-lactate-oxygen oxidoreductase was not reactivated to a significant extent. Although all activities except NADH oxidase and NADH-DICPIP oxidoreductase were reactivated by quinone readdition by a small amount, none of the NADH-dependent activities were reactivated to a significant percentage of the native membrane activities.

Benzyl-viologen-fumarate oxidoreductase was also reactivated by readdition of the n-pentane solution of quinone from 9.5% to 24% the native activity. This

destruction and reactivation is not consistent with the very small decrease of this activity in UV irradiated membranes (Figure 3.2.2.1,C), assuming that the major effect of both UV irradiation and n-pentane extraction is on the quinones. Secondary factors associated with n-pentane extraction are the effects of freeze drying and the removal of some lipid by n-pentane extraction. These two factors limit interpretation of results from n-pentane extracted membranes.

Possibly the most significant result from these experiments was the finding that D-lactate-fumarate oxidoreductase of n-pentane extracted membranes was significantly reactivated by readdition of quinone. The reactivation of this particular activity would merit further investigation.

#### 3.2.2.4: Conclusions from UV irradiation and n-pentane extraction experiments

The main conclusions from the ultraviolet irradiation and n-pentane extraction experiments are summarized below. For the sake of simplicity, the results for each donor will be discussed separately.

##### D-lactate

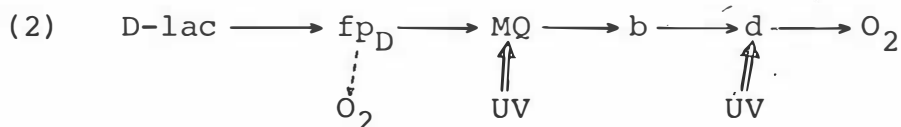
The site at which UV light acts on electron transport to fumarate is probably menaquinone. Since D-lactate-fumarate oxidoreductase was almost completely inhibited by UV light and since the semi-log plot of inactivation was linear, it



can be concluded that there is only one major site of inhibition and that menaquinone is an obligatory intermediate in electron transport to fumarate. Electron transport activity to fumarate with D-lactate as donor was also strongly inhibited by n-pentane extraction, and this was reversed to a significant extent by the addition of a quinone extract. In light of this, the following scheme must be part of any overall scheme for electron transport in *P. shermanii*.

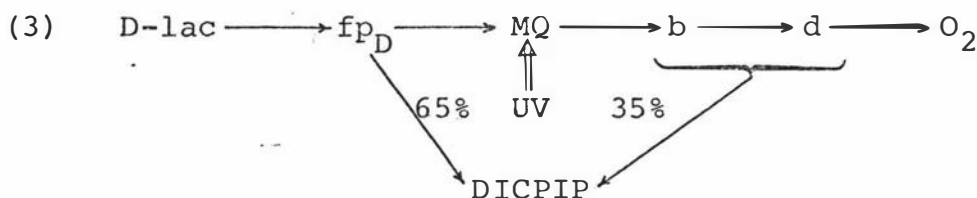


The much lower sensitivity of D-lactate-oxygen oxidoreductase to UV inactivation ( $t_{1/2} = 23$  min as compared with  $t_{1/2} = 8$  min for D-lactate-fumarate oxidoreductase) indicates that menaquinone is not an obligatory intermediate in D-lactate dependent oxygen consumption. The residual menaquinone after 80 min exposure to UV light should be about 0.1%, assuming that the  $t_{1/2}$  for menaquinone destruction is 8 min (as indicated by the inactivation of fumarate reduction). The non-linearity of the semi-log plot of loss of this activity on exposure to UV light also indicates that there is more than one site at which UV light acts. Possible sites are cytochrome d (Bragg, 1971) and flavin mononucleotide (Brodie, 1965). Partial destruction of cytochrome d is indicated by the spectra shown. The menaquinone-independent route may be due in part to direct transfer of electrons from the primary dehydrogenase to oxygen or to a bypass of menaquinone. These possibilities are summarised in the diagram below.

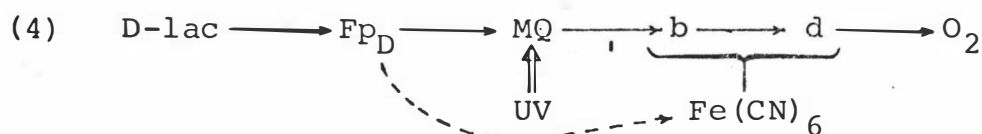


(NOTE: the cytochrome components are designated simply as b and d).

D-lactate-DICPIP oxidoreductase is decreased to a maximum of 35% after 10 min exposure to UV light. This indicates that a discrete portion of the DICPIP reductase activity (35%) involves a pathway requiring menaquinone, while the remaining 65% does not (and has no other site sensitive to UV light). The possible sites of interaction of DICPIP are indicated below.



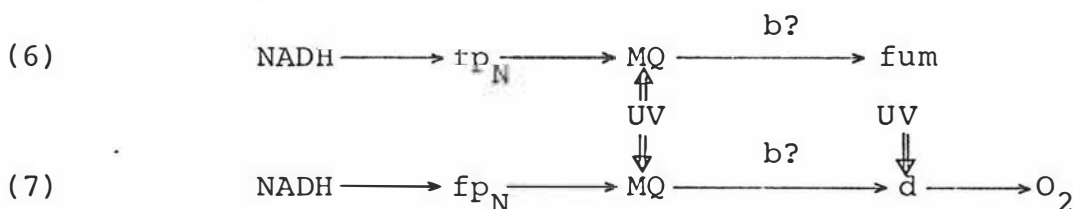
The inactivation of D-lactate- $\text{Fe}(\text{CN})_6$  oxidoreductase paralleled that of oxygen consumption. This indicates that the site(s) of UV inactivation of  $\text{Fe}(\text{CN})_6$  reductase and oxygen reductase activity is probably the same, so that  $\text{Fe}(\text{CN})_6$ , unlike DICPIP, is interacting with the electron transport pathway from D-lactate mainly at a site beyond menaquinone as diagrammed below.



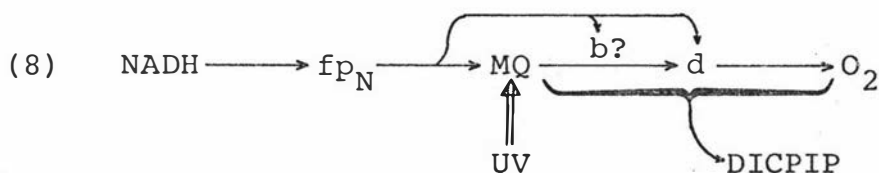
That the major inhibition of the electron transport by UV light is at a site before cytochrome b is clearly indicated by spectra of both steady-states and the substrate reduced state. This is consistent with schemes 2 and 4, above.

### NADH

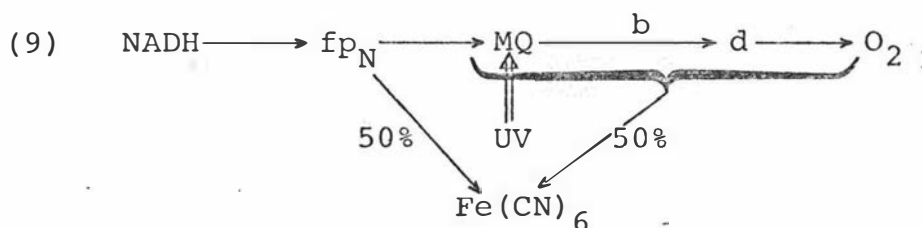
NADH-dependent fumarate and oxygen consumption decayed under UV light at rates similar to those of D-lactate dependent activities. The interpretation is thus the same as for D-lactate dependent activities.



NADH-DICPIP oxidoreductase was inactivated by UV light at the same rate as NADH oxidase, thereby indicating that DICPIP interacts with the electron transport chain from NADH principally after menaquinone.



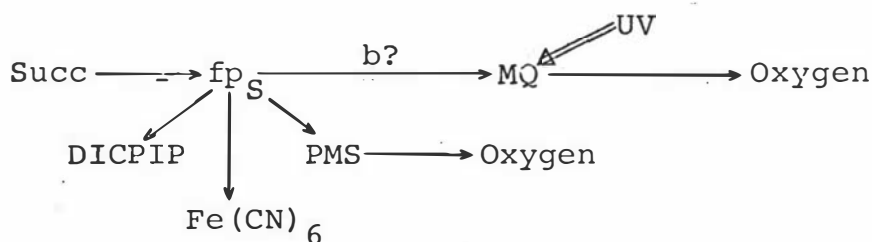
Irradiation with UV light inactivated only 50% of the NADH-Fe(CN)<sub>6</sub> oxidoreductase activity. The time course for the decrease of this UV-sensitive component was similar to that for inactivation of fumarate reductase, indicating that destruction of menaquinone is responsible for inhibition of this component of the total Fe(CN)<sub>6</sub> reductase activity.



The suggestion that  $\text{Fe}(\text{CN})_6$  can interact with the electron transport chain from NADH at the dehydrogenase site is consistent with the observation of Schwartz<sup>h</sup> & Krause<sup>g</sup> (1975) that  $\text{Fe}(\text{CN})_6$  is reduced more rapidly than DICPIP and other oxidants in a partially purified NADH dehydrogenase from *P. shermanii* PZ3.

### Succinate

Succinate-dependent activities were not significantly inhibited by UV irradiation except for the succinate-oxygen oxidoreductase activity in the absence of PMS. There was little difference between irradiated and unirradiated samples in the level of cytochrome reduction in the substrate reduced or in either steady-state. This indicates that cytochrome reduction by succinate is largely unaffected by the removal of quinone, but that the major route for the weak oxidase activity with succinate is via a quinone.



With succinate,  $\text{Fe}(\text{CN})_6$  and DICPIP probably react near the dehydrogenase since both activities are unaffected by UV irradiation.

### 3.2.3: Effects of dicumarol on electron transport

Dicumarol is an inhibitor of electron transport activity which acts as an antagonist of menaquinones or vitamin K (Lehninger (1965), of which it is an analogue (Harold, 1976). At lower concentrations ( $10^{-5}$  to  $10^{-6}$  M), it uncouples phosphorylation from oxidation of various substrates in *Mycobacterium phlei* (Brodie, 1965). The inhibition of oxidation is substrate specific in *M. phlei* where malate oxidation is inhibited but succinate oxidation is not inhibited (ibid). However, in mitochondrial particles, it has been demonstrated that dicumarol inhibits succinate oxidation by direct binding to succinate dehydrogenase (Wilson and Mertz, 1969).

Schwartz and Sporckenbach (1975) reported that dicumarol inhibited NADH, D, L-lactate and succinate dependent oxygen uptake by membrane particles of *P. shermanii* PZ3 at a concentration around  $10^{-7}$  moles per ng protein but no further data were given. Maximal inhibition was 70% and they found succinate oxidase activity to be more sensitive to inhibition than NADH or lactate oxidase. Sone (1972) reported 90% inhibition of both NADH-oxygen oxidoreductase and NADH-fumarate oxidoreductase by 0.2 mM dicumarol in *P. arabinosum* membrane particles.

Molinari and Lara (1960) reported that a partially purified lactate dehydrogenase from *P. pentosaceum* was inhibited by dicumarol (96% at 1 mM and 49% at 0.1 mM dicumarol).

### 3.2.3.1: Dicumarol inhibition of electron transport activities

The effects of dicumarol on D-lactate, L-lactate and NADH-dependent oxygen consumption were measured using the oxygen electrode (Figure 3.2.3.2,A). D-lactate-oxygen and NADH-oxygen oxidoreductases proved relatively insensitive to dicumarol, requiring about  $10^{-2}$  M to give 50% inhibition ( $pI_{50}/E = 1.5^{(a)}$ ). This is the upper limit of solubility of dicumarol in water so effects at higher concentrations could not be ascertained. This is much lower sensitivity than that reported by Schwartz and Sporkenbach (1975). L-lactate-oxygen oxidoreductase exhibited a much greater sensitivity, showing 50% inhibition at about  $10^{-5}$  M dicumarol ( $pI_{50}/E = 4.5$ ).

In contrast to the different oxygen oxidoreductase systems, reduction of fumarate by the three different reducing substrates showed much less difference in sensitivity to dicumarol inhibition. Half inhibition of fumarate reduction occurred at about  $10^{-5}$  M dicumarol ( $pI_{50}/E$  about 4.0). This is similar to the sensitivity of L-lactate-oxygen oxidoreductase, suggesting that a similar site might be involved in inhibition of both aerobic and anaerobic pathways from L-lactate.

NADH, D-lactate and L-lactate dehydrogenase activities measured using DICPIP or  $Fe(CN)_6$  as electron acceptors showed the same relative sensitivity to inhibition as did oxygen consumption. Half inhibition of L-lactate-DICPIP and L-lactate- $Fe(CN)_6$  oxidoreductases occurred at  $10^{-6}$  to  $10^{-7}$  M dicumarol ( $pI_{50}/E$  about 4.4 with  $Fe(CN)_6$  and 4.1 with

---


$$(a) \quad pI_{50}/E = \log_{10} \left( \frac{\text{inhibitor conc. giving 50\% inhibition (M)}}{\text{membrane protein conc. (g.l}^{-1}\text{)}} \right)$$

DICPIP), whereas for D-lactate and NADH dehydrogenases concentrations of  $10^{-4}$  to  $10^{-3}$  M dicumarol were required to give similar inhibition. Succinate dehydrogenase activity ( $\text{Fe}(\text{CN})_6$  or DICPIP) was least sensitive to dicumarol.

### 3.2.3.2: Effects of Dicumarol on Cytochrome Reduction

The effects of dicumarol on cytochrome<sup>0</sup><sub>λ</sub> reduction were determined using low temperature spectrophotometry of samples prepared as described previously (Section 3.2.1). The concentration of dicumarol used was the highest at which it was soluble in aqueous solution,  $2 \times 10^{-2}$  M (pI/E about 1.5). At this concentration, the L-lactate-dependent systems and the fumarate oxidoreductase systems should be almost completely inhibited.

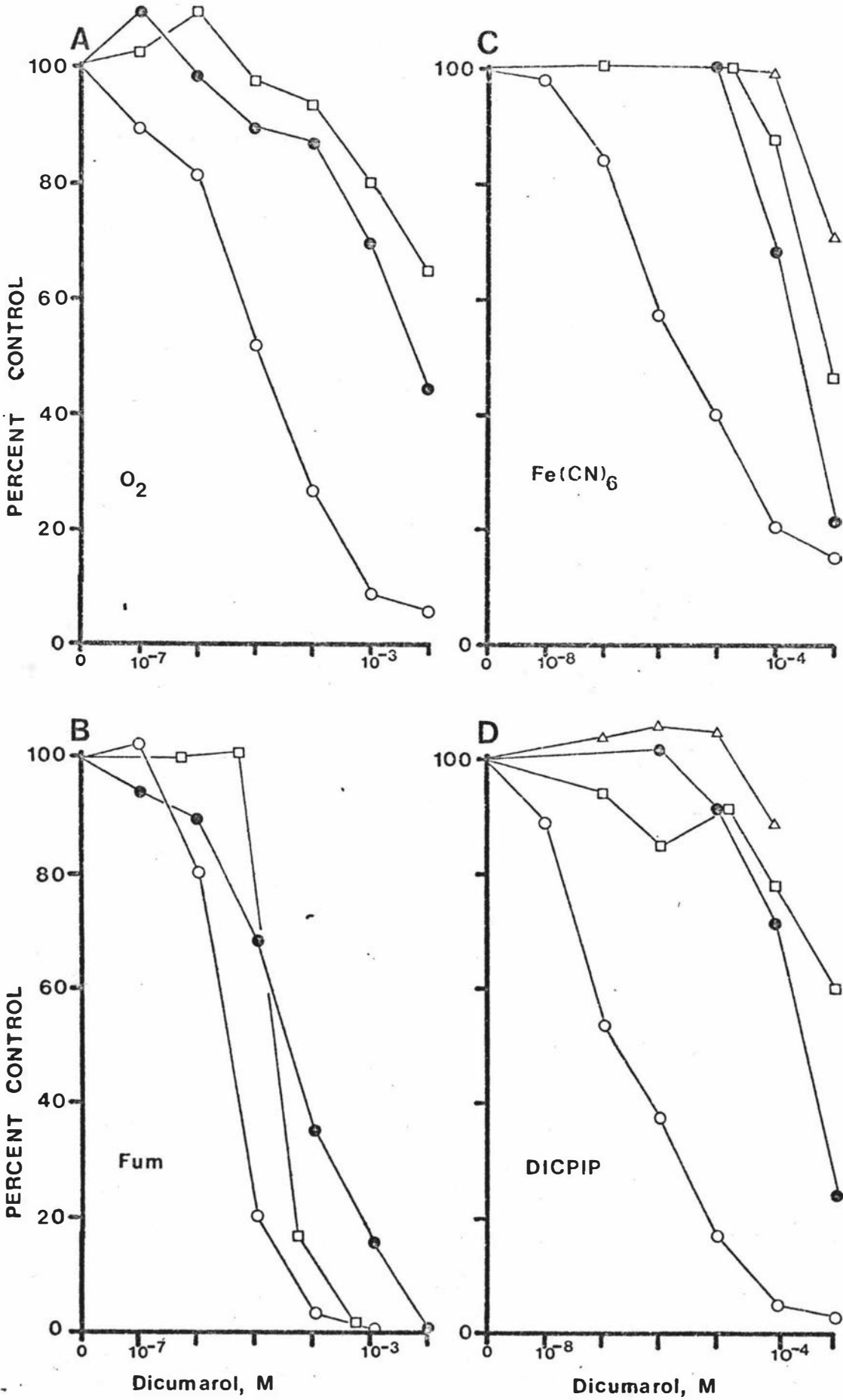
The effect of dicumarol on reduction of cytochrome b is shown in Figure 3.2.3.2,A. At the high concentration used, dicumarol inhibited reduction of cytochrome b in all cases. The difference between uninhibited and inhibited peak heights is similar for all four donors. It is rather surprising that a more complete inhibition of L-lactate-dependent cytochrome b reduction was not found in view of the high sensitivity of the L-lactate-dependent electron transport activity described above. The difference spectra between inhibited and uninhibited samples show some small qualitative differences in peak shape between the different reductants. In all cases a  $b_{556-7}$  is the major component in this difference spectrum with varying amounts of  $b_{553-4}$ . The very distinct shoulder on the short wavelength side of the b peak in the uninhibited minus inhibited difference spectrum

Figure 3.2.3.1: Dicumarol Inhibition of Electron Transport  
Activities of Membrane Vesicles of  
*P. shermanii*.

Values presented in these figures are the specific activities of electron transport processes in the presence of various concentrations of dicumarol expressed as a percentage of those of the uninhibited control. Each value is the average of duplicate determinations. A. Oxygen consumption measured with the oxygen electrode. B. Fumarate measured in anaerobic assay. C.  $\text{Fe}(\text{CN})_6$  reduction rate. D. DICPIP reduction rate. ●, D-lactate; ○, L-lactate; □, NADH; △, succinate.



Figure 3.2.3.1



for succinate (succ/DIC,succ) may be due to the 'c type' cytochrome with peak absorption at 548 nm. The reduction of  $b_{562-3}$  is apparently less affected by dicumarol than that of other cytochromes.

On the other hand, the anaerobic steady state difference spectra (S,fum/S,DIC,fum) consistently indicate that dicumarol inhibits reduction of cytochrome  $b_{556-7}$ , cytochrome  $b_{562-3}$  and the c type cytochrome. The most significant feature of the anaerobic steady-state spectra is the relatively small difference between the inhibited and uninhibited spectra, considering that the concentration of dicumarol used should, according to the data presented earlier (3.2.3.1), have completely inhibited the fumarate oxidoreductase activities. This can be explained if either the b-type cytochromes which are obligatory components of the anaerobic electron transport pathway to fumarate are only a small proportion of the total cytochrome b pool or if dicumarol inhibits fumarate oxidoreductase systems at two sites lying on either side of the cytochrome b in the anaerobic pathway, or both. This is possible considering the high concentration of dicumarol used. In retrospect, it would have been more informative to have also investigated the effect of a lower concentration of dicumarol.

Dicumarol causes a significant reduction in b peak size in the aerobic steady-state spectra (Fig. 3.2.3.2,C) indicating that inhibition is mainly on the reducing side of the b cytochromes. Again the difference between uninhibited and inhibited aerobic steady-states (S,H<sub>2</sub>O<sub>2</sub>/S,DIC, H<sub>2</sub>O<sub>2</sub> (Fig. 3.2.3.2,C)) indicates that the major

cytochromes which are less reduced in the inhibited state are  $b_{556-7}$  and to a lesser extent  $b_{562-3}$ .

### 3.2.3.3: Conclusions from experiments using dicumarol as an inhibitor

The major conclusions which may be drawn from the inhibition studies with dicumarol are as follows.

#### D-lactate and NADH

Dicumarol has similar effects on electron transport processes involving D-lactate and NADH. Electron transport from D-lactate or NADH to oxygen,  $\text{Fe}(\text{CN})_6$  or DICPIP was inhibited only at higher concentrations of dicumarol ( $pI_{50}/E$  of about 1.5). At these high concentrations of dicumarol, inhibition may be relatively non-specific. D-lactate and NADH-fumarate oxidoreductase systems were much more sensitive to dicumarol inhibition ( $pI_{50}/E$  of about 4.5). This concentration of dicumarol corresponds to a dicumarol to menaquinone ratio of about 2:1 (assuming menaquinone to be present at 16 nmol per mg protein (section 3.1.4)). If it is assumed that at this low concentration the effect of dicumarol is to inhibit menaquinone function (either reduction or oxidation or both) then the low sensitivity of the oxygen,  $\text{Fe}(\text{CN})_6$  and DICPIP oxidoreductases must be due to:

Figure 3.2.3.2: Effect of Dicumarol on Substrate Reduced and Steady State Low Temperature Spectra of Membrane Vesicles of *P. shermanii*.

Spectra of substrate reduced, anaerobic (fum) and aerobic ( $H_2O_2$ ) steady state membranes were prepared as described in methods and Section 3.2.1. Dicumarol, where indicated, was present at  $2 \times 10^{-2}$  M (pI/E = 1.5). D-lactate, L-lactate, succinate and fumarate were present at 6 mM. NADH and  $H_2O_2$  were present, where indicated, at 3 mM and 0.1%, respectively. Membranes were present at protein concentrations of  $0.60 \text{ mg ml}^{-1}$ ,  $0.61 \text{ mg ml}^{-1}$ ,  $0.65 \text{ mg ml}^{-1}$  and  $0.62 \text{ mg ml}^{-1}$  for D-lactate, L-lactate, NADH and succinate, respectively.

- A. Substrate reduced spectra
- B. Anaerobic-steady-state spectra
- C. Aerobic-steady state spectra.

FIGURE 3.2.3.2,A

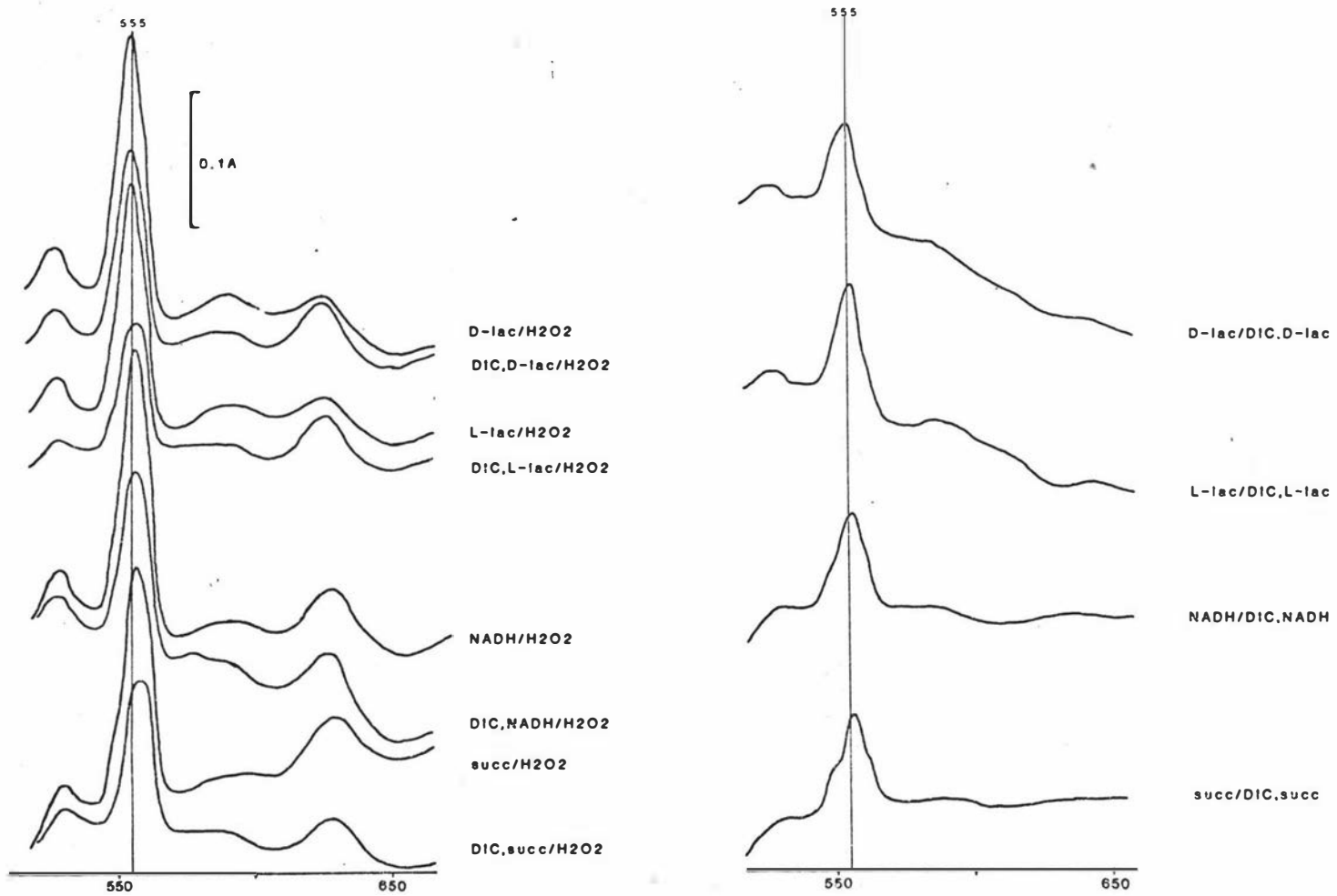


FIGURE 3.2.3.2,B

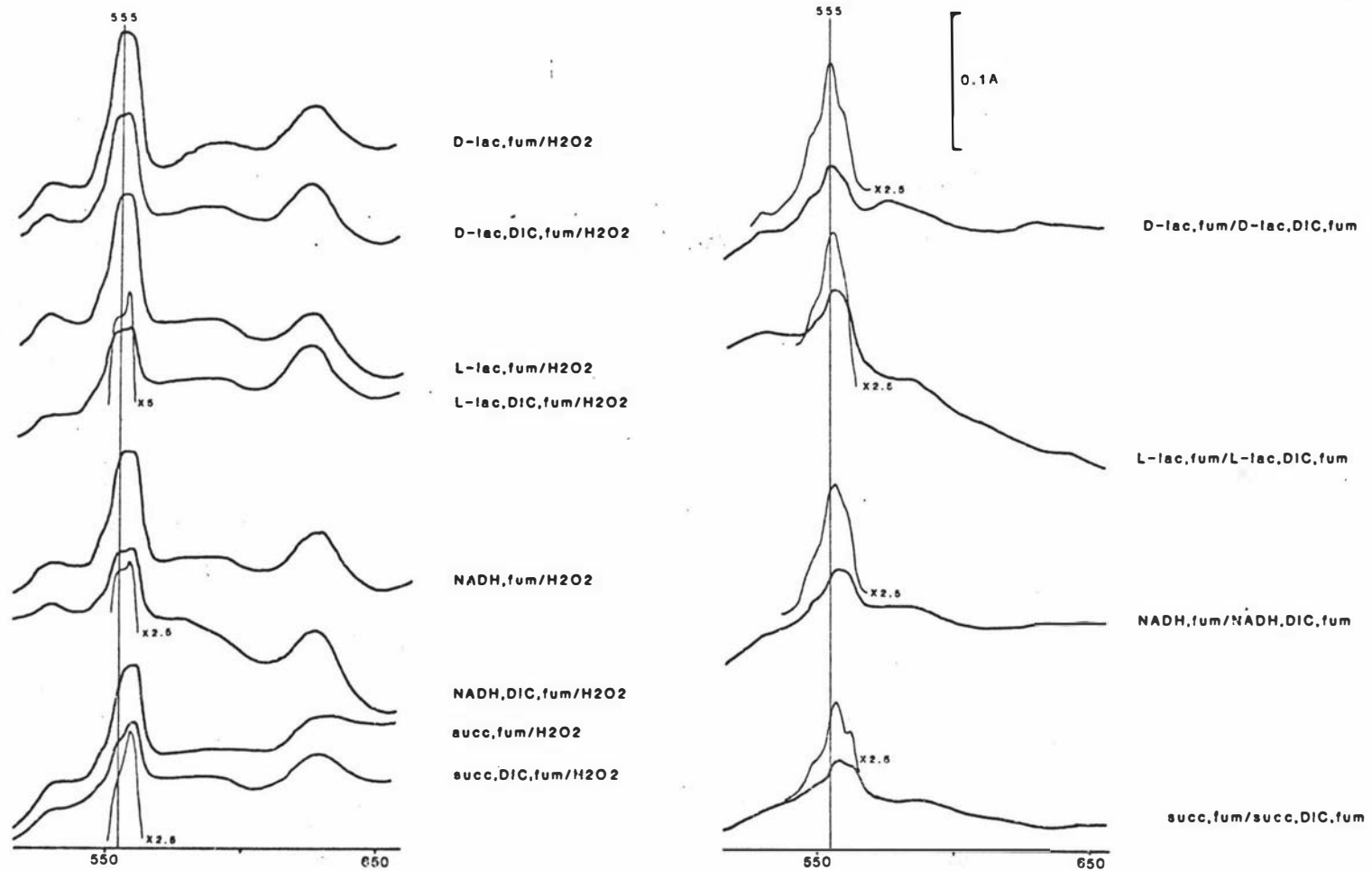
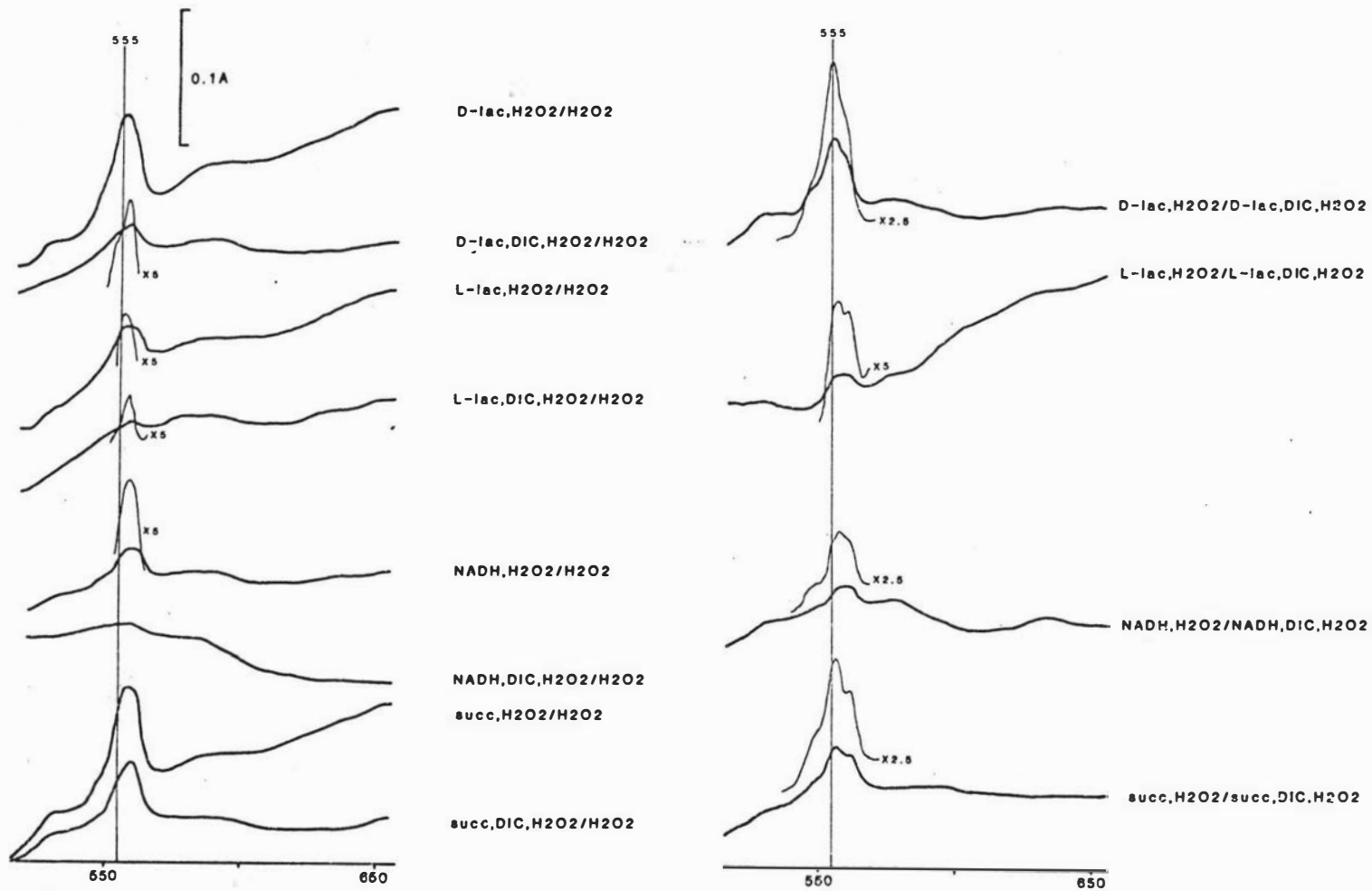
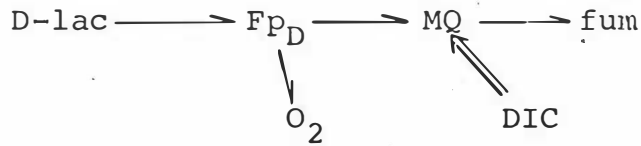


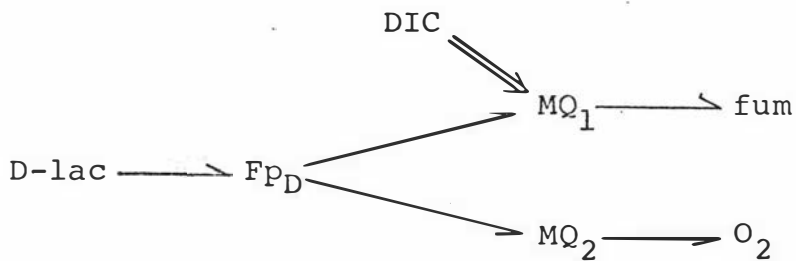
FIGURE 3.2.3.2,C



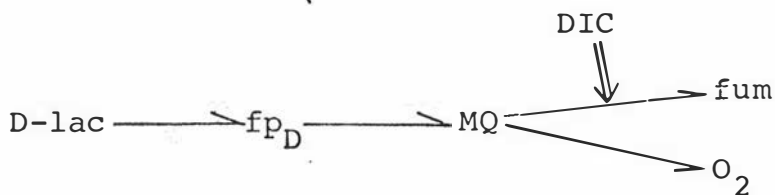
- (1) the existence of menaquinone-independent routes from NADH and D-lactate to oxygen, DICPIP and  $\text{Fe}(\text{CN})_6$ .



- (2) different pools of menaquinone showing different accessibility to dicumarol.



- (3) an inhibition of electron flow from menaquinone to fumarate but not to oxygen.



The data obtained by UV irradiation of membranes (Section 3.2.2) is consistent with the first of these possibilities.

At high concentrations, dicumarol inhibits primarily on the reducing side of the b-cytochromes. Since menaquinone ( $E'_0 = -70 \text{ mV}$ ) is likely to lie on the reducing side of cytochrome b, this observation is consistent with dicumarol acting as a menaquinone antagonist.



Anaerobic steady-state spectra (Fig. 3.2.3.2,B) show decreased reduction levels, especially of  $b_{556-7}$ ,  $b_{562-3}$  and the putative cytochrome c (548 cm). This again indicates a menaquinone function in the reduction of these b cytochromes. The decrease in peak height in the inhibited sample is relatively small, considering the high concentration of dicumarol used, and this may be due to a partial inhibition by dicumarol of the oxidation of these cytochromes by fumarate, as well as the inhibition of reduction.

The possibility of a dicumarol inhibition of the fumarate reductase system is supported by the observation of Wilson and Mertz (1969) that dicumarol binds to succinate dehydrogenase in mitochondria, and by the data of Schwartz and Sporkenbach (1975) that succinate oxidation was more sensitive to dicumarol inhibition than that of lactate or NADH.

### L-lactate

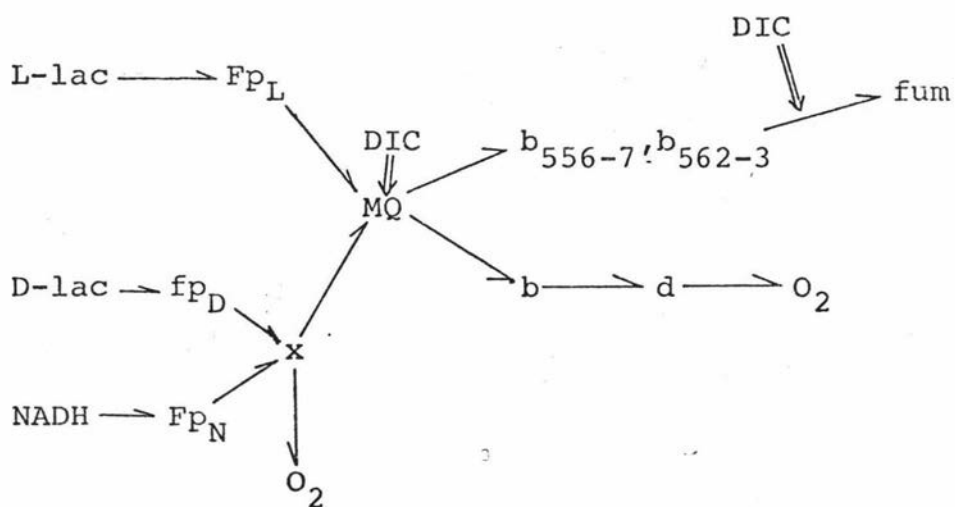
L-lactate-oxygen oxidoreductase is much more sensitive than D-lactate and NADH oxygen-oxidoreductase systems. The same difference applies to the  $\text{Fe}(\text{CN})_6$  and DICPIP oxidoreductases. Sensitivity of all these L-lactate dependent activities is similar to that of fumarate oxidoreductases. This suggests the following:

- (1) That menaquinone is an obligatory component of all electron transport processes from L-lactate, and
- (2) that the site of inhibition by dicumarol is the same for fumarate, oxygen,  $\text{Fe}(\text{CN})_6$  and DICPIP oxidoreductase systems.

## Succinate

Reduction of  $\text{Fe}(\text{CN})_6$  and DICPIP is not inhibited by dicumarol except at very high concentrations. Spectra indicate that the reduction of  $b_{556-7}$  and  $b_{562-3}$  is slightly inhibited by dicumarol. However this inhibition may be due to the inhibition of succinate dehydrogenase by dicumarol at high concentrations as mentioned above. It would have been of interest to examine the effects of dicumarol on cytochrome oxidation and reduction at a low concentration of inhibitor.

Taken in conjunction with the schemes presented in the previous sections, the results of the study of the effects of dicumarol are consistent with the following scheme.



#### 3.2.4: Inhibitory effects of p-chloromercuribenzoate on electron transport processes

The sulfhydryl reagent p-chloromercuribenzoate (pCMB) inhibits many enzymes by reacting with essential sulfhydryl groups. It reacts with iron-sulfur proteins, causing the loss of characteristic EPR signals (Orme-Johnson and Orme-Johnson, 1978). In propionibacteria it has been shown to inhibit glycerol phosphate dehydrogenase (Sone, N., 1973), lactate<sup>de</sup> hydrogenase (Molinari and Lara, 1958) and NADH dehydrogenase but not succinate dehydrogenase (Sone, 1972). Sone reported that both NADH-oxygen oxidoreductase and NADH-fumarate oxidoreductase activities were inhibited strongly by pCMB. However, a partially purified NADH dehydrogenase from *P. shermanii* which was assayed using  $\text{Fe}(\text{CN})_6$ , was inhibited only 20% by p-hydroxy mercuriphenyl sulfonate at 2 mM (Schwartz and Krause, 1975).

##### 3.2.4.1: Inhibition of electron transport activities

The rate of oxygen consumption with D- and L-lactate and NADH was inhibited by pCMB but the extent of inhibition differed according to whether lactate (D- or L-) or NADH was the electron donor (Figure 3.2.4.1,A). While both D- and L-lactate-dependent oxygen consumption were inhibited almost completely by  $10^{-3}$  M pCMB, NADH-dependent consumption was only inhibited to about 50% at this concentration of pCMB, and at higher concentrations appeared to approach a maximum of 50 to 60% inhibition. Succinate-

dependent oxygen consumption was not inhibited to nearly the same extent, only 20% inhibition being attained at  $10^{-3}$  M pCMB. The finding that NADH oxidase activity was only partially inhibited in membranes from *P. shermanii* contrasts with the report by Sone (1972) of 98% inhibition of NADH oxidase by  $2 \times 10^{-5}$  M pCMB in *P. arabinosum*.

In contrast to the inhibition of oxidase activity fumarate reduction with D-lactate, L-lactate and NADH was inhibited similarly for all three substrates (Figure 3.2.4.1,B). Inhibition of fumarate reduction with these substrates reached 100% at  $10^{-4}$  M pCMB. Expressed in terms of  $pI_{50}/E$ , the concentration of pCMB required for complete inhibition of NADH, D- or L-lactate-fumarate oxidoreductases ( $pI_{50}/E$  of about 4.0) was comparable to that for complete inhibition of the D- and L-lactate oxygen oxidoreductases. The portion of NADH oxidase activity which was sensitive to inhibition by pCMB was inhibited at a similar  $pI_{50}/E$  (about 4.0).

Inhibition of ferricyanide and DICPIP reductases by pCMB (Figure 3.2.5.1,C & D) paralleled the results obtained for inhibition of oxygen uptake, i.e., concentrations of pCMB giving 100% inhibition of activities with D-lactate inhibited NADH dependent activities only partially and succinate dependent activities very little (20 to 30%).

The presence of PMS did not affect the inhibition curves. The  $pI_{50}/E$  values for pCMB inhibition of D-lactate dependent oxidoreductases were similar in the presence or absence of PMS (Table 3.2.5.1).

Table 3.2.5.1: pCMB  $pI_{50}/E$  Values for D-lactate Oxidoreductases.

	Oxygen	Fumarate	Fe(CN) <sub>6</sub>	DICPIP
+PMS	-	-	3.9(10 <sup>-5.2</sup> )	2.6(10 <sup>-5.1</sup> )
-PMS	3.8	4.2	4.3(10 <sup>-5.7</sup> )	2.6(10 <sup>-4.5</sup> )

Values presented are  $pI_{50}/E$  (moles per gram). Bracketted figures are the  $I_{50}$  (molar).

The data presented in Table 3.2.5.1 indicate that D-lactate-DICPIP oxidoreductase is much less sensitive to pCMB than the other activities if this inhibition is expressed in terms of  $pI_{50}/E$ , although the difference was somewhat less when expressed as the actual concentration of pCMB in solution at which half inhibition occurred (bracketted figures in Table 3.2.5.1).

Inhibition of benzyl viologen-fumarate oxidoreductase by pCMB could not be measured, due to the reduction of pCMB by the  $KBH_4$  used to reduce the benzyl viologen.

#### 3.2.4.2: Effects of pCMB on the reduction level of cytochromes

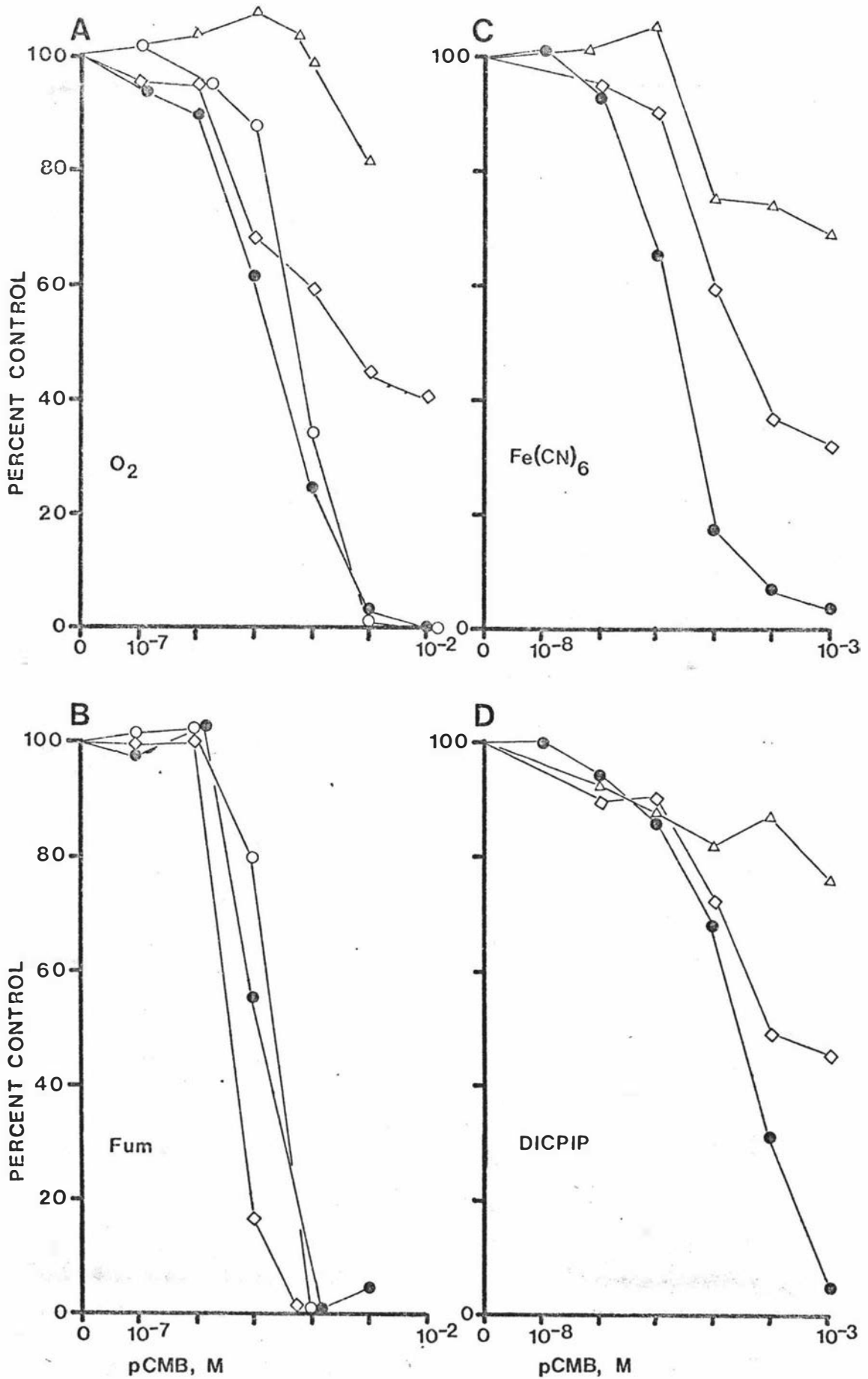
The substrate-reduced spectra indicate that reduction of cytochrome b by D-lactate and NADH was inhibited by pCMB (Figure 3.2.4.2,A) whereas that by succinate was not inhibited to any significant extent (Figure 3.2.5.2,A). In the case of D-lactate, the b-peak area of the inhibited

Figure 3.2.4.1: pCMB Inhibition of Oxidoreductases  
Present in Membranes of *P. shermanii*.

In these figures, the specific activity of the various electron transport reactions is expressed as percentage of uninhibited control. D-lactate as donor, ●; L-lactate as donor, ○; NADH as donor, ◇; succinate as donor, △.

- A. Inhibition of Oxygen consumption as measured with the oxygen electrode.
- B. Inhibition of fumarate reduction measured anaerobically at 270 nm.
- C. Inhibition of  $\text{Fe}(\text{CN})_6$  reduction measured aerobically at 420 nm.
- D. Inhibition of DICPIP reduction measured aerobically at 600 nm.

Figure 3.2.4.1



substrate-reduced spectra (D-lac,pCMB/H<sub>2</sub>O<sub>2</sub>) is similar to that of the uninhibited aerobic steady state spectrum (D-lac, H<sub>2</sub>O<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> (Fig. 3.2.5.2,C)) and cytochrome d is only partially reduced, indicating that the membranes were frozen before all the oxygen had been consumed. However, in the case of NADH, the inhibited substrate-reduced b peak is much larger than that of the inhibited aerobic steady state (Fig. 3.2.5.2,C), possibly indicating that the reduction of some or all of the b-type cytochromes by NADH is less strongly inhibited by pCMB than it is with D-lactate.

The inhibition by pCMB on the reducing side of the b cytochromes is supported by the anaerobic steady state spectra (Figure 3.2.5.2,B). That pCMB inhibits reduction is indicated by the lower reduction level of the b peak with all reducing substrates in the presence of inhibitor as compared to that in its absence.

Comparison of the relative heights of the b peak in the inhibited anaerobic steady-state spectra for D-lactate and NADH suggests that the pCMB has inhibited reduction less in the case of NADH than with D- or L-lactate. This suggestion is made on the basis of the peak heights of the spectra, S,pCMB,fum/H<sub>2</sub>O<sub>2</sub> as compared with S,fum/H<sub>2</sub>O<sub>2</sub> (Fig. 3.2.5.2,B), where the inhibited peak area is a much greater percentage of the uninhibited peak area with NADH than with D- or L-lactate.

The uninhibited minus inhibited difference spectra (S,fum/S,pCMB, fum in Fig. 3.2.5.2,B - right hand spectra) indicate that the reduction of b<sub>562-3</sub> and, to a lesser extent, b<sub>556-7</sub> is inhibited by pCMB, whereas reduction of



Figure 3.2.4.2: Effects of pCMB on Reduction Level of  
Cytochromes in Membrane Vesicles of  
*P. shermanii*.

Spectra presented in these figures are of membrane suspensions prepared as described in the text. Membranes were present at protein concentrations of 0.58 mg/ml, 0.72 mg ml<sup>-1</sup>, 0.92 mg ml<sup>-1</sup> and 0.62 mg ml<sup>-1</sup> for spectra prepared with D-lactate, L-lactate, NADH and succinate, respectively. pCMB was present where indicated, at a concentration of 5 mM. Fumarate, D- and L-lactate and succinate were added, where indicated, to 6 mM. NADH was present, where indicated, at 3 mM. H<sub>2</sub>O<sub>2</sub> was added to 0.1%.

- A. Substrate - reduced spectra
- B. Anaerobic steady-state spectra
- C. Aerobic steady-state spectra

FIGURE 3.2.4.2,A

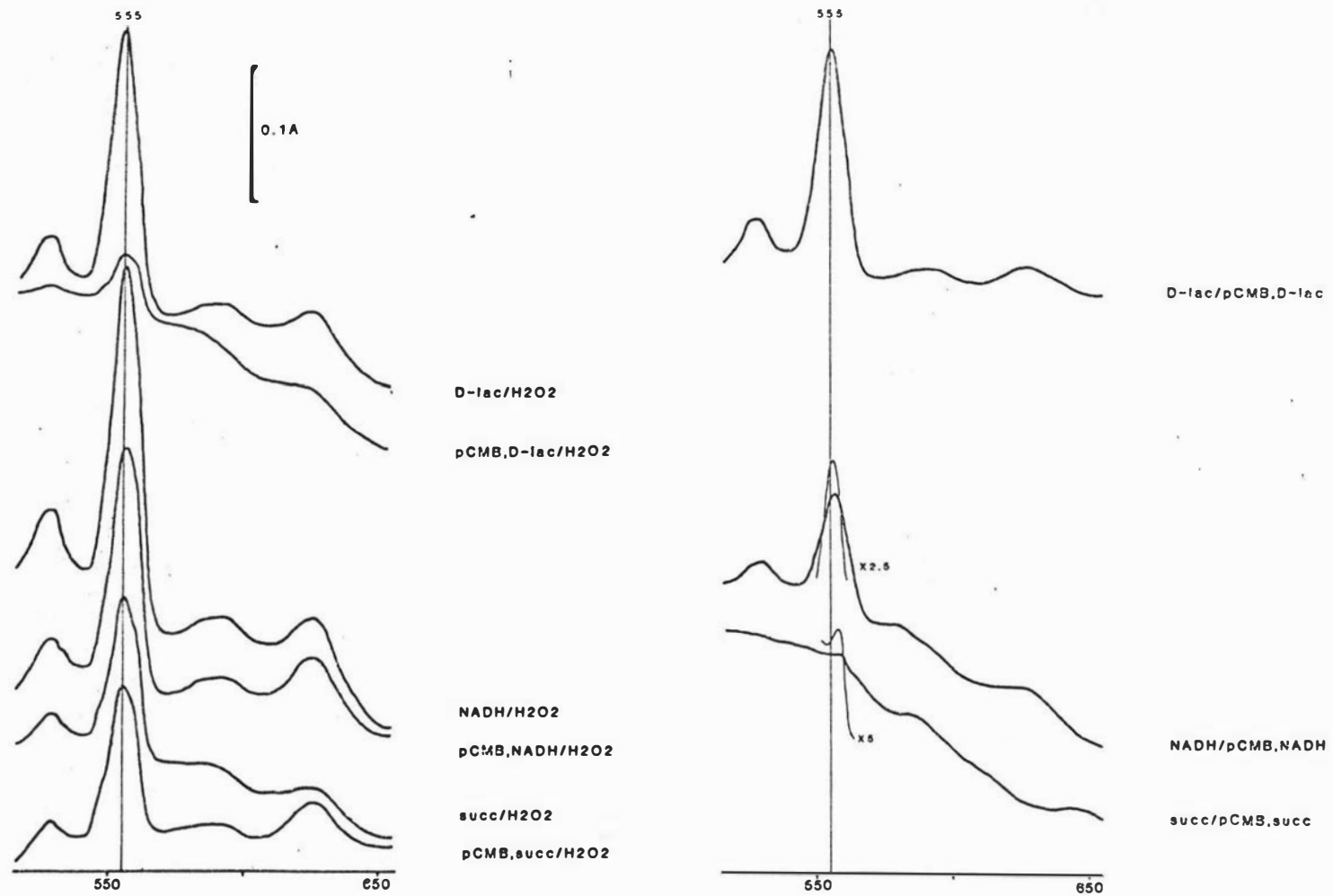


FIGURE 3.2.4.2,B

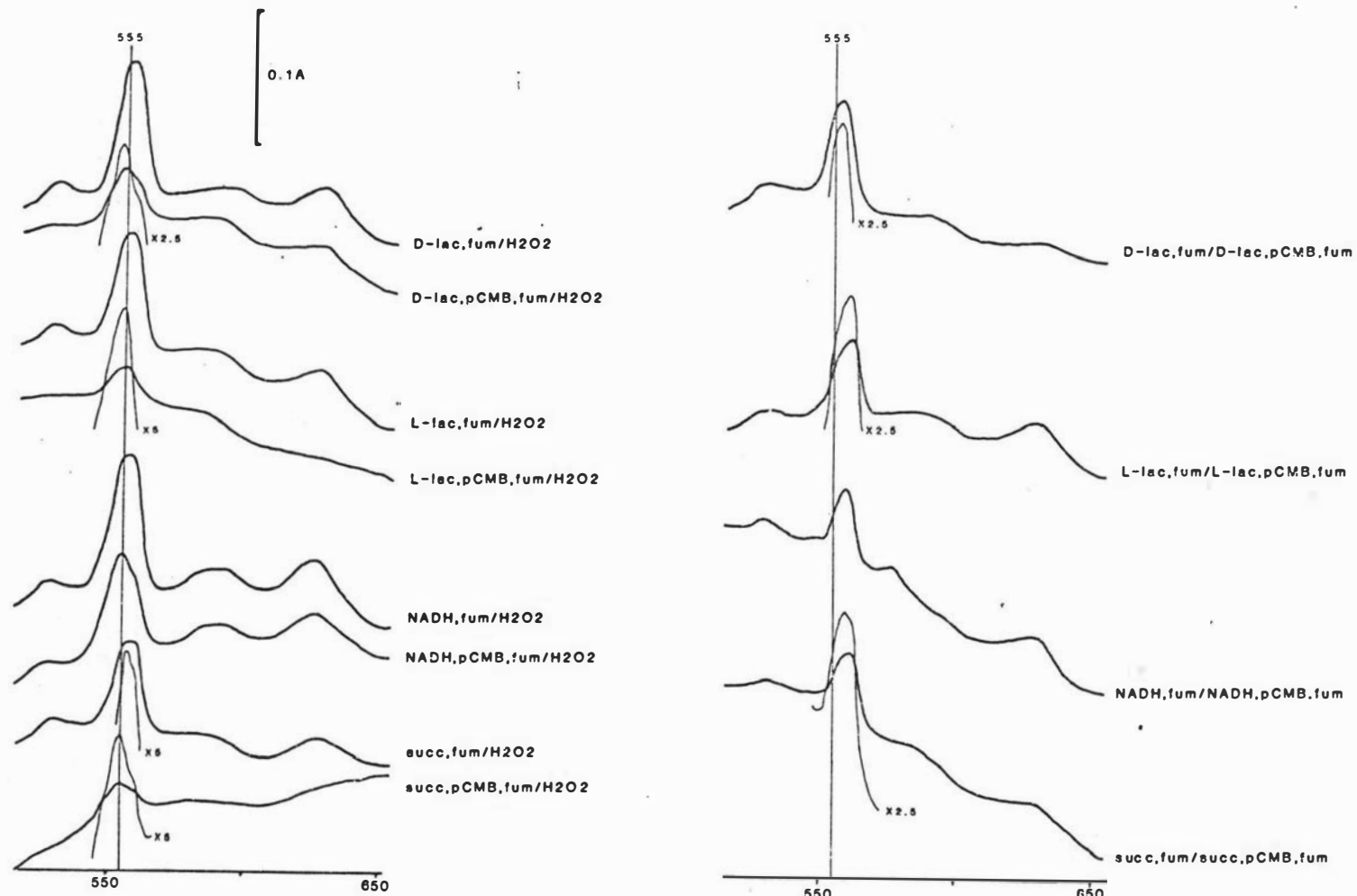
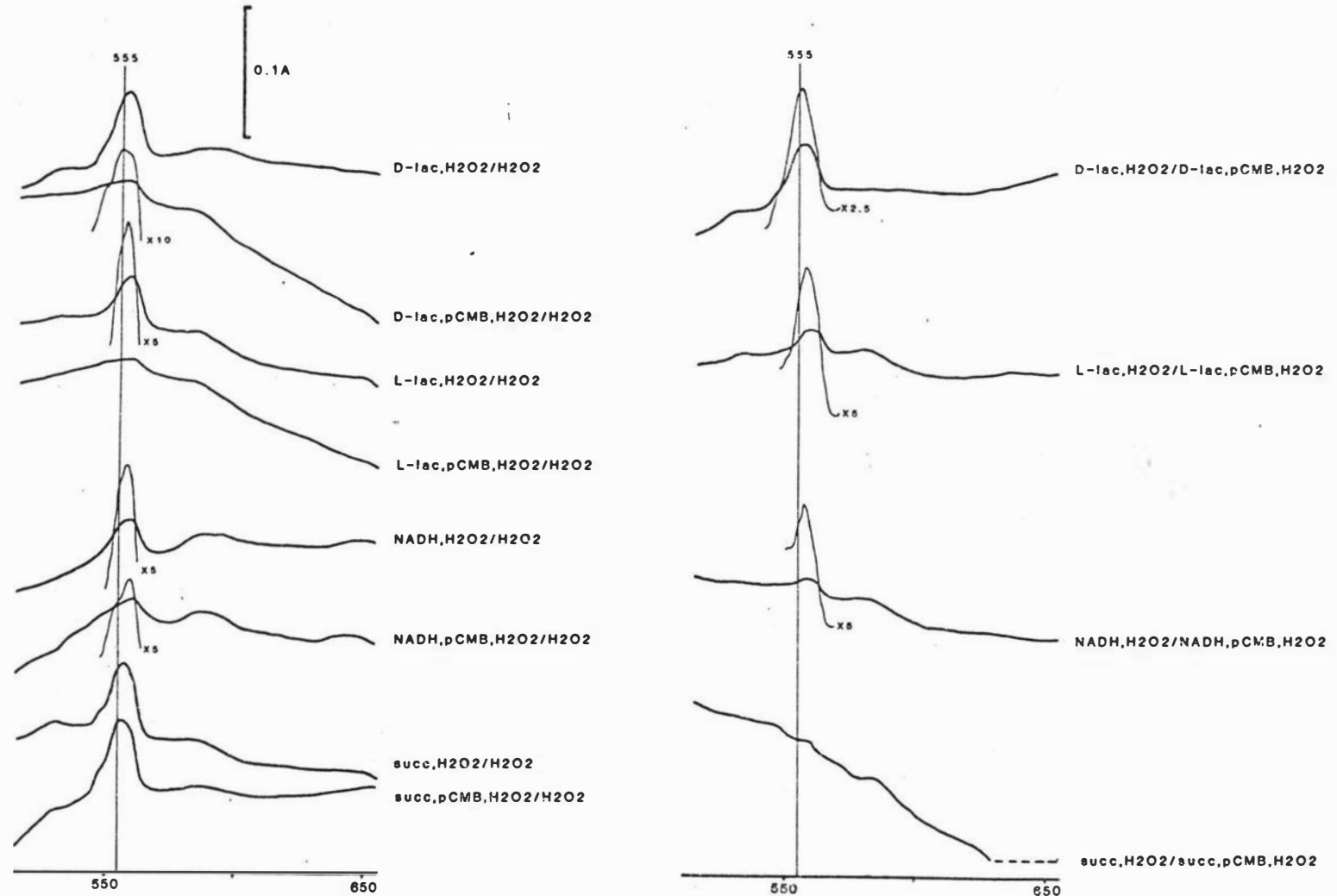


FIGURE 3.2.4.2,C



$b_{553-4}$  is much less sensitive to inhibition. The presence of a  $b_{553-4}$  component in the inhibited steady state spectra is evident in the case of D-lactate and NADH. Since the concentration of pCMB used should have been sufficient to inhibit electron transport to fumarate almost completely, the relatively small effect of pCMB on the reduction level of cytochrome  $b_{553-4}$  in the anaerobic steady-state may indicate that this cytochrome does not lie on the direct pathway from substrate to fumarate. In the case of NADH, the proportion of the  $b_{553-4}$  component in the inhibited anaerobic steady state spectrum (NADH, pCMB, fum/ $H_2O_2$ ) suggests that this component may be almost fully reduced, since the redox titration of the b type cytochromes (Section 3.1.5) indicated that  $b_{553-4}$  accounted for only about 15% of the total fully reduced peak. While undue weight cannot be placed on this observation in view of the difficulties in estimating the relative quantities of the b type cytochromes in the complex peak, it is consistent with the view that  $b_{553-4}$  is not on the direct path to fumarate.

The marked effect of pCMB in the succ, fum/ $H_2O_2$  spectrum is difficult to explain in view of the clear cut indications from both electron transport rates and the substrate reduced and aerobic steady state that succinate-dependent activities are less sensitive to pCMB. The fact that the inhibited aerobic steady state peak (succ, pCMB,  $H_2O_2/H_2O_2$  (Fig. 3.2.4.2,C)) is much larger than the inhibited anaerobic steady state peak (succ, pCMB, fum/ $H_2O_2$ ) indicates that the small size of the latter cannot be due to the introduction of oxygen into the cuvette.

That the major site of inhibition by pCMB is on the reducing side of the b cytochromes is also indicated by the decreased b peak of the aerobic inhibited steady states ( $S, pCMB, H_2O_2/H_2O_2$ ) compared to the uninhibited aerobic steady state ( $S, H_2O_2/H_2O_2$ ). This does not apply to succinate where there is virtually no difference between inhibited and uninhibited aerobic steady-states.

In light of the marked difference between pCMB inhibition of NADH dependent oxygen consumption and that with D- and L-lactate (Figure 3.2.4.2,A) it was expected that there might have been a difference between the inhibited aerobic steady state with NADH as compared with that of D- or L-lactate. The aerobic steady state provides little indication of this, possibly due to the low level of cytochrome b reduction in the aerobic steady state.

### 3.2.4.3: Conclusions from the study of pCMB inhibition

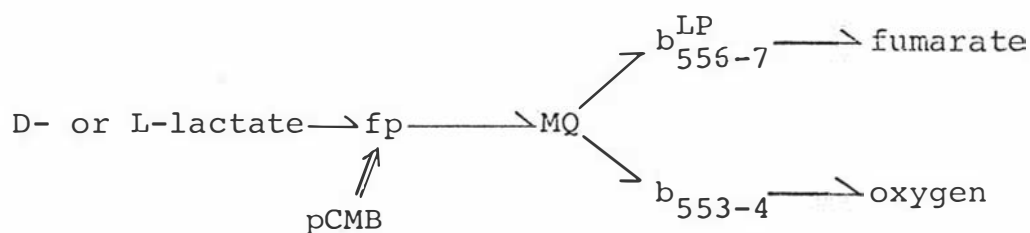
#### D- and L-lactate dependent electron transport

D-lactate dependent electron transport to oxygen, fumarate,  $Fe(CN)_6$  and DICPIP was almost completely inhibited by  $10^{-3}$  M pCMB. The inhibition curves give an approximate  $pI_{50}/E$  of 4 (except for the DICPIP oxidoreductase which has a  $pI_{50}/E$  of about 2.5). This implies a common site of inhibition for D-lactate-oxygen,  $-Fe(CN)_6$  and -fumarate oxidoreductase systems. The same is true for the L-lactate dependent activities which were investigated.

Inhibition occurs on the reducing side of the b cytochromes in the aerobic and anaerobic steady states and also in the substrate reduced

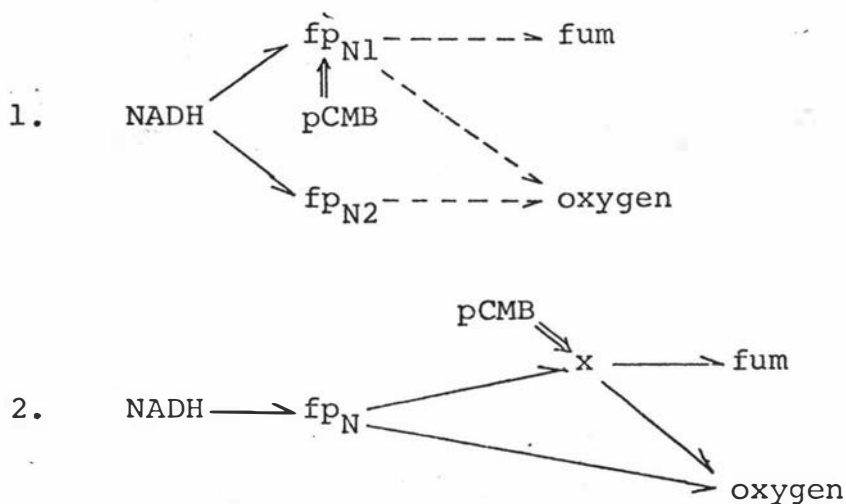
membranes. Membranes in the anaerobic steady state in the presence of pCMB revealed that cytochrome  $b_{553-4}$  was still largely in the reduced state and its reduction level was not markedly affected by pCMB, suggesting that this cytochrome is not on the pathway to fumarate. The difference spectra between uninhibited and inhibited anaerobic steady states (D-lac,fum/D-lac,pCMB,fum) indicate that cytochromes  $b_{556-7}$  and  $b_{562-3}$  are oxidizable by fumarate.

The following diagram (based partly on evidence presented earlier) indicates a pathway consistent with these results.



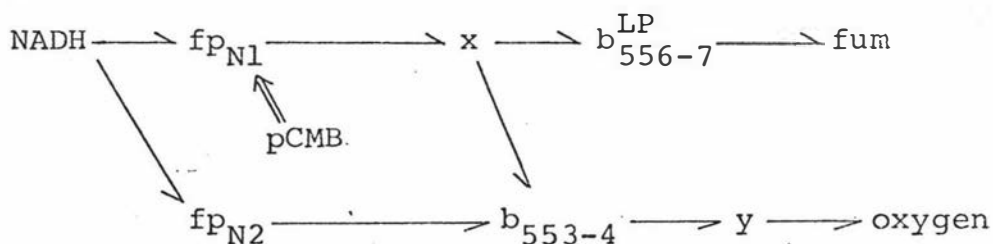
#### NADH dependent electron transport

Activities coupled to NADH all showed a maximum inhibition of 50 to 60%, except NADH-fumarate oxidoreductase, which was completely inhibited by pCMB. Two possible schemes can be suggested to account for this result.



In scheme 1. inhibition is at the dehydrogenase level and two separate NADH dehydrogenases as postulated, one of which is insensitive to pCMB. In scheme 2. the inhibition site must be placed at a site on the path to fumarate but after a branch to oxygen.

Since the cytochrome  $b_{553-4}$  appears to be largely reduced in the inhibited anaerobic steady state whereas cytochromes  $b_{562-3}$  and  $b_{556-7}$  are less fully reduced, a scheme similar to that for D- and L-lactate can be postulated, with the addition of a pCMB insensitive route allowing reduction of cytochrome  $b_{553-4}$  on an aerobic pathway.



#### Succinate dependent electron transport

Electron transport activities coupled to succinate were not strongly inhibited by pCMB, the maximum inhibition being 20 to 30% at  $10^{-3}$  M pCMB. It was not possible to test benzyl viologen-fumarate oxidoreductase directly due to reduction of pCMB by  $\text{KBH}_4$ .

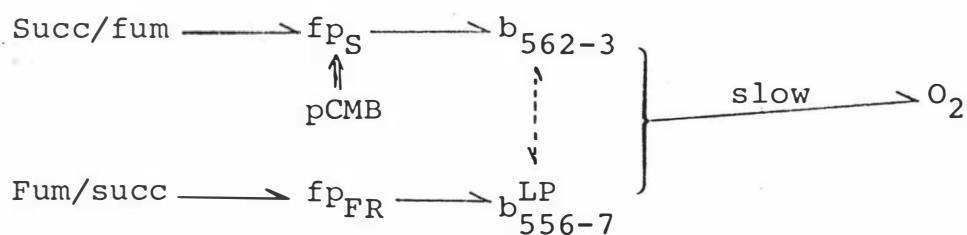
The anaerobic steady state spectra provide no indication of a pCMB sensitive site between the b type cytochromes and fumarate. Moreover, there was no difference between inhibited and uninhibited reduction levels of cytochrome b in the substrate reduced or aerobic steady states with



succinate as reducing substrate. Thus there is no indication from these observations that succinate dehydrogenase and fumarate reductase activities are due to different enzyme systems. This contrasts with the results of Kroger (1975) who found that succinate- $\text{Fe}(\text{CN})_6$  oxidoreductase activity in *Vibrio succinogenes* was sensitive to p-chloromercuriphenyl-sulfonate but that fumarate reductase was not.

The one observation from the experiments using pCMB as an inhibitor which suggests distinct succinate dehydrogenase and fumarate reductase systems is the marked effect of pCMB on the anaerobic steady state spectrum with succinate as reductant. This effect of pCMB on the reduction level of  $b_{562-3}$  is consistent with the suggestion made earlier that this cytochrome is closely associated with a succinate dehydrogenase system but can also be oxidized by a separate fumarate reductase.

In order to reconcile the scheme presented below with the finding that pCMB has had no effect on the reduction level of  $b_{562-3}$  in the aerobic steady state, it is necessary to postulate that the rate of oxidation of  $b_{562-3}$  by oxygen is slow relative to the rate of which it can be reduced by succinate either via fumarate reductase or a separate succinate dehydrogenase.



### 3.2.5: Inhibitory effects of HOQNO on electron transport processes and cytochrome reduction

As described in the introduction (Section 1.2), the inhibitors HOQNO and NOQNO have been widely used to study electron transport processes in bacteria. HOQNO was used in one of the earliest studies of anaerobic electron transport in propionibacteria by deVries *et al* (1973) who stated that it was 'an inhibitor of cytochrome b function' citing Cox *et al*, (1970) as the basis of the statement. In fact, Cox *et al* did not state that HOQNO was an inhibitor of cytochrome b function. They suggested, on the basis of mutant studies indicating that ubiquinone functioned in two different parts of the electron transport chain, that HOQNO inhibited both the oxidation and reduction of cytochrome b. The exact site and mode of action of the alkyl-hydroxyquinoline-N-oxides appears to be still uncertain. The earliest studies of these compounds (Lightbown and Jackson, 1956) indicated a site between cytochrome b and  $c_1$  in the succinoxidase system of mitochondria. However, subsequent work with bacterial systems indicates that it acts by blocking quinone function. Thus Singh and Bragg (1975) found that it blocked electron transport to fumarate in a cytochrome-less mutant of *E. coli*. Kroger and Dadak (1969) also concluded that HOQNO acted at the quinone site in the electron transport chain of *B. megaterium*. The conflicting findings of deVries *et al* (1973 and 1977) and of Schwartz and Sporkenbach (1975) from studies of the effects of these inhibitors on electron transport in propionibacteria have been described in the

introduction. The structural similarities of HOQNO with naphthoquinones (Cornforth and James, 1956) suggest that it may act as an antagonist of reactions involving menaquinone.

#### 3.2.5.1: Inhibition of electron transport activities

Inhibition by HOQNO of electron transport activities in membranes prepared from *P. shermanii* as described previously is shown in Figure 3.2.5.1. Oxygen and fumarate reductases were inhibited similarly for all substrates with an  $I_{50}$  about  $10^{-5}$  M ( $pI_{50}/E$  about 3.6). This concentration of HOQNO is considerably higher than the concentration of NOQNO required to give comparable inhibition in the work of Schwartz and Sporkenbach (1975) who observed a  $pI_{50}/E$  for NADH and D-lactate-oxygen oxidoreductases of about 5 to 6.

The  $Fe(CN)_6$  and DICPIP oxidoreductase systems were even less sensitive to inhibition by HOQNO than the oxygen and fumarate oxidoreductase systems. At the highest concentration of HOQNO attainable, only D-lactate and NADH- $Fe(CN)_6$  oxidoreductase were significantly inhibited and even then only by about 20 to 30%. Interestingly the NADH-DICPIP oxidoreductase activity was stimulated by up to 2.3 fold by the presence of HOQNO (Fig. 2.3.5.1,D). A similar stimulation was not found for the other donors. That the stimulation of NADH-DICPIP oxidoreductase is due to removal of competition with oxygen is evident from Table 3.2.5.1 which shows that no such stimulation occurred in the absence of oxygen.

Figure 3.2.5.1: Effects of HOQNO on Electron Transport Activities.

The activities of various electron transport reactions are expressed as a percentage control in the absence of inhibitor. D-lactate, ● (+PMS, ■); L-lactate, ○; NADH, ◇; succinate, △(+PMS ▲).

- A. Oxygen consumption
- B. Fumarate Reduction
- C.  $\text{Fe}(\text{CN})_6$  Reduction
- D. DICPIP Reduction

Figure 3.2.5.1

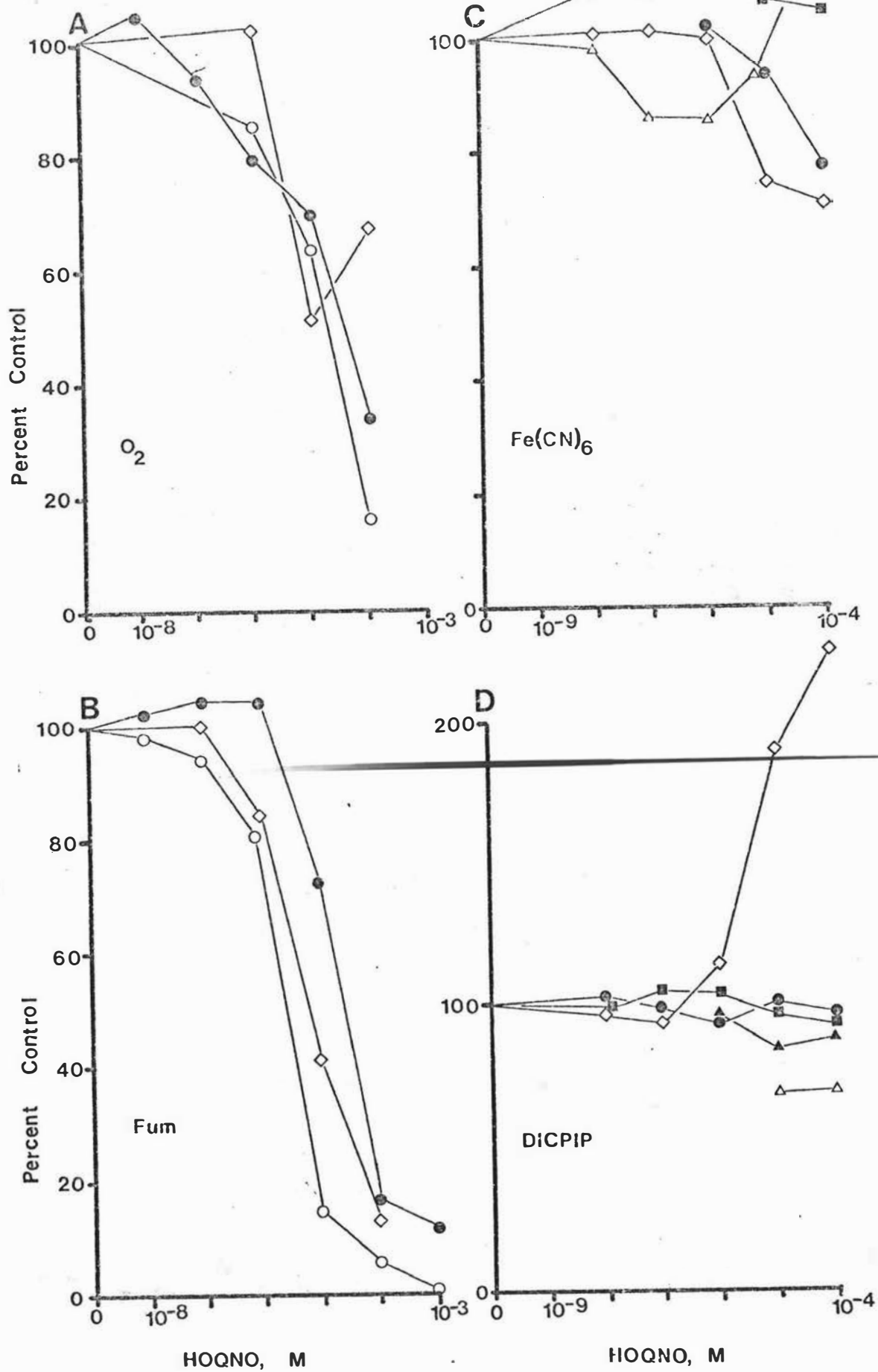


Table 3.2.5.1: The Effect of Oxygen on HOQNO Stimulation

Oxygen (a)	HOQNO (b)	NADH-DICPIP oxidoreductase $\mu\text{mol min}^{-1}\text{mg}^{-1}$	R (c)
+	-	3.67	0.02
+	+	5.47	0.03
-	-	7.20	0.10
-	+	7.40	0.02

(a) + indicates the reagents were prepared equilibrated with air, - indicates the reagents were prepared under nitrogen.

(b) assay concentration of  $10^{-3}$  M.

(c) R is the difference between duplicates.

### 3.2.5.2: Effects of HOQNO on cytochrome reduction

The effects of HOQNO on cytochrome reduction were measured by use of low temperature spectrophotometry with methods described previously. Not all the spectra observed are shown because the effects of HOQNO on cytochrome reduction were very minor for all substrates. Only the spectra observed when NADH was reducing substrate are shown in Figure 3.2.5.2, where  $pI/E$  was 0.065 (HOQNO present at 0.645 mM). Spectra with other reducing substrates were similar. The concentration of HOQNO used is near the aqueous limit of solubility and gave strong inhibition of oxidase and fumarate reductase activities (Fig. 3.2.5.1).

HOQNO caused a decreased in the b peak of substrate-reduced membranes but showed virtually no effect on the aerobic or anaerobic steady state b or d peaks.

### 3.2.5.3: Conclusions from studies with HOQNO

It appears from these results that electron transport processes in membrane particles from *P. shermanii* as prepared in this study are less sensitive to HOQNO inhibition than in the study of Schwartz and Sporckenbach (1975). No satisfactory explanation can be offered for this discrepancy. It is possible that the carbohydrate material present with the membrane particles used in this work may have limited accessibility of HOQNO in some way.

Schwartz and Sporckenbach used NOQNO rather than HOQNO and it is known that some electron transport processes are much more sensitive to NOQNO than HOQNO (Lightbown and Jackson, 1956). DeVries *et al* (1977), however, used HOQNO at a concentration of about  $10^{-5}$  moles  $\text{gm}^{-1}$  and observed a small decrease in the level of reduction of cytochrome b in both the aerobic and anaerobic steady states (from 53% reduction in uninhibited membranes to 47% in inhibited membranes).

Thus there are three conflicting findings concerning the effect of HOQNO or NOQNO on electron transport in membranes from propionibacteria:

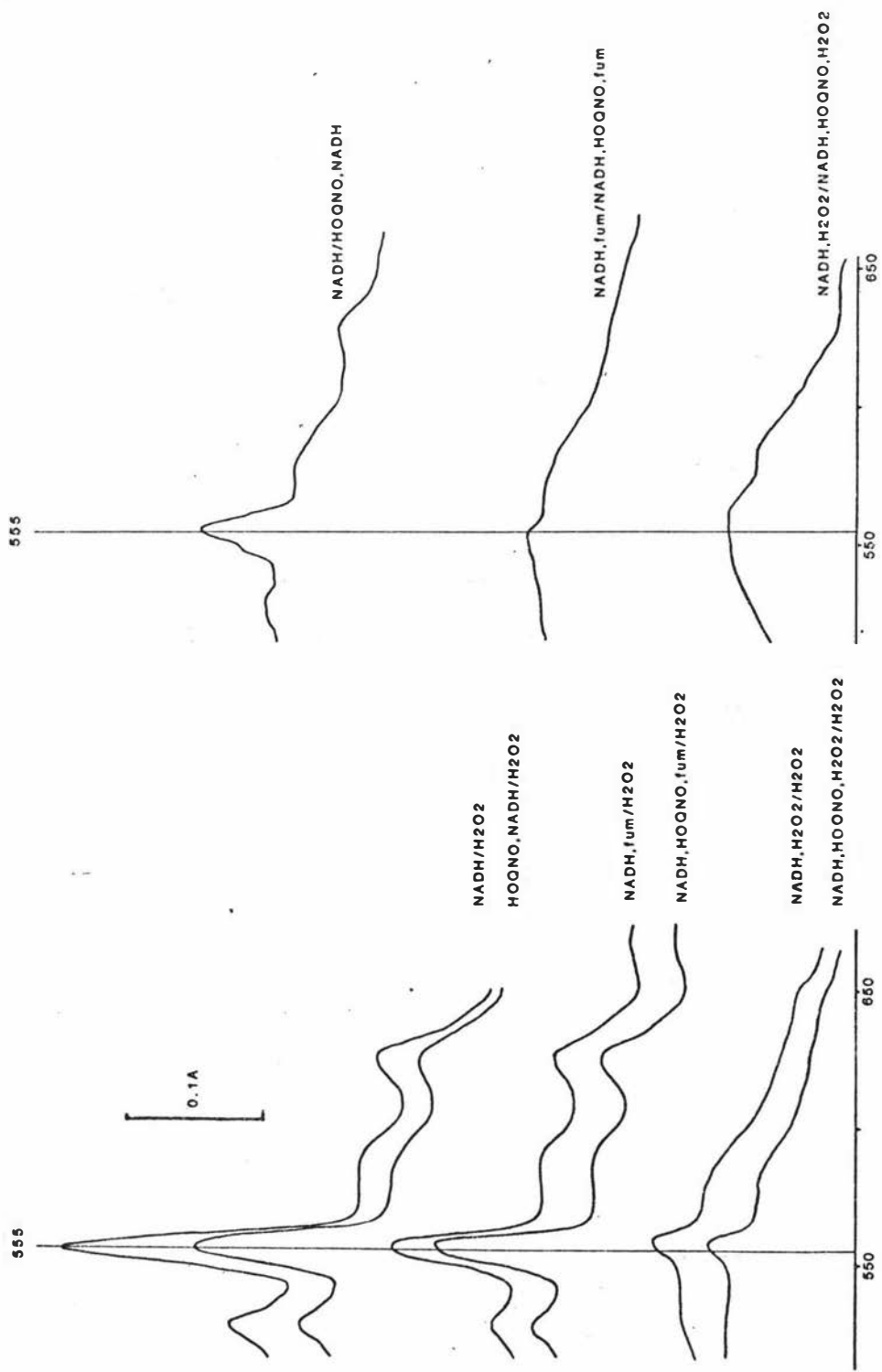
1. HOQNO causes a small decrease in the anaerobic steady state reduction level of cytochrome b (deVries *et al* (1977)).
2. NOQNO causes an increase in the anaerobic steady state reduction level of cytochrome b (Schwartz and Sporckenbach, 1975).

Figure 3.2.5.2: Effects of HOQNO on Cytochrome Reduction

Spectra presented were measured at low temperature as described in the methods section (2.4.9) and section 3.2.1. Membranes were present at a protein concentration of  $0.75 \text{ mg ml}^{-1}$ . NADH, fumarate and  $\text{H}_2\text{O}_2$  were added to final concentrations of 3 mM, 6 mM and 0.1%. HOQNO was present at 0.645 mM.



FIGURE 3.2.5.2



3. HOQNO has little effect on the reduction level of the cytochrome b peak in the anaerobic (or aerobic) steady state (this study).

Sone (1972) also found that NOQNO did not inhibit either NADH-fumarate oxidoreductase or NADH-oxygen oxidoreductase activities in membrane particles from *P. arabinosum*.

It is clear that the effects of HOQNO and NOQNO require a more extensive study to resolve these conflicting findings.

#### 3.2.6: Effects of cyanide on electron transport

Cyanide has been widely used as an inhibitor of terminal oxidases. It binds to cytochrome aa<sub>3</sub> (Van Buuren *et al*, 1972), cytochrome d (Pudek and Bragg, 1974) and cytochrome o (Webster and Liu, 1974). Cytochrome d has been shown to be less sensitive to cyanide than cytochrome o in several bacteria (Oka and Arima, 1965; Jones, 1973; Pudek and Bragg, 1974).

Sone (1972), working with *P. arabinosum*, reported that NADH-oxygen oxidoreductase was inhibited 74% and 89% by KCN concentrations of 1 mM and 2 mM, respectively, whereas with NADH-fumarate oxidoreductase, the inhibition was only 13% and 43% respectively. He also reported that lactate (presumably D-lactate) dependent oxygen consumption in membranes from *P. arabinosum* was practically insensitive to 1 mM cyanide (3% inhibition) whereas NADH dependent oxygen consumption was almost completely inhibited, which

suggested that different substrates may be oxidized via different terminal oxidases. Bonartseva *et al* (1973) also reported complete inhibition of NADH oxidase activity by 1 mM cyanide in freshly prepared membrane particles from *P. shermanii* and *P. petersonii* although the inhibitory effect decreased on storage of the membrane preparation. However, Schwartz and Sporkenbach (1975) reported that oxidase activity in membranes of *P. shermanii* was inhibited by cyanide only at concentrations of 10 mM or greater.

Cyanide at 3.3 mM has been reported to stimulate a partially purified lactate-DICPIP oxidoreductase from *P. pentosaceum* by a factor of 1.25 (Molinari and Lara, 1960).

#### 3.2.6.1: Effects of cyanide on electron transport activities

The inhibition of oxygen consumption in membranes of *P. shermanii* was investigated over the range 0 to 100 mM cyanide (Figure 3.2.5.1,A). These results indicate that NADH dependent oxygen reduction is inhibited to a maximum of about 92% at 20 mM cyanide. D-lactate-oxygen oxidoreductase is less completely inhibited by cyanide than that of NADH. However, the difference in cyanide sensitivity of the NADH and D-lactate oxidase systems is very much less than that reported by Sone (1972). In the presence of PMS, D-lactate dependent oxygen uptake is even less strongly inhibited by cyanide. Analysis of the data for D-lactate oxidase inhibition by use of Dixon plots (Figure 3.2.6.1,B) suggests that there are two binding sites for cyanide. Cyanide inhibition at the less sensitive binding site ( $K_i$  about 22 mM) appears to be responsible for inhibition of D-lactate

-dependent oxygen consumption in the presence of PMS, indicating that PMS bypasses the more sensitive site. L-lactate-dependent oxygen consumption was also inhibited by cyanide, but to an even lesser extent than D-lactate-oxygen oxidoreductase, and in the presence of PMS there was virtually no inhibition of L-lactate dependent oxygen consumption. Succinate-dependent oxygen consumption was also very little affected by cyanide, being inhibited to a maximum of 20% by 20 mM cyanide.

In contrast to the effect on oxygen consumption, there was no inhibition of fumarate consumption at 10 mM cyanide. However, there was almost complete inhibition at 100 mM cyanide with NADH and D-lactate and 75% inhibition with L-lactate (Figure 3.2.6.1,C). The inhibition of fumarate consumption at this high cyanide concentration may possibly be due to a non-specific effect of high ionic strength. However, choline chloride (100 mM) or potassium chloride (100 mM) did not cause a similar inhibition (30% inhibition for the former and none with the latter).

Cyanide also inhibited D- and L-lactate- and NADH-DICPIP oxidoreductase activity (Figure 3.2.6.1,D), but the inhibitory effect again varied according to the reducing substrate. D-lactate-DICPIP oxidoreductase was most sensitive, 2.5 mM cyanide giving 50% inhibition. L-lactate-DICPIP oxidoreductase showed only a small decrease in activity at low concentrations and then strong inhibition at 100 mM cyanide.  $\text{NAD}^{\text{H}}\text{-DICPIP}$  oxidoreductase showed a twofold rise in specific activity with increasing cyanide concentrations up to 10 mM; inhibition occurred at higher concentrations. The

stimulation of NADH-DICPIP oxidoreductase activity is probably due to removal of oxygen competition. As reported earlier (Section 3.1.2), NADH-DICPIP oxidoreductase (unlike D-lactate-DICPIP oxidoreductase) is considerably higher under anaerobic conditions than aerobic conditions and this indicates that there is competition between DICPIP and oxygen for electrons from NADH. This is also a possible explanation for the stimulation observed by Molinari and Lara (1960).

#### 3.2.6.2: Effect of cyanide on stoichiometry of oxygen consumption

Total oxygen consumption per mole of substrate oxidized was also measured at cyanide concentrations varying between 0 and 100 mM (Table 3.2.6.1). The ratio of oxygen consumed to substrate oxidized is expected to vary between 0.5 and 1.0, depending on the proportion of oxygen reduced to  $\text{H}_2\text{O}$  or  $\text{H}_2\text{O}_2$ . In the case of an oxidase system catalysing reduction of oxygen to water, the ratio of oxygen consumed to substrate oxidized would be 0.5 ( $2\text{H}^+ + 2\text{e}^- + \frac{1}{2}\text{O}_2 \rightarrow \text{H}_2\text{O}$ ), whereas oxidase systems reducing oxygen to hydrogen peroxide would give a ratio of 1.0 ( $2\text{H}^+ + 2\text{e}^- + \text{O}_2 \rightarrow \text{H}_2\text{O}_2$ ). For all three substrates, the ratio is higher in the presence of cyanide than in the absence of cyanide (Table 3.2.6.1), suggesting that in the presence of cyanide an increased proportion of the oxygen uptake is mediated by a peroxygenic oxidase. Further evidence for a change in the nature of the terminal oxidase in the presence of cyanide is evident from the shape of the oxygen uptake curves (Figure 3.2.6.1,F).

Table 3.2.6.1: Effect of cyanide on the stoichiometry of mole of oxygen consumed per mole of substrate oxidised by membrane particles from *P. shermanii*.

Cyanide Concentration (mM)	Mole of oxygen per mole substrate		
	NADH (a)	D-lactate	L-lactate
0	0.49	0.52	0.62
2.5	0.51	0.54	0.60
5.0	0.50	0.56	0.685
10.0	0.545	0.60	0.71
25.0	0.575	0.61	0.77
50.0	0.69	0.66	0.75
75.0	0.60	0.59	-
100	0.685	0.57	0.78

(a) All substrates were used at a final concentration of 0.2 mM. Total oxygen uptake was measured with an oxygen electrode using the reaction mixture described in the text (Section 2.5.3) and allowing oxygen uptake to go to completion.

The rate of oxygen consumption in the absence of cyanide was zero order (linear with time) with respect to oxygen concentration until very low oxygen concentration was reached. In the presence of 50 mM cyanide the rate curve is first order with respect to oxygen concentration over the whole range of oxygen concentration. This indicates that the  $K_m$  values for oxygen of the oxidase system operating in the presence and absence of cyanide are very different.

Figure 3.2.6.1: Effects of Cyanide on Electron Transport Activities

A. Effect of cyanide on rate and total oxygen consumption.

Membrane particles suspensions were preincubated in open oxygen electrode chambers with the appropriate concentration of cyanide and 0.01  $\mu\text{mol}$  reducing substrate for 3-5 min before inserting the electrode and adding further substrate. All substrates were present at a final concentration of 3 mM. Substrates used were: NADH,  $\odot$ ; D-lactate,  $\blacksquare$ ; D-lactate + 0.5 mM PMS,  $\square$ ; L-lactate,  $\blacktriangle$ ; L-lactate + PMS,  $\triangle$ ; Succinate, X.

B. Dixon plots of some of the data presented in Figure 3.2.6.1,A.

The data for L-lactate have been included but the regression lines have been omitted for clarity. Symbols are as in Figure 3.2.6.1,A.

Figure 3.2.6.1

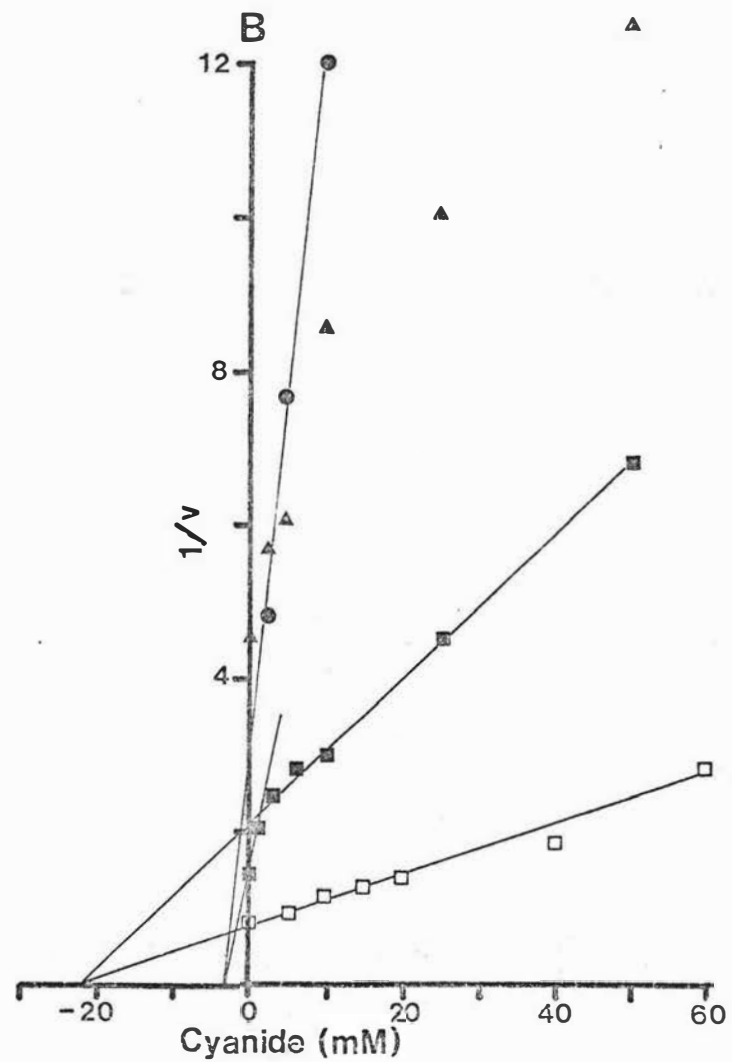
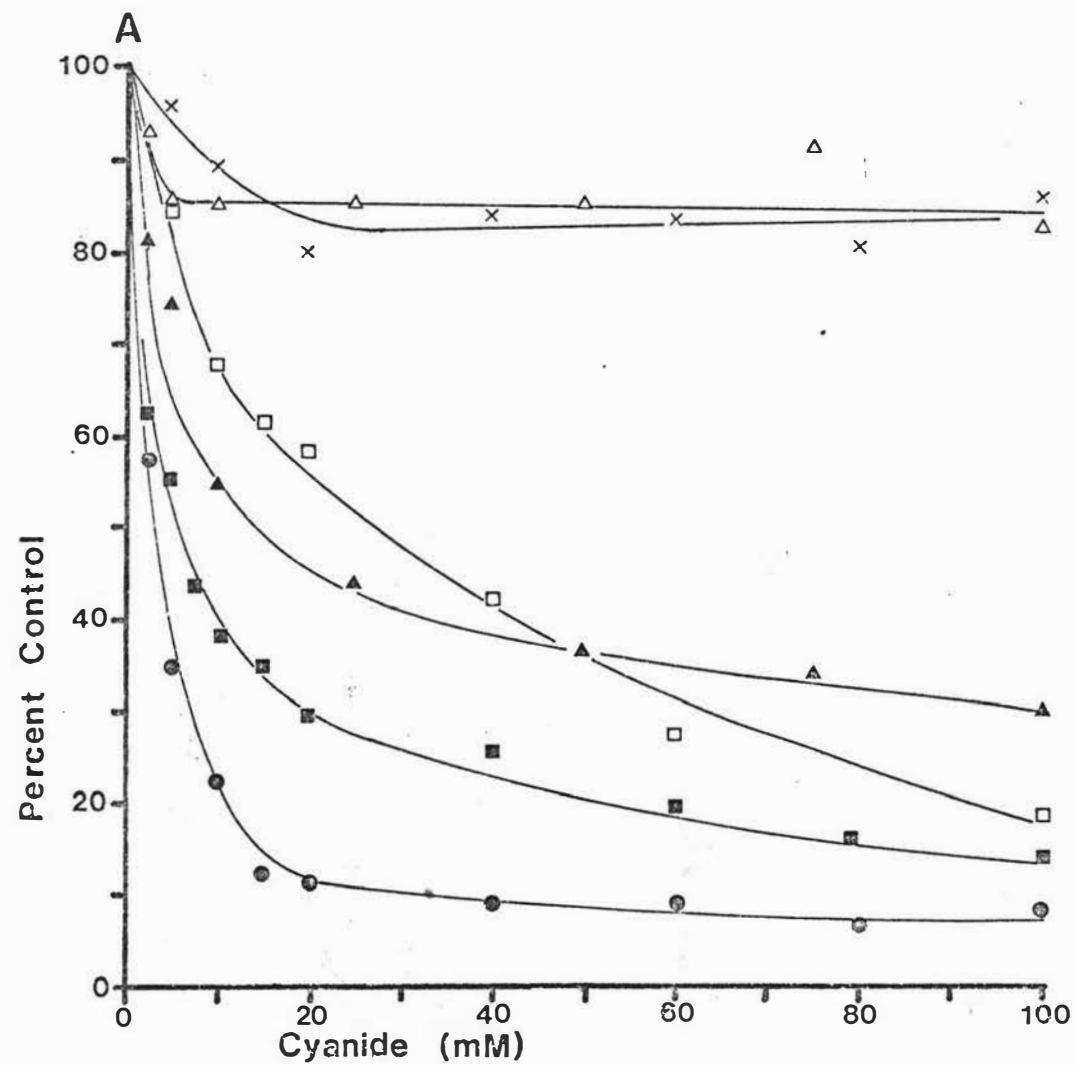




Figure 3.2.6.1: Effects of Cyanide on Electron Transport  
Activities (continued)

C. Cyanide inhibition of fumarate reduction

Substrates and fumarate were present at final concentrations of 1 mM. Activities are recorded as % control determined in the absence of cyanide. D-lactate,  $\blacksquare$ ; L-lactate,  $\blacktriangle$ ; NADH,  $\bullet$ .

D. Cyanide inhibition of DICPIP reductase

Activities of D-lactate-DICPIP oxidoreductase ( $\blacksquare$ ), L-lactate-DICPIP oxidoreductase ( $\blacktriangle$ ) and NADH-DICPIP oxidoreductase ( $\bullet$ ) were measured against cyanide concentrations of  $10^{-5}$  to  $10^{-1}$  M and recorded as a percent control determined in the absence of cyanide.

Figure 3.2.6.1

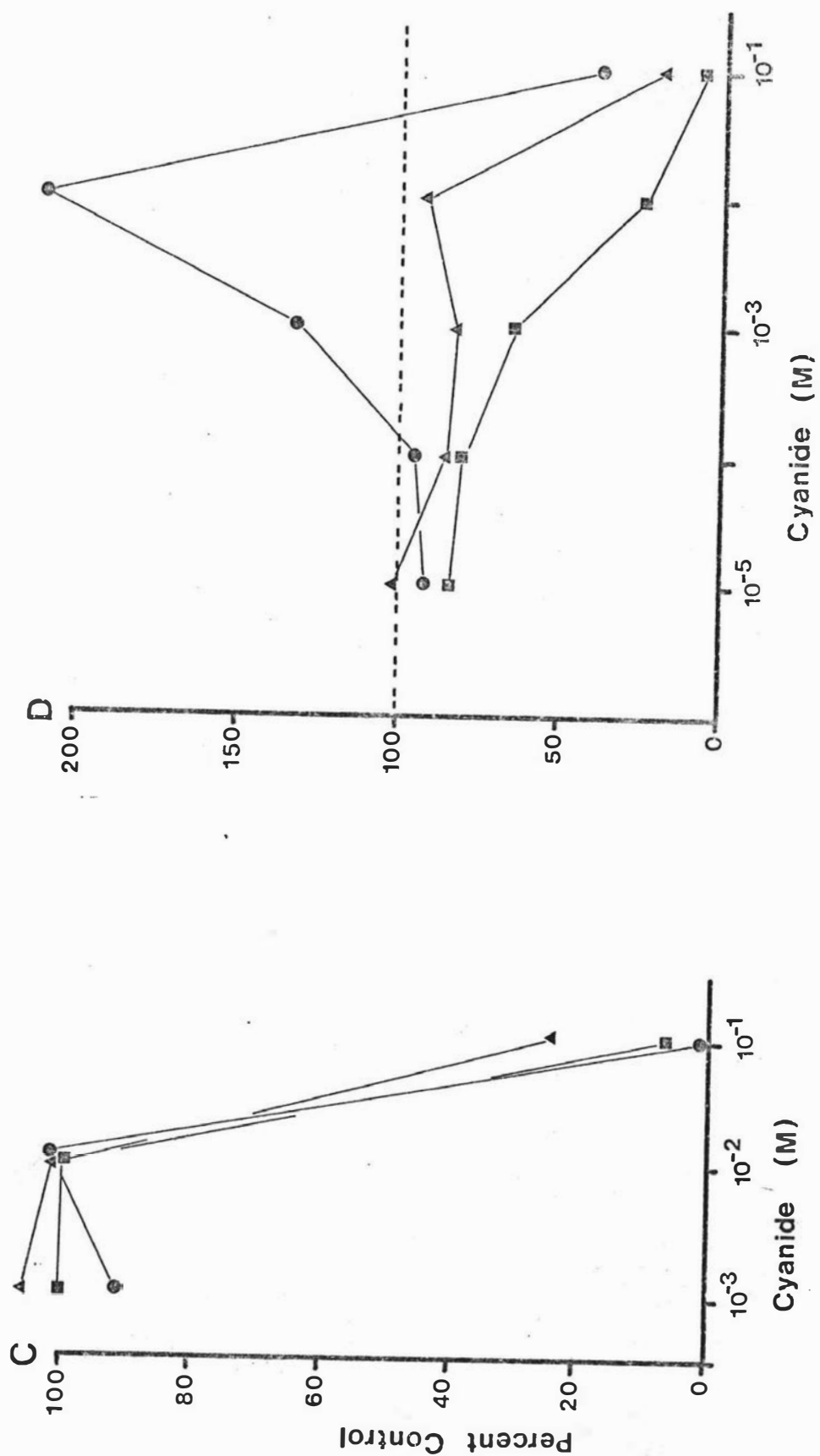
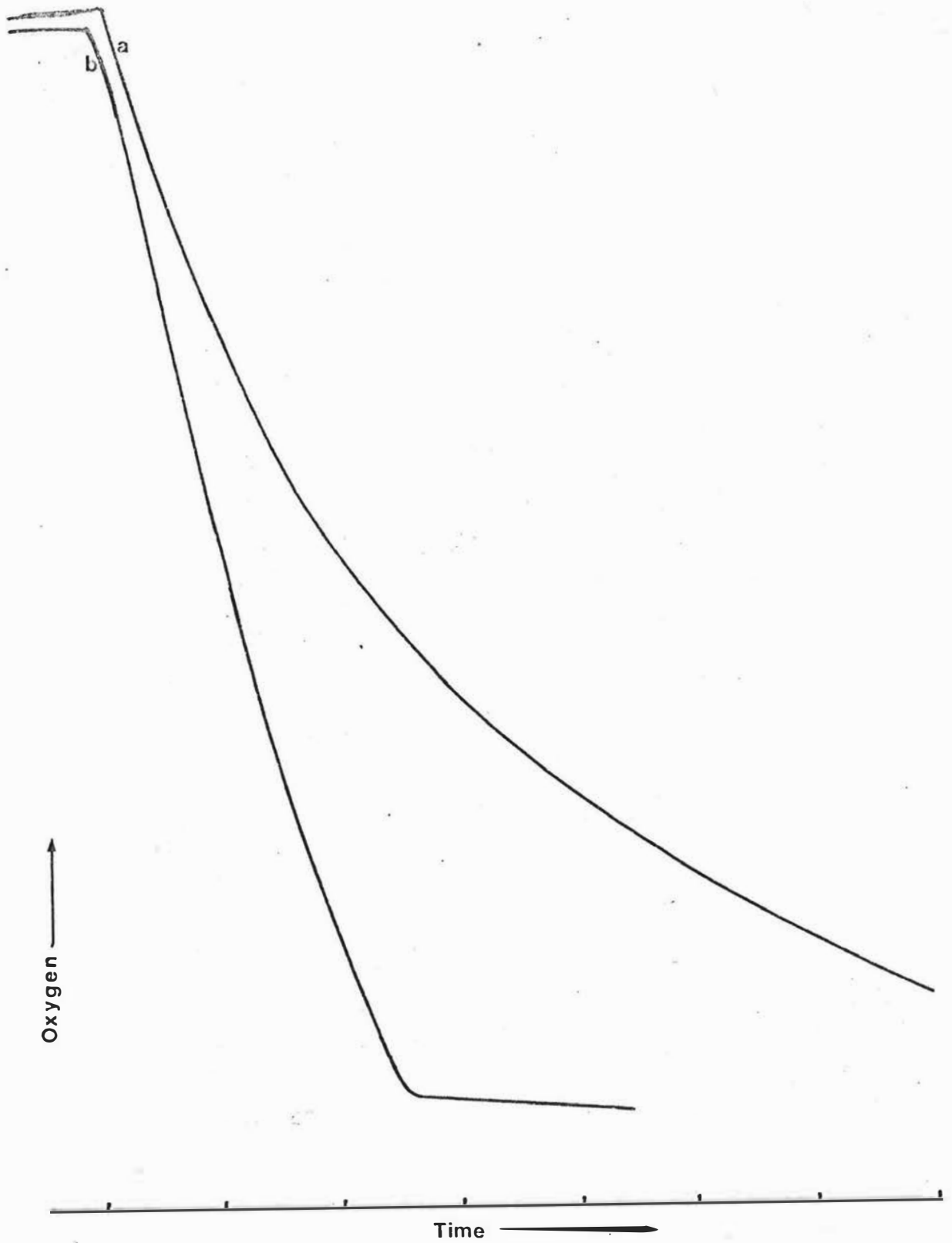


Figure 3.2.6.1: Effects of Cyanide on Electron  
Transport Activities

E. Oxygen <sup>uptake</sup> tracing in the presence and absence of  
cyanide

Tracings shown are for D-lactate oxidase in the  
presence ( a ) and absence ( b ) of cyanide (50 mM)  
assayed using the oxygen electrode as described in  
methods (2.5.3).

Figure 3.2.6.1,E



In the case of L-lactate, the ratio of oxygen consumed to substrate oxidized in the absence of cyanide is markedly greater than 0.5 which could be interpreted to indicate that a significant proportion of L-lactate dependent oxygen uptake by membrane particles is due to a peroxigenic flavoprotein oxidase or to direct autoxidation of the primary dehydrogenase. This would be consistent with the high cyanide-insensitive component of the L-lactate oxygen oxidoreductase activity (Figure 3.2.5.1,A). However, such an explanation is difficult to reconcile with the presence of catalase activity in the membrane preparation (Table 3.2.6.2) since in the absence of cyanide this should immediately decompose any  $H_2O_2$  formed, giving an oxygen: substrate ratio of 0.5.

Table 3.2.6.2: Cyanide Inhibition of Catalase

Cyanide (mM)	% Activity
0	100
0.333	25
0.667	14.7
1.667	7.9
5.0	0

Catalase was assayed by measuring the rate of  $O_2$  production from  $H_2O_2$  using the oxygen electrode. Oxygen-free buffer under  $N_2$  gas plus membrane ( $0.07 \text{ mg ml}^{-1}$ ) were preincubated in the electrode cell at  $30^\circ\text{C}$  and the assay was initiated by addition of  $0.1 \text{ ml } H_2O_2$  (final

concentration about 0.05 mM). Uninhibited specific activity was  $0.551 \mu\text{mol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ . Specific activities for oxygen consumption of this same membrane preparation were 0.75, 3.9 and  $2.4 \mu\text{mol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$  for L-lactate, D-lactate and NADH, respectively.

That  $\text{H}_2\text{O}_2$  is actually produced during substrate oxidation in the presence of cyanide was confirmed by iodometric determination of  $\text{H}_2\text{O}_2$  in reaction mixtures containing membrane suspension, substrate and 50 mM cyanide which had been incubated for a sufficient period to allow oxidation of substrate. In Table 3.2.6.1, the measured values for  $\text{H}_2\text{O}_2$  produced per mole of substrate oxidized are compared with those calculated from the values for the stoichiometry of oxygen consumed per mole of substrate oxidized given in Table 3.2.6.3 at 50 mM cyanide.

Table 3.2.6.3: Production of  $\text{H}_2\text{O}_2$  in the presence of 50 mM Cyanide

Substrate	$\mu\text{mol O}_2/\mu\text{mol}$ measured	substrate calculated <sup>(a)</sup>
D-lactate	0.30	0.32
L-lactate	0.18	0.50
NADH	0.155	0.38

(a) the basis for calculation of  $\text{H}_2\text{O}_2$  production is as follows. If  $x$  is the mole fraction of substrate oxidized via a peroxigenic oxidase and  $1-x$  the mole fraction oxidized via a water producing oxidase, then the total  $\text{O}_2$  uptake per mole of substrate oxidized will be  $x + 0.5(1-x)$ . Using the values for  $\text{O}_2$  uptake per mole of substrate oxidized at 50 mM cyanide given in Table 3.2.6., the value of  $x$  can be found for each substrate.

Apart from D-lactate, the measured peroxide production is less than that calculated from the  $O_2$ :substrate stoichiometry. However, the errors in estimating small quantities of  $H_2O_2$  titrimetrically are considerable, so undue significance should not be attached to the lack of agreement between measured and calculated values. The significant finding is that  $H_2O_2$  production does occur in the presence of cyanide, which is consistent with the proposed explanation for the increase in  $O_2$ :substrate stoichiometry in the presence of cyanide. No  $H_2O_2$  was detectable in the absence of cyanide with any substrate.

#### 3.2.6.3: Effects of cyanide on cytochrome reduction levels

The effects of cyanide on reduction and oxidation of the cytochromes in membranes of *P. shermanii* were measured using low temperature spectrophotometry in systems identical to those previously described. Because cyanide did not inhibit fumarate consumption at 10 mM but did at 100 mM, these two concentrations were used in the hope that this would permit the resolution of those cytochromes involved in aerobic and anaerobic respiration. Because two different concentrations of cyanide were used, the figures (3.2.6.2, A and B) presenting these spectra are necessarily more complicated. To simplify the discussion comments on these spectra, only the anaerobic and aerobic steady state spectra are shown (the substrate reduced spectra do not add any further information). Also, due to limitations of space, difference spectra of membranes in the anaerobic steady state

and the aerobic steady state versus oxidized membranes are not shown for experiments in which NADH was the reducing substrate. However, the difference spectra shown on the right hand side of Figures 3.2.5.2, A and B indicate the effects of cyanide on the absorption spectra for all three substrates under the conditions tested.

The most obvious effect of cyanide can be seen in the aerobic steady state spectra (Figure 3.2.6.2, B). These spectra clearly indicate that cyanide, at 10 and 100 mM, inhibits oxidation of cytochrome b.

With D-lactate, the cytochrome b peak in the aerobic steady state is more reduced in the presence of 10 mM cyanide than with 100 mM, presumably because the higher concentration inhibits both reduction and oxidation of cytochrome b. The difference spectrum (D-lac, 10/100 (Figure 3.2.5.2, B) indicates that it is the reduction of  $b_{553-4}$  which is mainly inhibited by 100 mM cyanide.

With NADH, cyanide also inhibits oxidation of the b cytochromes, but in the presence of 100 mM cyanide the cytochromes (mainly  $b_{553-4}$  and  $b_{556-7}$ ) are more reduced than in 10 mM cyanide (spectrum 10/100, NADH,  $H_2O_2$  (Figure 3.2.6.2, B) showing that the relative effects of cyanide on the oxidizing and reducing sides of the b cytochromes are different for D-lactate and NADH.

With succinate, for which cyanide inhibition of oxygen consumption was very minor, inhibition is again primarily on the oxidizing side of the b cytochromes (Figure 3.2.6.2, C). Cyanide at 100 mM increased the level of aerobic steady state cytochrome b reduction over that observed with 10 mM cyanide.



Figure 3.2.6.2: Effects of Cyanide on Cytochrome  
Reduction

Membranes were prepared and observed using low temperature spectrophotometry as described in 2.6.1 and 3.2.1 in the presence of 0, 10 and 100 mM cyanide. Spectra for each reducing substrate were prepared in separate experiments. Membranes were present at a protein concentration of 0.83 mg ml<sup>-1</sup> with spectra for NADH, L-lactate and succinate, and 0.6 mg ml<sup>-1</sup> with spectra for D-lactate.

Since two different concentrations of cyanide have been used, a slightly different convention has been adopted for labelling the spectra. The numerals 0, 10 and 100 refer to the millimolar concentration of cyanide used. Thus, the spectrum labelled 0/10, for example, is the difference spectrum obtained when the uninhibited steady-state sample is in the sample cuvette, while the steady-state sample containing 10 mM cyanide is in the reference cuvette.

- A. Anaerobic steady-state membranes
- B. Aerobic steady-state membranes

FIGURE 3.2.6.2, A

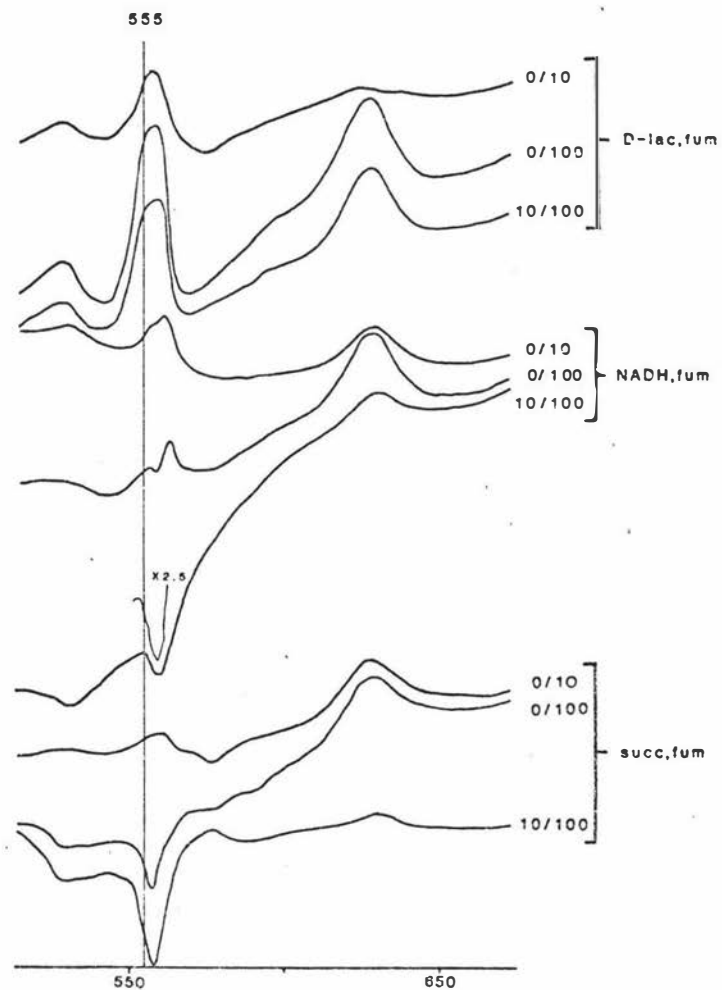
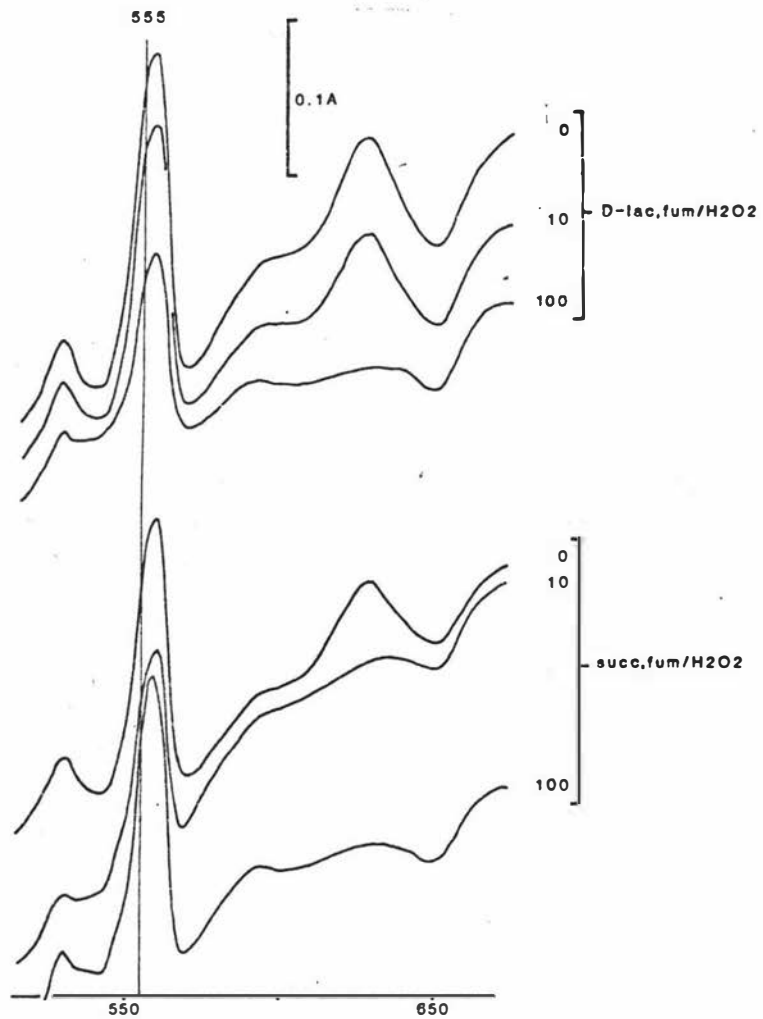
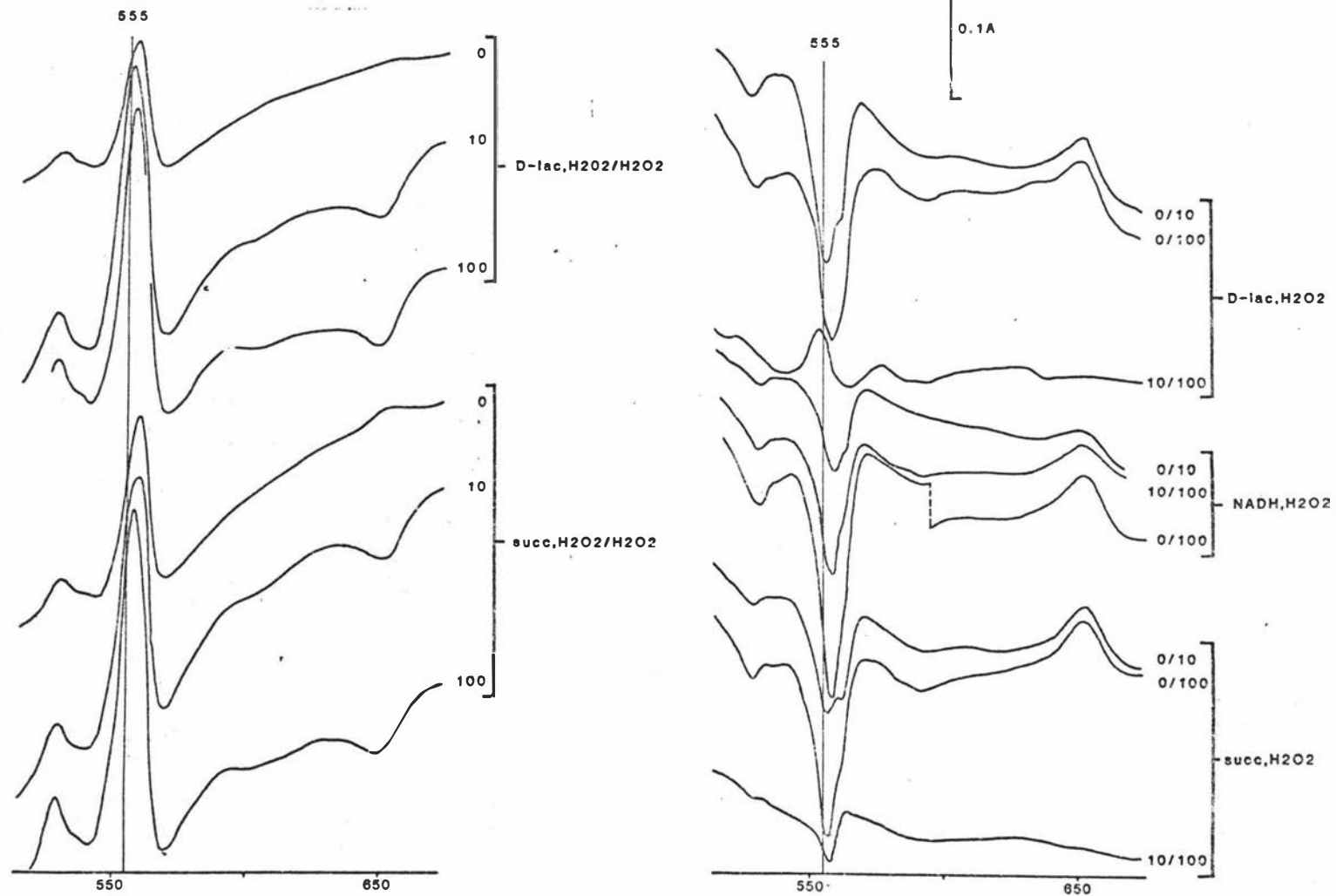


FIGURE 3.2.6.2, B



The reduced cytochrome d peak has disappeared in all the aerobic steady-state spectra, however, in the inhibited spectra, the disappearance has not been accompanied by an increase in oxidized cytochrome d since there is a trough in the spectrum at 650 nm (due to oxidized cytochrome d in the reference cuvette). Note that there is no similar trough in the uninhibited spectrum. This difference can be attributed to the formation of cyanocytochrome d in the presence of cyanide (Pudek and Bragg, 1974; Kaufmann and van Gelder, 1974). This indicates that cytochrome d is a site of cyanide binding with a  $K_1$  value that is less than 10 mM.

The anaerobic steady-state spectra (Fig. 3.2.6.2,B) show inconsistencies with respect to the effect of cyanide on cytochrome d in that there is a reduced cytochrome d peak at 10 mM cyanide but not at 100 mM cyanide with D-lactate. Cyanide should not react with reduced cytochrome d under anaerobic conditions (Pudek and Bragg, 1974), so the disappearance of the d peak at 100 mM cyanide may indicate that a trace of oxygen was added with either cyanide or the fumarate. Alternatively, it may be that at very high concentrations (100 mM) cyanide can bind to cytochrome d even under anaerobic conditions.

The anaerobic steady-state difference spectra obtained with D-lactate indicate that 10 mM <sup>and 100 mM</sup> cyanide both inhibit reduction of the b type cytochromes, but that inhibition by 100 mM cyanide is much more significant. Such an inhibition on the reducing side of cytochrome b could account for the inhibition of D-lactate-fumarate oxidoreduc-

tase activity at 100 mM cyanide (Figure 3.2.6.2,A).

With NADH in the anaerobic steady state (Figure 3.2.6.2,B), the effect of cyanide is much less marked and there is little difference between the spectra at 10 and 100 mM cyanide. This is consistent with other data indicating that inhibition at a site other than the terminal oxidase at high cyanide concentrations is much more marked with D-lactate than with NADH.

#### 3.2.6.4: Conclusions from experiments using cyanide

A summary of conclusions and indications from inhibitor studies with cyanide are as follows:

1. Cyanide inhibits membrane-coupled reduction of oxygen at concentrations of cyanide between 1 and 10 mM and inhibits fumarate reduction at higher concentrations (10 to 100 mM).
2. The inhibition of oxygen consumption with D-lactate as reducing substrate appears to have two sites of inhibition, with  $I_{50}$  values, as estimated from Dixon plots, of approximately 2 and 22 mM cyanide. The low affinity site is presumably responsible for inhibition of D-lactate dependent oxygen consumption in the presence of PMS since the Dixon plot for this activity only indicates a single site with an  $I_{50}$  value of 22 mM. With NADH and L-lactate, the existence of a low affinity site is less evident.

The high affinity site, bypassed in the presence of PMS, is presumably the terminal oxidase. Spectral evidence indicates that cyanide binds to cytochrome d and the

relatively high concentration of cyanide required for inhibition of the terminal oxidase is consistent with cytochrome d being the major terminal oxidase, since it is known to be much less sensitive to cyanide inhibition than other cytochrome oxidases in other bacteria (Jones, 1973; Pudek and Bragg, 1974).

3. The oxidation of the b cytochromes is blocked by 10 mM cyanide. In particular, the reduction levels of  $b_{556-7}$  and  $b_{562-3}$  are much higher in the aerobic steady state with cyanide than in the absence of cyanide.

4. Cyanide also inhibits reduction of cytochrome b, but the inhibiting effect is different for different reducing substrates. High cyanide concentrations (100 mM) have a much stronger inhibitory effect on reduction of cytochrome b by D-lactate than with other donors, as indicated by the anaerobic steady state spectra. This is consistent with the greater sensitivity of D-lactate-DICPIP oxidoreductase and D-lactate oxidation in the presence of PMS than the corresponding activities with other donors. The one result not consistent with this interpretation is the apparently complete inhibition of NADH-fumarate oxidoreductase by 100 mM cyanide (Figure 3.2.6.1,C).

5. Oxygen consumption per mole of substrate oxidized increases in the presence of cyanide. This can be accounted for by a shift in the nature of the oxidase mediating oxygen uptake from a cytochrome terminal oxidase (cytochrome d) to a peroxigenic oxidase since the increase in oxygen uptake is

shown to be accompanied by the production of hydrogen peroxide. The peroxigenic oxidase may be a flavoprotein oxidase (possibly electron transfer from the primary dehydrogenase flavoprotein to oxygen (Sone, 1973).

Alternatively, some cytochrome oxidases produce peroxide in the presence of cyanide e.g., the purified NADH-cytochrome oxidoreductase from *Vitreoscilla* sp. (Webster and Liu, 1974).

6. With D-lactate in the aerobic steady state, increased inhibition at 100 mM cyanide over that at 10 mM cyanide was reflected mainly in an increased inhibition of reduction of  $b_{553-4}$  (10/100, D-lac,  $H_2O_2$ ; Figure 3.2.6.2,C). This indicates that  $b_{553-4}$  is on the oxidizing side of an inhibition site of low cyanide affinity.

### 3.2.7: Effects of Fumarate

Sone (1972) observed in oxygen electrode experiments with membranes from *P. arabinosum* that the rate of  $\alpha$ -glycerol phosphate-dependent oxygen consumption was inhibited 71% by fumarate, and he concluded from this that 'the electron transport system seems to use fumarate in preference to oxygen as the terminal electron acceptor'. No further explanation was offered by Sone (1972) and there are no other published studies of the interaction between oxygen and fumarate in propionibacteria.

At an early stage of the present study, it was noticed that oxygen consumption was slightly inhibited by fumarate. This effect was further characterized by studying this inhibition over a range of fumarate concentrations, by examining the effect of cyanide on fumarate inhibition of oxygen uptake, and by observing the effects of fumarate on the reduction level of cytochrome b in the aerobic steady-state.

#### 3.2.7.1: Effects of fumarate on oxygen consumption

Initial experiments performed at an early stage of work for this thesis showed that D-lactate, L-lactate and NADH-oxygen oxidoreductase activities measured with the oxygen electrode were inhibited by 20 to 30% in the presence of 2 mM fumarate. To further characterize the site at which fumarate was acting, this inhibitory effect was measured as a function of fumarate concentration. Both the rate of oxygen uptake and the amount of oxygen consumed per mole of substrate were measured. For the measurement of total oxygen consumption, it was necessary to use lower concentrations of reducing substrate (0.2 mM) so that the amount of substrate supplied was less than the total amount of oxygen available in solution in the reaction mixture.

The presence of 1 mM fumarate inhibited both the rate of oxygen uptake and the amount of oxygen consumed by about 20% for D-lactate, L-lactate (not shown) and NADH (Figure 3.2.7.1). At concentrations above 10 mM there was a further marked inhibition of the rate of oxygen uptake, while at



concentrations greater than 20 mM, the total oxygen uptake was also further inhibited. Both the rate and total oxygen uptake per mole of substrate approached zero at a fumarate concentration of 40 mM. Thus there appear to be two separate inhibitory effects of fumarate on oxygen uptake: one at low concentrations giving a maximum inhibition of about 20% and a second at concentrations of fumarate greater than 10 to 20 mM which gives complete inhibition at about 40 mM. Examination of the inhibitory effect over the range 0 to 1 mM fumarate (Fig. 3.2.7.1,B) indicates an approximate  $I_{50}$  of 0.1 to 0.2 mM for inhibition at the high affinity site. This is less than the  $K_{m(\text{fum})}$  of benzyl viologen-fumarate oxidoreductase (0.53 mM fumarate, Figure 3.2.7.2). Although the values of these two parameters are rather different, they are sufficiently close to be consistent with the possibility that the inhibition of oxygen consumption is due to fumarate binding to the fumarate reductase and thus competing with oxygen for electrons from the substrate.

The percent inhibition of succinate (+PMS) dependent oxygen consumption caused by fumarate was much greater than that with D-lactate, L-lactate or NADH (Figure 3.2.7.1). This inhibitory effect was further investigated by measuring the  $K_{i(\text{fum})}$  for succinate (+PMS)-DICPIP oxidoreductase. Dixon plots indicate that fumarate inhibits this activity competitively with a  $K_i$  of 0.53 mM fumarate (Figure 3.2.7.3), exactly equal to the  $K_{m(\text{fum})}$  of fumarate reductase (benzyl viologen-fumarate oxidoreductase). This suggests that succinate dehydrogenase and fumarate reductase activities are due to the same enzyme system.

A study of the effect of fumarate on DICPIP reductase activity showed that fumarate also competed with DICPIP in a manner similar to that with oxygen (Table 3.2.7.1).

Table 3.2.7.1: Competition of Fumarate with DICPIP Reductase

Fumarate	PMS	D-lactate	L-lactate	NADH	Succinate
-	-	22.0	8.92	10.1	1.01
+	-	17.4	7.3	6.0	0.333
-	+	38.0	10.5	-	6.68
+	+	31.4	7.52	-	1.44

Values presented are specific activities of DICPIP reduction as  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ . All assays were performed in the absence of oxygen as described in 2.4.6.

### 3.2.7.2: Effect of cyanide on fumarate competition

Previous work (Section 3.2.6) has shown that cyanide inhibits oxygen consumption largely by binding at the terminal oxidase site. Inhibition of this oxidase by cyanide should allow fumarate to compete more effectively with oxygen for electrons, thus decreasing the total oxygen consumption per mole of substrate oxidized when both oxygen and fumarate are present. The effect of varying cyanide concentration on oxygen uptake in the presence of 2 mM fumarate and in the absence of fumarate was investigated using the oxygen electrode and measuring both the initial

Figure 3.2.7.1: Fumarate Inhibition of oxygen consumption

Values presented in this figure were determined using the Clarke oxygen electrode as described in section 2.5.3 and the text, and are recorded here as % of control values determined in the absence of fumarate. Final substrate concentrations were 0.4 mM for D-lactate and NADH and 2.0 mM for succinate. PMS was present at 0.5 mM, where indicated. D-lactate-oxygen oxidoreductase, ○ ; NADH-oxygen oxidoreductase □ ; total oxygen consumption with D-lactate, ● ; total oxygen consumption with NADH, ■ ; succinate (+PMS)-oxygen oxidoreductase, △ . Specific activities for oxygen uptake in the control samples were 3.11, 2.40, and 1.11  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  for D-lactate-, NADH- and succinate (+PMS)-oxygen oxidoreductases, respectively.

- A. Fumarate concentration varied between 1 and 40 mM.
- B. Fumarate concentration varied between 0 and 1 mM.

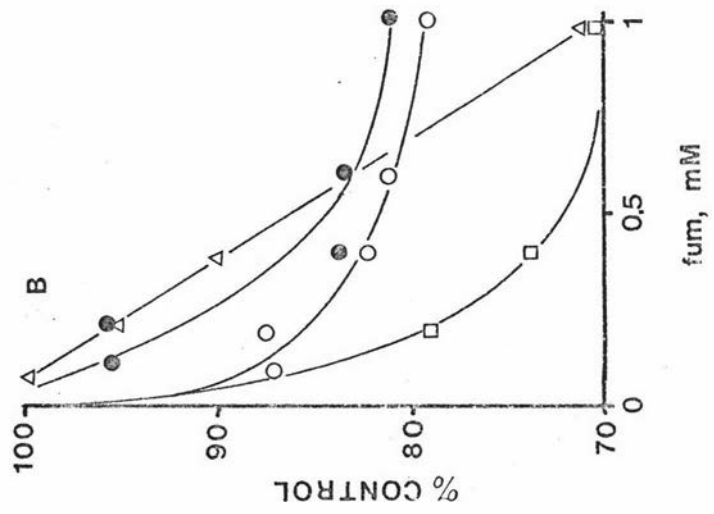
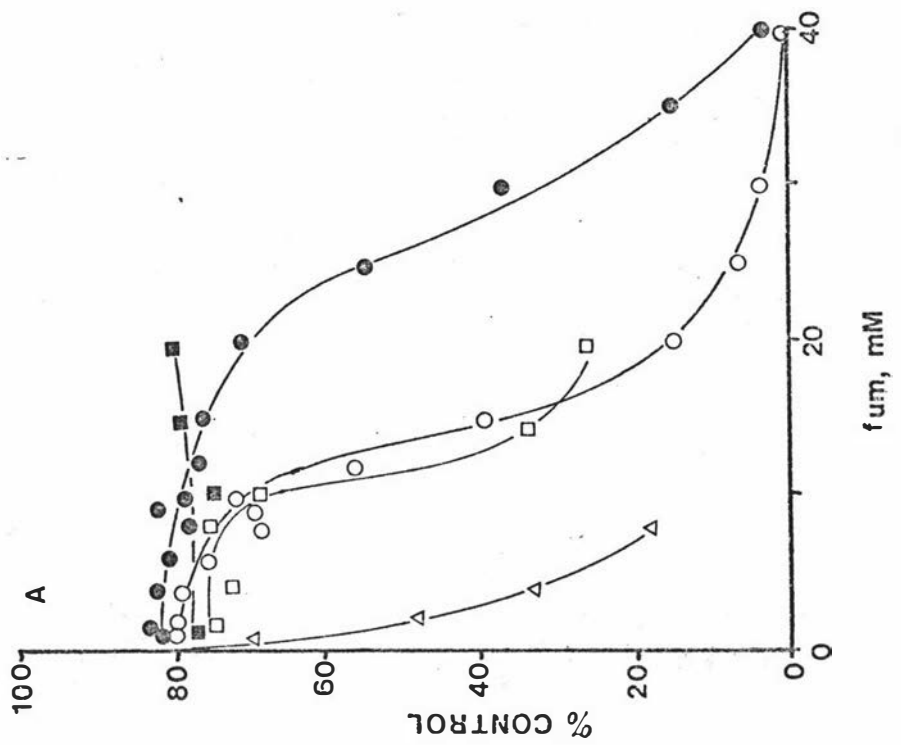


Figure 3.2.7.1

Figure 3.2.7.2: Apparent  $K_{m(\text{fum})}$  for benzyl viologen-fumarate oxidoreductase.

Specific activities of benzyl viologen-fumarate oxidoreductase were determined as described in section 2.5.2. The velocity,  $v$ , is expressed as the change in  $A_{555} \text{ min}^{-1}$ . A linear regression of the data in this figure gives the equation  $v^{-1} = 0.655 + 0.347S^{-1}$ , where  $n = 10$  and  $r = 0.976$ . This gives estimated values for  $K_{m(\text{fum})}$  and  $V_{\text{max}}$  of 0.529 mM and 33.9  $\mu\text{mol min}^{-1} \text{ mg}^{-1}$  respectively.

Figure 3.2.7.2:  $K_{i(\text{fum})}$  for Succinate(+PMS)-DICPIP oxidoreductase

Succinate(+PMS)-DICPIP oxidoreductase activity was determined as described in section 2.5.1 at a PMS concentration of  $10^{-4} \text{ M}$ . Linear regressions of the data shown have three intercepts with an average value of -0.526, indicating the value of  $K_{i(\text{fum})}$  is 0.53 mM. Succinate concentrations of 0.7 mM ( $\nabla$ ), 2.0 mM ( $\blacktriangledown$ ) and 10 mM ( $\square$ ) were used.

Figure 3.2.7.2

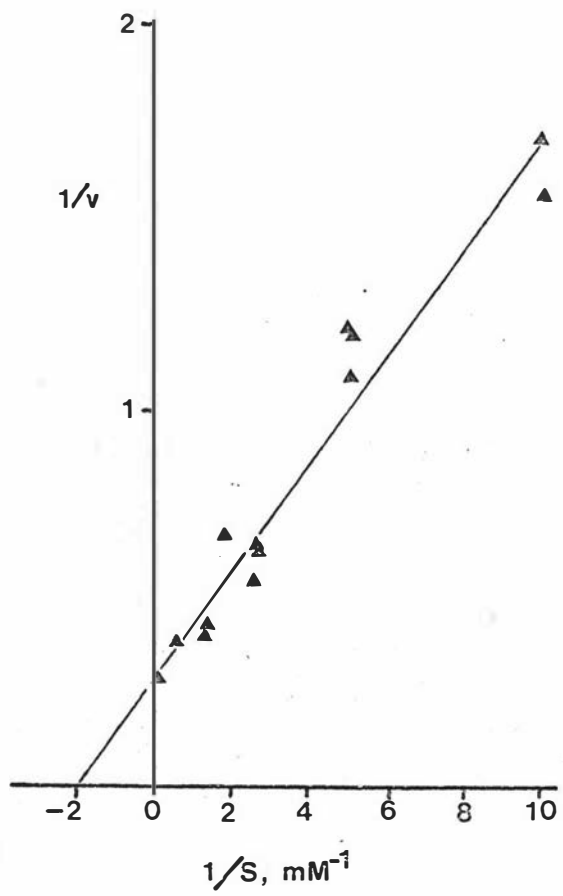
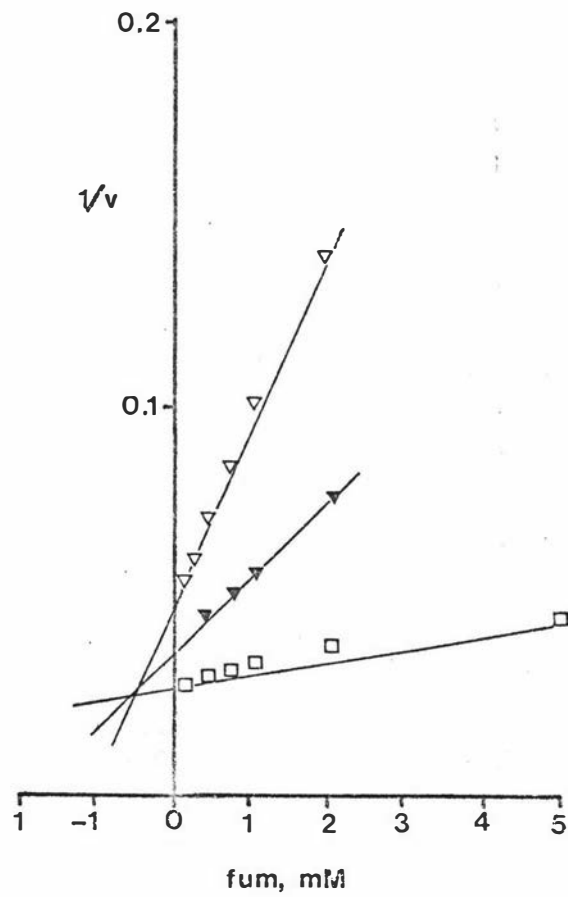


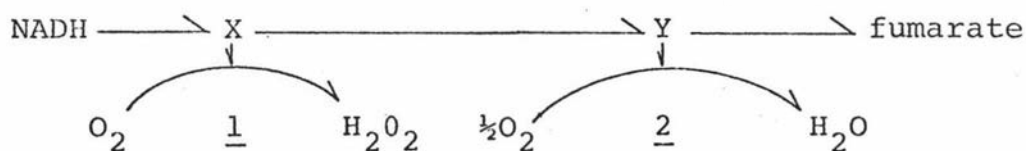
Figure 3.2.7.3



rate of oxygen consumption and the total oxygen consumption. Results of these experiments are shown in Figure 3.2.7.4.

The effect of fumarate on the relationship between cyanide concentration and total oxygen uptake was very different for NADH and D- and L-lactate dependent oxygen consumption. For all substrates, with increasing cyanide, the oxygen uptake per mole of substrate increased in the absence of fumarate. This confirms the effect of cyanide reported earlier (Section 3.2.6.2) where this increase in oxygen uptake per mole of substrate oxidised was attributed to an increasing proportion of the oxygen uptake proceeding via a cyanide insensitive peroxigenic oxidase as the cyanide concentration increases.

In the case of NADH in the presence of fumarate, a substantial decrease in the oxygen uptake per mole of substrate occurred with increasing cyanide. Oxygen consumption in the presence of fumarate decreased from 77% of the control in the absence of cyanide to 18% at 40 mM cyanide. This result is consistent with the idea that the inhibitory effect of fumarate is due to competition between the two electron acceptors for electrons from the substrate. A possible scheme consistent with these results is shown below.



1. A cyanide-insensitive oxidase
2. A cyanide-sensitive oxidase

Figure 3.2.7.4: Effect of Cyanide on Fumarate Competition  
with Oxygen

Oxygen consumption was measured using the Clarke electrode as described in section 2.4.8. Substrate concentrations were 0.4 mM for D- and L-lactate and NADH. Fumarate was present at 2 mM final concentration (solid symbols).

Values plotted are the percent activity of total oxygen consumption in the absence of fumarate. Control values were 3.93, 0.746 and 2.40  $\mu\text{mol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$  and 0.555, 0.318 and 0.406  $\text{mol O}_2 (\text{mol S})^{-1}$  for D-lactate, L-lactate and NADH, respectively.

Total oxygen consumption  $\square$

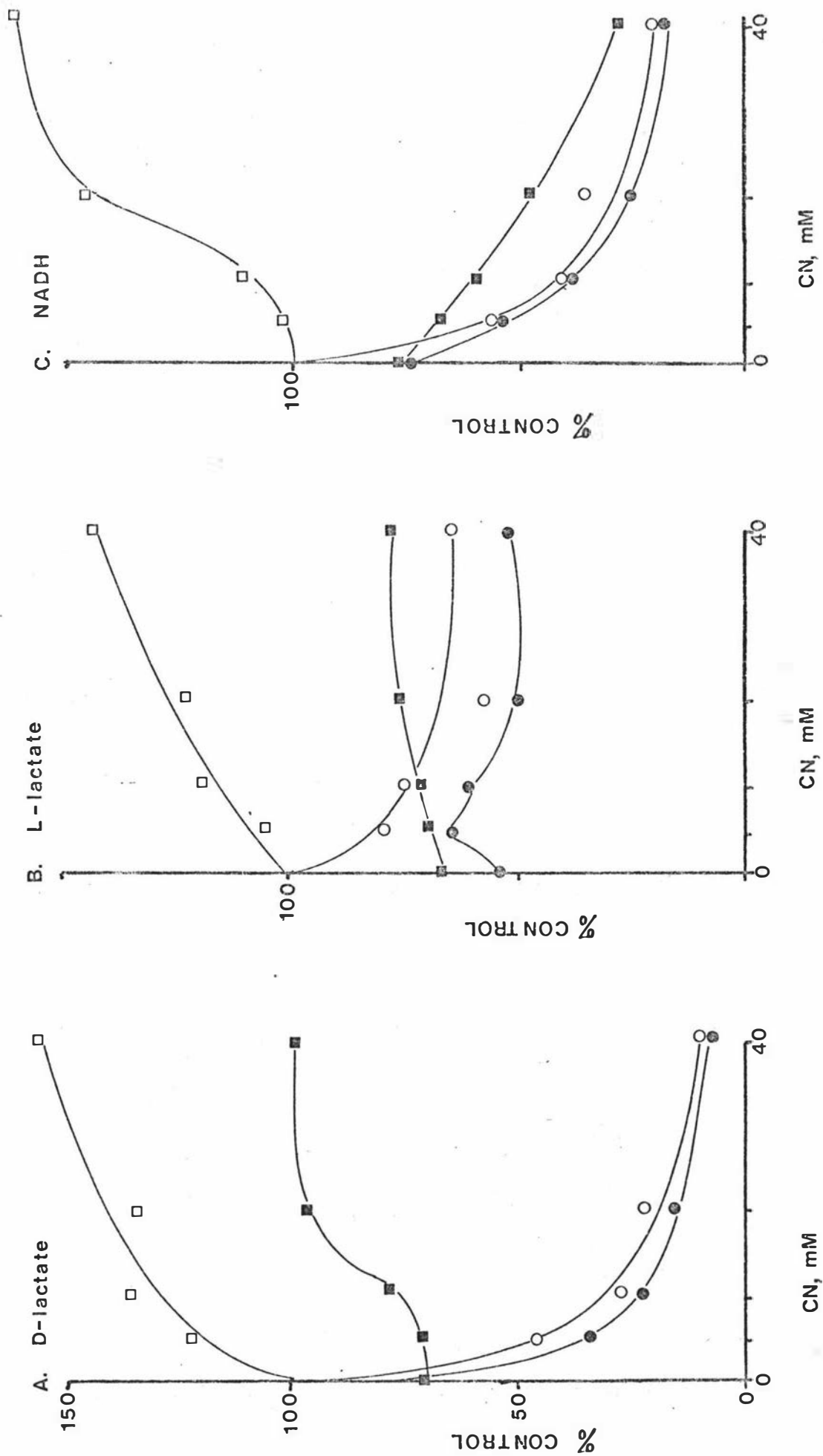
Total oxygen consumption in the presence of fumarate  $\blacksquare$

Rate of oxygen consumption  $\circ$

Rate of oxygen consumption in the presence of  
fumarate  $\odot$

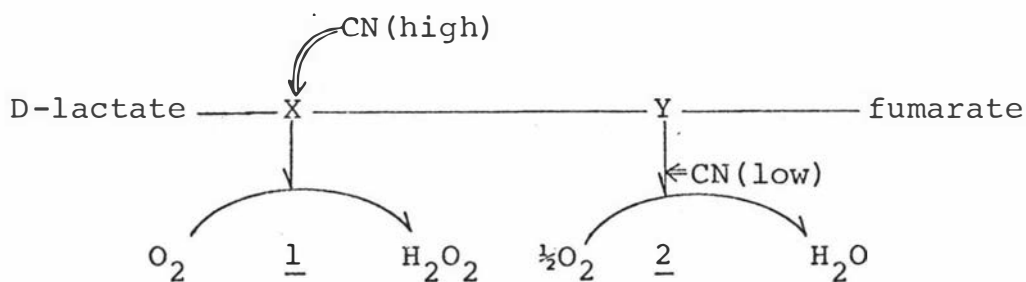


Figure 3.2.7.4



As electron flow to the cyanide-sensitive oxidase is decreased, an increased proportion of electrons from the substrate will flow to fumarate so that the amount of oxygen reduced per mole of substrate decreases. Since the oxygen uptake mediated by the cyanide insensitive oxidase is only a small proportion of the total, the competing effect of fumarate on oxygen consumption more than offsets the increase in oxygen to substrate stoichiometry at high cyanide in the absence of fumarate.

With D-lactate, however, the oxygen consumption per mole of substrate in the presence of fumarate remains at about 60 to 70% of that in the <sup>of fumarate</sup> absence at all cyanide concentrations. This must mean that with D-lactate, cyanide must be affecting oxygen consumption not only at the terminal oxidase, but also at a site in the electron transport chain before the branch point to fumarate or oxygen (see scheme below). Evidence for such an inhibition at high cyanide concentrations in the case of D-lactate was reported in section 3.2.6.



The apparently lower sensitivity of NADH-dependent DICPIP reductase activity and NADH-dependent cytochrome b reduction to inhibition by high cyanide concentrations may correlate with the difference between the effects of

cyanide on NADH and D-lactate-dependent activities in the present study. The possibility that there are two NADH-dependent dehydrogenases, as suggested by the pCMB inhibition data (section 3.2.4.), one of which bypasses a cyanide-sensitive site prior to the branch point may also explain this result.

### 3.2.7.3: Low temperature spectra of membranes in the aerobic steady state with fumarate

If the inhibitory effect of fumarate (over the range 0 to 2 mM) on both the rate of oxygen uptake and the total amount of oxygen consumed is due to competition between oxygen and fumarate for electrons, then it may be possible to detect a change in the level of reduction of one or more of the b-type cytochromes in the aerobic steady state when fumarate is present in addition to oxygen.

The effect of fumarate on the aerobic steady state with D-lactate, NADH and succinate is shown in Figure 3.2.7.5,A. With all three substrates there is a decrease in the reduction level of the b cytochromes in the aerobic steady state caused by fumarate. With D-lactate, the difference spectrum D-lac, $H_2O_2$ /D-lac, $H_2O_2$ ,fum (Figure 3.2.7.5,A) shows a peak at 556 nm. This peak may be composed of small portions of b cytochromes other than  $b_{556-7}$ , but  $b_{556-7}$  is probably the major component. Fumarate also caused a decrease in the aerobic steady-state reduction level of the b cytochromes with NADH. With NADH the peak absorption appeared at 558.5 nm, indicating that the peak was composed of portions of  $b_{562-3}$  and  $b_{556-7}$ .

The difference in peak shape with D-lactate and NADH is reproducible, indicating that there may be a distinct difference between the two reducing substrates in their interaction with the cytochromes b.

The differences observed in the spectra of Figure 3.2.7.5,A were very small. It was expected that an amplification of the effect of fumarate on the cytochrome b peak might occur if the rate of cytochrome b oxidation by oxygen was decreased. As was shown in section 3.2.6, cyanide inhibited the rate of oxygen consumption at low concentrations by binding to cytochrome d and this caused an increase in reduction level of the b peak. It was thus expected that in the presence of 10 mM cyanide, which inhibited oxygen consumption but not fumarate reduction coupled to D-lactate and NADH, the effect of fumarate would be increased. That this is the case is shown in Figure 3.2.7.6,B. With both D-lactate and NADH, the proportional oxidation of the b cytochromes, caused by addition of fumarate to the aerobic steady state, is increased over that in the absence of cyanide (compare  $S, CN: \frac{H_2O_2}{H_2O_2+fum}$  with  $S; \frac{H_2O_2}{H_2O_2+fum}$  (Figure 3.2.7.6,B and A, respectively).

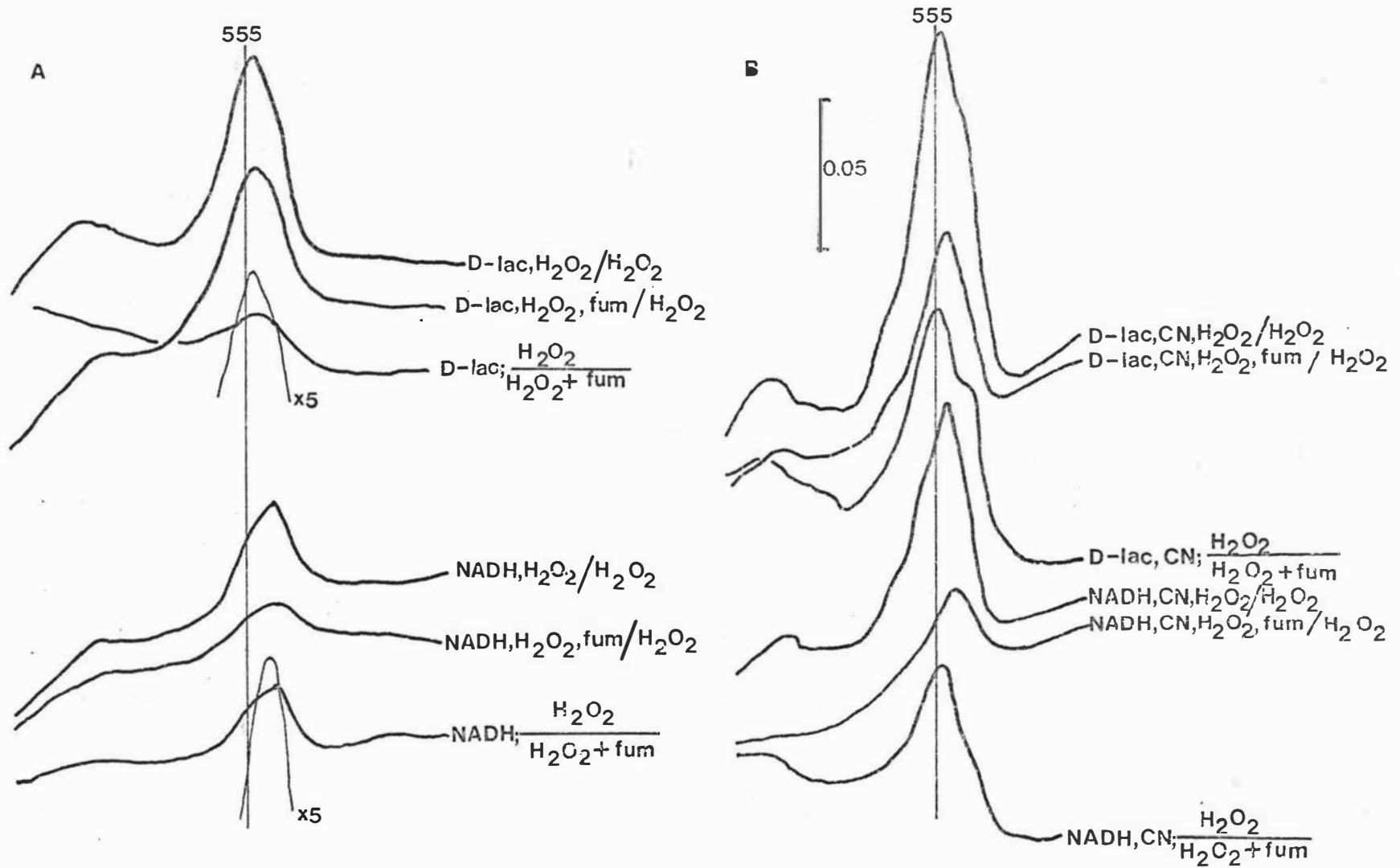
In contrast to the situation in the absence of cyanide, the difference spectrum showing the effect of fumarate on the cyanide inhibited aerobic steady-state ( $S, CN; \frac{H_2O_2}{H_2O_2+fum}$  (Figure 3.2.7.6,B)) shows a peak absorption at 555 nm, indicating that fumarate is oxidising a greater portion of the cytochrome  $b_{553-4}$ , as well as  $b_{556-7}$ , when cyanide is present than when it is absent.

Figure 3.2.7.5: Effect of Fumarate on the Aerobic Steady State Reduction Level of the b Cytochromes in the Absence and Presence of 10 mM Cyanide.

Samples were prepared as described in section 2.6.1 and Section 3.2.1. D-lactate and fumarate were present at final concentrations of 6 mM. NADH was present at 3 mM.

- A. Spectra in the absence of cyanide. Membranes were present at a protein concentration of  $0.59 \text{ mg ml}^{-1}$ .
- B. Spectra in the presence of 10 mM cyanide. Membranes were present at a protein concentration of  $0.8 \text{ mg ml}^{-1}$ .

Figure 3.2.7.5



These results are consistent with the schematic arrangement of the cytochromes presented in earlier sections (i.e., section 3.2.4.3) but do not provide clear support for a role of cytochrome  $b_{556-7}^{LP}$  in electron transport to fumarate.

#### 3.2.7.4: Conclusions from the investigation of fumarate competition with oxygen consumption

Fumarate inhibits oxygen consumption by membrane particles of *P. shermanii* when D-lactate, L-lactate and NADH are reducing substrates. Concentrations of fumarate between 1 and 10 mM inhibited both the rate and total oxygen consumption by about 20%. The inhibitory effect of fumarate over the range 0 to 1 mM has an  $I_{50(fum)}$  of 0.1 to 0.2 mM. This value is only 20 to 40% of the values of  $K_m(fum)$  of fumarate reductase (benzyl viologen-fumarate oxidoreductase) and of  $K_i(fum)$  of succinate dehydrogenase (succinate(+PMS)-DICPIP oxidoreductase) both of which were 0.53 mM. In view of this discrepancy, the suggestion that fumarate is affecting oxygen consumption by acting as an alternative electron acceptor via fumarate reductase is not conclusively established.

If fumarate is competing with oxygen for electron flow, it would be expected that redox intermediates would be less reduced in the presence of both terminal acceptors than with either oxygen or fumarate alone. If, on the other hand, the effect of fumarate is due to direct inhibition of an oxidase activity, then redox components preceding the inhibited oxidase should be more reduced when in the steady-state with

both terminal acceptors in comparison to the steady state in the presence of oxygen alone. Fumarate, when added to the aerobic steady state with D-lactate and NADH, caused a decrease in reduction level of the b cytochromes over that in its absence, thus ruling out the possibility of direct inhibition of oxidase activity. With D-lactate, the b cytochromes further oxidized by fumarate showed a peak at 556 nm, and, with NADH, showed a peak at about 558 nm.

Concentrations of fumarate greater than 10 mM caused a further inhibition of rate and total oxygen consumption with  $I_{50}(\text{fum})$  values of about 13 mM and 26 mM, respectively. To explain the mechanism by which higher concentrations of fumarate inhibit oxygen consumption would require further investigation. Such an investigation would only be worthwhile if it could be shown that the intracellular concentration of fumarate ever reached 10 mM or greater.

### 3.2.8: Other inhibitors of electron transport

As mentioned in section 3.2.1.1, several other inhibitors were examined in a preliminary survey of compounds which might be useful in these studies. Of these, the only one that warrants additional comment is CO. The effects of this were examined only very superficially, but the result is possibly of significance in the light of other work. CO does produce a change in the dithionite reduced spectrum indicative of an o-type cytochrome (Fig. 3.1.5.2). If CO is binding to an o-type cytochrome



which acts as a terminal oxidase, then CO should cause some inhibition of oxygen consumption as measured with the Clarke electrode. Two efforts were made to demonstrate inhibition of oxygen consumption in the presence of CO. In both cases, two aliquots of membrane suspension in buffer were prepared in closed 15 ml tubes. Carbon monoxide (5 ml) was injected to one vial and one hour at room temperature was allowed for the CO to react with the membranes before assaying for oxidase activity as normally. This was done on the first occasion with normal room lighting and subsequently in a darkened room to avoid photodecomposition of the CO-reacted cytochrome. There was no effect of carbon monoxide on either the specific activity of oxygen consumption or on the competition by fumarate for oxygen consumption. The absence of an effect on fumarate competition indicates that CO does not inhibit fumarate reductase.

This experiment was not repeated and hence cannot be considered a firm result. Also, the concentration of CO was not well defined, the time allowed for reaction may be insufficient, and the lighting conditions may have been sufficient to allow reversal of CO-inhibition. While these possibilities must be kept in mind, the absence of effect of carbon monoxide suggests that the CO-binding cytochrome is not active in the electron transport reactions measured under these conditions.

#### 4: DISCUSSION

The general aim of the work described in this thesis was to investigate the electron transport system in *Propionibacterium shermanii* by use of a membrane fraction prepared from bacterial cells grown anaerobically on lactate. The membrane fraction used in these studies showed a composition similar to that reported by Schwartz and Sporckenbach (1975) for membranes from a different strain of *P. shermanii* in respect to both the proportions of electron transport components (menaquinone and cytochrome b) and the specific activities of various electron transport processes.

Particular aspects of the electron transport system which were investigated were:

1. The composition of the anaerobic and aerobic pathways and the relationship between them.
2. The distinction between the pathways involved in the oxidation of the four reducing substrates, D- and L-lactate, NADH and succinate.
3. The role of the cytochrome components in these pathways.

#### 4.1: Anaerobic Electron Transport to Fumarate

As described in the Introduction, the proposal that energy could be conserved for cellular synthesis (as, for instance, ATP) during the oxidation of lactate coupled by an electron transport system to the reduction of fumarate is not a novel idea. However, while some of the earliest studies which lead to the recognition of the importance of this coupled system in the metabolism of anaerobes were

performed with species of propionibacteria (Barker and Lipmann, 1949; Bauchop and Elsdon, 1960; Allen *et al*, 1974), subsequent work on the elucidation of this system has largely been done with other anaerobes such as *Vibrio succinogenes* (Kroger 1975), *Proteus rettgeri* (Kroger *et al* 1974) and *Bacillus megaterium* (Kroger *et al*, 1971).

The composition and organization of the anaerobic electron transport system in propionibacteria was neglected until relatively recently, when Sone (1972, 1973, 1974) DeVries *et al* (1973, 1977) and Schwartz and Sporckenbach (1975) established that this system was contained in membranes prepared from propionibacteria and that these membranes contained menaquinone, flavoproteins and cytochromes.

Sone (1974) and Schwartz (1975) isolated and characterized the major quinone present in membranes from *P. arabinosum* and *P. shermanii* as II,III-tetrahydromenaquinone-9. Ultraviolet absorption spectra of n-pentane extracts of freeze dried membranes prepared from *P. shermanii* ATCC 9614 in this study (Figure 3.1.4.1) confirm the presence of a menaquinone in quantities similar to that reported by Schwartz and Sporckenbach (1975).

Sone (1974) provided evidence for the involvement of this quinone in the anaerobic electron transport path to fumarate in *P. arabinosum* by examining the effect of UV light on the rate of fumarate reduction by NADH, lactate and glycerol phosphate. In the present study, it was confirmed that, in membranes prepared from *P. shermanii* ATCC 9614, fumarate reduction coupled to the oxidation of NADH, D- and L-lactate was almost completely inhibited by

UV light, n-pentane extraction and dicumarol, i.e., with treatments which could be expected to interfere primarily with the function of quinones. D-lactate and NADH-dependent reduction of fumarate in membranes were more sensitive to UV light than were the corresponding activities with oxygen as electron acceptor. Semi-log plots of the decay of D-lactate and NADH-fumarate oxidoreductase activities (Figure 3.2.2.1) were very nearly linear with half-decay times of about 7 minutes. The linearity of the semi-log plot indicates that UV light is probably acting mainly at a single site. The work of Brodie and Ballantyne (1960) on membrane particles from *Mycobacterium phlei* showed that naphthoquinones were much more rapidly destroyed by UV light at 360 nm than other components of the electron transport pathway. Although the destruction of menaquinone by UV light has not directly been investigated it seems most probable that the site of UV inhibition of electron transport to fumarate in membranes of *P. shermanii* is the menaquinone.

Brodie and Ballantyne (1960) provided evidence for the specificity of the UV effect by showing that vitamin K<sub>1</sub> (the menaquinone present in *M. phlei*) could restore activity to the inactivated particles. Sone (1974) was also able to demonstrate reactivation of NADH, lactate and glycerol phosphate dependent fumarate reductase activities in UV treated particles from *P. arabinosum*. Attempts in the present study to reactivate fumarate reductase activities in UV-irradiated particles by addition of quinone extracts from *P. shermanii* were not successful.

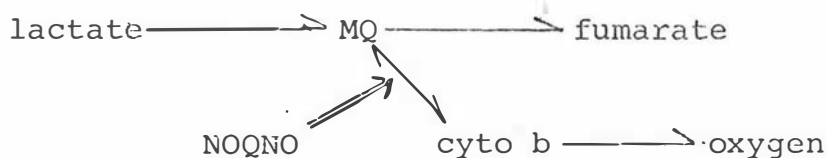
Extraction of *P. shermanii* membranes with n-pentane, another treatment which has been used to demonstrate the involvement of quinones in electron transport systems of bacteria (Kroger *et al*, 1974), was found in the present study to give virtually complete loss of membrane-coupled fumarate reduction. Restoration of activity to the levels in unextracted particles upon addition of quinone extracts of *P. shermanii* did not occur, except for a partial recovery of D-lactate-fumarate oxidoreductase activity. Possible reasons for the low recoveries of activity may have been damage caused by freeze drying, removal of material other than quinone by n-pentane extraction and the inability to maintain fully dessicated conditions during extraction and re-addition of the quinone.

However, reactivation of D-lactate fumarate oxidoreductase by addition of quinone did occur to a significant extent (to 50% of the activity in unextracted particles). This result may reflect the inherent stability of the D-lactate dehydrogenase, since this system was more stable than other dehydrogenase systems during prolonged storage of membrane suspensions or following treatment with detergents (K. Scott and G.G. Pritchard, personal communication).

Inhibition of membrane-coupled fumarate reduction by low concentration of dicumarol (Figure 3.2.3.1) also indicated a role for menaquinone in this system. The concentration of dicumarol giving 50% inhibition of fumarate reduction occurred at a dicumarol to menaquinone ratio of about 2:1, which is consistent with the view that dicumarol inhibits by interfering with menaquinone function. As in

the case of UV inhibition, fumarate reduction was more sensitive to dicumarol inhibition than was oxygen reduction.

One objective of the study of the anaerobic pathway was to determine whether or not cytochrome b was involved in electron transport to fumarate, since controversy exists in the literature in regard to this point. Schwartz and Sporckenbach (1975) using membranes prepared from *P. shermanii* PZ3 concluded that cytochrome b was not on the pathway to fumarate. They found that NOQNO inhibited reduction of cytochrome b by lactate, the degree of reduction of cytochrome b in the aerobic steady state being lower than in uninhibited membranes. At the same time the percentage reduction of menaquinone was found to be increased, indicating that one site of NOQNO inhibition was between menaquinone and cytochrome b. They also observed that, whereas fumarate reoxidized the lactate-reduced cytochrome b to about 50% in uninhibited membranes (anaerobic steady state), if NOQNO was added to membranes in which cytochrome b was reduced by lactate, then fumarate addition caused only a 5% reoxidation of the cytochrome b. In contrast to this, oxygen reoxidized cytochrome b to the same level as that observed in the absence of NOQNO. Since the site of NOQNO inhibition occurred between cytochrome b and menaquinone and cytochrome b was not significantly reoxidized by fumarate in the presence of NOQNO but was by oxygen, they concluded that menaquinone was the branch point to fumarate or oxygen and that cytochrome b functioned only on the pathway to oxygen, as diagrammed below.



Schwartz and Sporkenbach (1975) suggest that the rapid reoxidation of cytochrome b by anaerobic fumarate in the absence of NOQNO found in their work and previously reported by deVries *et al* (1973) and Sone (1974) was due to equilibration of the cytochrome b with the menaquinone pool.

In contrast to this, deVries *et al* (1977) found that addition of oxygen-free fumarate to HOQNO-inhibited, reduced membrane particles from *P. pentosaceum* and *P. freudenreichii* resulted in immediate reoxidation of cytochrome b to a steady state level below that in the uninhibited particles. They concluded from this that cytochrome b functions on the electron transport pathway to fumarate. Apart from the use of HOQNO rather than NOQNO, the use of different buffers (tris rather than phosphate) and the use of different strains of propionibacteria, there is no obvious explanation for the discrepancy between the observations of these two groups.

The data obtained using HOQNO in the present study do not resolve this disagreement since the inhibition of electron transport activities was found to be much less than that reported by Schwartz and Sporkenbach (1975) using comparable concentrations of NOQNO. Subsequent examination of the literature on the alkyl quinoline-N-oxides indicates that NOQNO is a more potent inhibitor of electron transport than HOQNO (Lightbown and Jackson, 1956). While this would not account for the difference between the findings of Schwartz and Sporkenbach (1975) and deVries *et al* (1973 and



1977) it may partially account for the less pronounced inhibition found in the present study. A comparison of relative inhibition between the present data and those of deVries *et al* (1973 and 1977), who also used HOQNO, is not possible since they did not report on the sensitivity of electron transport activities to the inhibitor.

However, the electron transport scheme proposed by Schwartz and Sporkenbach in which all the cytochrome b lies on the pathway to oxygen is not supported by results presented in this thesis. In the presence of dicumarol or using UV-irradiated membranes, the substrate-reduced b peak reduction level is substantially decreased by the addition of fumarate to a level lower than that in untreated membranes. This could not occur if menaquinone functioned between fumarate and all of the cytochrome b.

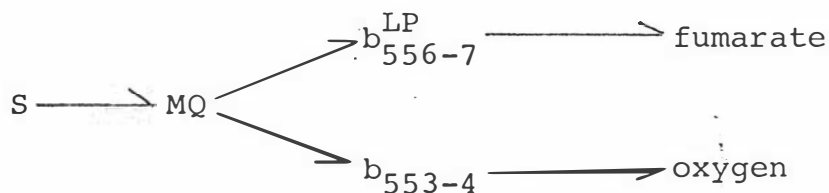
Evidence from the present study on the number and midpoint redox potentials of the b cytochromes is of significance in relation to this controversy.

*P. shermanii* ATCC 9614 contains at least four b-type cytochromes (Table 3.1.5.1), two of which have midpoint redox potentials which would permit a functional role in electron transport to fumarate from lactate and NADH. These are  $b_{553-4}$  and  $b_{556-7}^{LP}$ , both of which have midpoint potentials at pH 7 ( $E_{m7}$ ) of about -20 mV.

Comparison of the anaerobic and aerobic steady states indicated that a large proportion of the b cytochrome remains reduced in the anaerobic steady state, whereas only a small proportion is reduced in the aerobic steady state (Figure 3.2.1.2). It seems likely that those b

cytochromes which are not completely oxidized by fumarate will include  $b_{556-7}^{HP}$  and  $b_{562-3}$ , since the midpoint potentials of these two cytochromes are about 50 to 90 mV above that of the succinate/fumarate couple (+33 mV). This is consistent with the observation that the anaerobic steady state peaks are composed of mainly  $b_{562-3}$  and  $b_{556-7}$  (presumably the HP form).

Comparison of uninhibited anaerobic steady state spectra indicated that fumarate oxidized both  $b_{553-4}$  and  $b_{556-7}$  (presumably the LP form). Evidence from the effect of pCMB on the anaerobic steady state level of cytochrome b reduction (Section 3.2.4) indicated that  $b_{553-4}$  was largely reduced in the inhibited anaerobic steady state, but not the inhibited aerobic steady state. This suggests that this cytochrome may function in electron transport to oxygen rather than to fumarate. This leaves  $b_{556-7}^{LP}$  as the most likely cytochrome component of the anaerobic pathway. This possible arrange is diagrammed below.



Additional evidence supporting a role for cytochrome b in electron transport to fumarate is provided by the observation that when fumarate was added to membrane particles in the aerobic steady state, thereby decreasing the rate of oxygen consumption, there was a small decrease in reduction of  $b_{556-7}$  when D-lactate was reducing substrate and of both  $b_{556-7}$  and  $b_{562-3}$  when NADH was reducing substrate

(Figure 3.2.7.3). This, however, is not conclusive evidence that  $b_{556-7}^{LP}$  is on an electron transport pathway to fumarate. In fact, the evidence related above can only indicate that if a b cytochrome is involved in electron transport to fumarate then the most likely candidate is cytochrome  $b_{556-7}^{LP}$ .

The presence of a low potential b type cytochrome on the electron transport pathway to fumarate has been demonstrated by the studies of Unden, Hackenberg and Kroger (1980) for the fumarate reductase of *Vibrio succinogenes*. The fumarate reductase was purified and it was observed that reduction of fumarate by naphtho-quinone analogues was cytochrome b-dependent while benzyl viologen oxidation was not. The cytochrome b involved had an  $E_{m7}$  of -20 mV. Succinate dehydrogenase complexes from other organisms have been found to have a b-type cytochrome as part of the complex (Davis and Hatefi, 1971; Davis *et al*, 1969; Hederstedt *et al*, 1979). It is thus possible that a similar system occurs in *P. shermanii* which utilizes cytochrome  $b_{556-7}^{LP}$  in this role.

#### 4.2: The Aerobic Electron Transport Pathway

Although the propionibacteria are usually regarded as anaerobic bacteria because visible colonies do not appear on the surface of agar plates incubated aerobically, their ability to use oxygen as an electron acceptor for the oxidation of a wide range of substrates has been well established (Schwartz, 1973; deVries *et al*, 1972;

Bonartseva *et al*, 1973; Van Gent-Ruijters *et al*, 1976; Pritchard *et al*, 1977). However, very little is known about the aerobic pathway and one of the aims of the present study was to provide further information on the components of this pathway and its relationship to the anaerobic pathway. Other questions relevant to the data presented in this thesis are the nature of the terminal oxidase and the differences between pathways for aerobic oxidation of NADH, D-lactate, L-lactate and succinate.

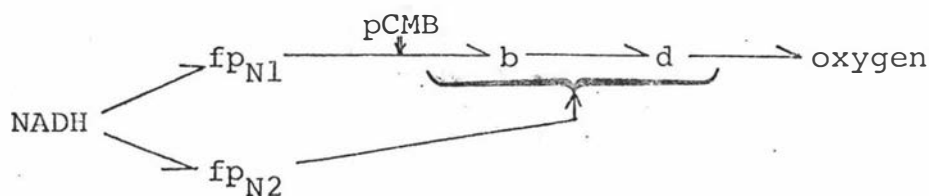
#### 4.2.1: Differences between reducing substrates in electron transport to oxygen

The rates of oxygen consumption dependent on D-lactate and NADH were approximately equivalent and remained stable for several days. The rate of oxygen consumption dependent on L-lactate was at best equivalent to that with D-lactate but was usually much less due to the instability of the L-lactate dependent activity. The rate of oxygen consumption with succinate was much less than that with either D-lactate or NADH (about 3% for both). The relative rates of oxygen uptake with these substrates are similar to those reported by Schwartz and Sporckenbach (1975).

The factor by which PMS stimulated oxygen consumption varied with the reducing substrate. Succinate oxidase was stimulated by the greatest factor (19x) whereas D-lactate oxidase was least stimulated (2.6x) and the stimulation of the L-lactate oxidase was intermediate (6x). The large PMS stimulation with succinate indicates that succinate dehydrogenase is not the rate-limiting step in succinate oxidase activity.

A further difference between L-lactate and other reducing substrates emerged from studies of oxidase inhibition by dicumarol (Section 3.2.3). L-lactate oxidase activity was much more sensitive to dicumarol inhibition than D-lactate or NADH oxidases. This sensitivity also occurred with L-lactate-DICPIP and L-lactate- $\text{Fe}(\text{CN})_6$  oxidoreductases, indicating either that coupling of the dehydrogenase to reduction of these dyes and to oxygen was dependent on menaquinone or that the L-lactate dehydrogenase was itself inhibited by dicumarol.

NADH oxidase also differed from D-lactate and L-lactate oxidases in its sensitivity to inhibition by pCMB. NADH-oxidase was inhibited to a maximum of 50% whereas D-lactate and L-lactate oxidases were completely inhibited by pCMB (Figure 3.2.4.1). This indicates the presence of two routes of electron transport to oxygen from NADH. Since NADH-DICPIP and NADH- $\text{Fe}(\text{CN})_6$  oxidoreductases were also inhibited by pCMB to a maximum of approximately 50% (Section 3.2.4), the presence of a pCMB-insensitive dehydrogenase was indicated. However, the oxidoreductase systems initiated by these dehydrogenases must share the same terminal oxidase, since total NADH oxidase was inhibited about 90% by cyanide, apparently at a single site (Section 3.2.6). These results are summarized in the diagram below.



#### 4.2.2: The nature of the terminal oxidases

Cytochromes present in *P. shermanii* which may function as terminal oxidases are cytochromes a, d and a CO-binding cytochrome tentatively identified as cytochrome o (Sone, 1972; Schwartz and Sporkenbach, 1973; Pritchard *et al*, 1977). Only the cytochrome d and the CO-binding b cytochrome are present in significant quantities, so that considerations of the major cytochrome terminal oxidase in *P. shermanii* under conditions of growth used in this study can be restricted to these two.

Cytochrome o, a carbon monoxide binding pigment, was first observed in *Staphylococcus albus* (Chance, 1954; Smith, 1954). Photoreversal of CO binding was used to demonstrate its function as a terminal oxidase (Castor and Chance, 1955). Cyanide at  $10^{-5}$  M is usually effective in inhibiting cytochromes of the o-type (Okunuki, Kamen and Sekuzu, 1968; Jones, 1973).

Cytochrome d functions as terminal oxidase in many bacteria (Jurtshuk, Mueller and Acord, 1975). It is slowly destroyed by UV light (Bragg, 1971) and may be inhibited by cyanide (Pudek and Bragg, 1974; Jones, 1973). Reported values for the cyanide inhibition constant ( $k_i$ ) vary with the organism (approximately 15  $\mu$ M with *Azotobacter vinelandii* (Jones, 1973) and 7.4 mM with *E. coli* (Pudek and Bragg, 1974)) but they are generally at least an order of magnitude greater than the  $K_i(\text{CN})$  for cytochrome o in any one organism.

In membranes from *P. shermanii*, cyanide at 10 mM inhibited oxidases of NADH, D-lactate and L-lactate by between 50 and 90% while having no effect on the corresponding

fumarate oxidoreductase activities (Figure 3.2.6.1, A & C). As mentioned above, NADH oxidase was almost completely inhibited by cyanide with an  $I_{50}$  for cyanide of about 2.2 mM (Figure 3.2.6.1,B). With D-lactate there appear to be two sites of inhibition, one with an  $I_{50}$  of about 2 mM, presumably the terminal cytochrome oxidase, and one with an  $I_{50}$  value of 20-25 mM due to inhibition at a site between the D-lactate dehydrogenase and cytochrome b. Cytochrome d appears to be totally reacted with cyanide at 10 mM concentration since the d peak is absent from low temperature spectra of membranes in the aerobic steady state with 10 mM cyanide (Figure 3.2.6.2,B). The disappearance of cytochrome d is due to formation of cyanocytochrome d (Pudek and Bragg, 1974; Kauffman and Van Gelder, 1973) which does not absorb light at 620-650 nm.

Two attempts were made to examine the effect of CO on oxygen consumption, but no CO inhibition could be demonstrated. While further studies with CO would be necessary to substantiate this finding, it is consistent with the idea that cytochrome o, if it does occur in this bacterium, does not have a significant role in oxygen consumption in membranes prepared from *P. shermanii*, and that the major terminal oxidase is cytochrome d.

While the above evidence indicates that cytochrome d is the major terminal oxidase, accounting for about 90% of the rate of oxygen consumption with NADH, it appears, on the basis of cyanide inhibition studies, to account for a smaller percentage of the oxygen consumption with D-lactate, less still with L-lactate and only about 20% with succinate.

The actual specific activities of the cyanide-resistant component of succinate oxidase and L-lactate oxidase are very small, since the initial uninhibited activities of these two oxidases are small relative to those of NADH and D-lactate. If the residual oxidase activity determined were entirely due to a peroxygenic oxidase, then the ratio of oxygen consumed per mole of substrate oxidized should approach unity, whereas it only reached about 0.68 with NADH and D-lactate and 0.75 with L-lactate, indicating that peroxide production accounted for a maximum of 40-50% of the cyanide resistant electron flow. The origin of the cyanide-insensitive component of the oxygen uptake is not clear. It could be due to direct electron transfer from the primary dehydrogenase to oxygen as found by Sone (1973) for the purified glycerophosphate dehydrogenase from *P. arabinosum*. It could also be due to peroxygenic reactions at the terminal oxidase similar to that observed with a purified NADH oxidase (containing cytochrome o) from a *Veillonella* species which displayed the same ratio of oxygen consumed to NADH (0.68) as did inhibited membranes from *P. shermanii* (Webster, 1975).

#### 4.3: The Presence of Steps Common to Both Aerobic and Anaerobic Pathways

Two different pieces of evidence presented in this thesis indicate that some steps in electron transport pathways to fumarate and oxygen are shared. The first of these is the similarity of effect of some inhibitors on



electron transport to these two acceptors and the second is the competition between fumarate and oxygen for electrons from the reducing substrate.

The inhibitor pCMB inhibited both fumarate and oxygen reduction at a similar concentration and inhibited reduction of the b cytochromes in steady states with either oxygen or fumarate. This indicated that inhibition of oxygen or fumarate reduction occurred at similar sites, presumably at the dehydrogenases for the respective reducing substrates.

Both fumarate reduction and oxygen consumption were inhibited by UV light and dicumarol treatment of membranes although the less complete inhibition of oxygen oxidoreductases suggests that only part of the total oxygen consumption has an absolute requirement for menaquinone. The extent to which peroxygenic reactions at the dehydrogenases may account for the component of oxygen consumption which was less sensitive to inhibition by UV light or dicumarol cannot be determined from results presented in this thesis, since  $H_2O_2$  determination during oxygen uptake experiments with UV-irradiated membranes was not performed.

Sone (1972) demonstrated a decrease in oxygen consumption when fumarate was added to respiring membranes of *P. arabinosum*. This same effect was demonstrated in membranes from *P. shermanii* and further characterized by showing that both the rate and total of oxygen consumption were decreased by the same proportion at fumarate concentrations up to 10 mM. This result indicates that the electron transport pathways to oxygen and fumarate share common steps up to an intermediate located at a branch point.

A possible candidate for the common intermediate at the branch point is menaquinone. One view of the role of quinones is that they act as a freely mobile pool of redox intermediate in the lipid phase of the membrane which is reduced by dehydrogenase complexes and oxidized by separate oxidase complexes (Li *et al*, 1981). According to this view, the flow of electrons via fumarate reductase to fumarate would cause a decrease in the reduction level of the menaquinone pool, thus decreasing the concentration of reduced substrate available to the oxidase and consequently causing a decrease in the observed rate of oxygen consumption and in the total oxygen consumed per mole of substrate.

#### 4.4: Interaction of DICPIP and $\text{Fe}(\text{CN})_6$ With the Electron Transport System

The effect of inhibitors on the rate of reduction of the electron acceptors DICPIP and  $\text{Fe}(\text{CN})_6$  was studied since it was expected that these acceptors would intercept electrons at a step (or steps) prior to the terminal oxidase or fumarate reductase and thus provide further evidence on the sites of action of the inhibitors. However, the data obtained suggest that the interaction of these acceptors with the electron transport system is very complex in that:

1. The sites of interaction are different depending on the particular reducing substrate used.

2. DICPIP and  $\text{Fe}(\text{CN})_6$  may act at different sites.
3. One or both acceptors may act at more than one site in the electron transport chain.

Identification of the site(s) at which these two acceptors act was not a primary aim of this study, but the data which bear on this question are discussed below. Since the site(s) of interaction appears to differ for each reducing substrate, the results are discussed separately for each of the four reducing substrates used.

#### 4.4.1: D-lactate

Specific activities of oxidoreductases for D-lactate-DICPIP and D-lactate- $\text{Fe}(\text{CN})_6$  were similar, which may indicate that the two acceptors interact mainly at the same site when D-lactate is the reducing substrate (Table 4.4.1.1.).

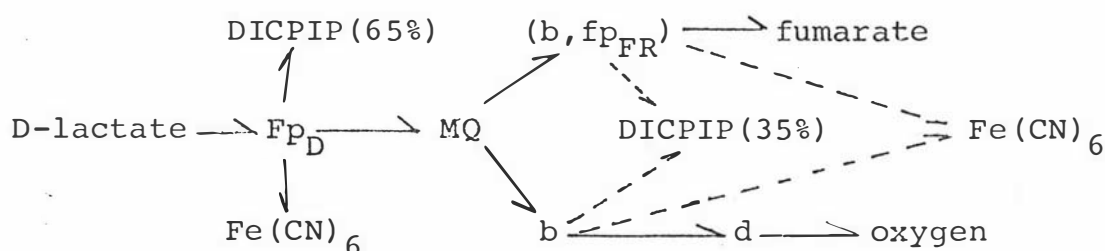
Table 4.4.1.1: Specific Activities of D-lactate Oxidoreductases

Oxygen	PMS	$\text{Fe}(\text{CN})_6$	DICPIP	Oxygen	Fumarate
+	-	30.8	43.4	7.67	-
+	+	94.1	67.2	19.7	-
-	-	33.2	39.5	0	4.02
-	+	91.0	71.9	-	-

Values presented are specific activities in  $\mu\text{mol min}^{-1}$   $\text{mg protein}^{-1}$ . See Tables 3.1.2.2 and 3.1.2.3 for details of preparation

Significantly, oxygen did not affect the rate with either acceptor whether or not PMS was present, and the specific activity with the dyes was much greater than that with oxygen. This suggests that these electron acceptors do not interact at a site near the terminal oxidase of the electron transport system.

Ultraviolet light inhibited the reduction of ferricyanide and D-lactate oxidase similarly whereas only 35% of D-lactate-DICPIP oxidoreductase was sensitive to UV light, the residual 65% being insensitive to UV light (Fig. 3.2.2.1). This indicates that DICPIP is acting at two distinct sites in the D-lactate dependent electron transport pathways. One of these sites must be on the high redox potential side of menaquinone, possibly the fumarate reductase or a component of the aerobic pathway. The UV-insensitive reduction of DICPIP is probably due to interaction of this acceptor at the level of the primary D-lactate dehydrogenase. The greater sensitivity of  $\text{Fe}(\text{CN})_6$  reduction to UV indicates that the major site of interaction of  $\text{Fe}(\text{CN})_6$  is at a point on the oxidizing side of menaquinone. However, the lack of any effect of oxygen on the rate of  $\text{Fe}(\text{CN})_6$  or DICPIP reduction indicates that none of the site(s) is at the terminal oxidase. The following scheme summarizes these conclusions.



#### 4.4.2: L-lactate

Because of the instability of the L-lactate coupled activities, reactions with this donor were less thoroughly investigated than other donors. Before clear conclusions regarding L-lactate-coupled activities can be drawn, stabilization of these activities by some alteration of the procedure for membrane preparation is necessary. There are, however, several results which do bear on the site(s) at which  $\text{Fe}(\text{CN})_6$  and DICPIP react with the electron transport system when L-lactate is donor. These are listed below:

1. Oxygen competes with DICPIP and  $\text{Fe}(\text{CN})_6$  for electron flow (removal of air stimulates activity approximately 5 and 2.3 fold, respectively). (Table 3.1.2.4).
2. PMS stimulates activity 1.6x when  $\text{Fe}(\text{CN})_6$  is acceptor but has no effect when DICPIP is acceptor.
3. Maximum activities (anaerobic in the presence of PMS) are approximately the same for DICPIP and  $\text{Fe}(\text{CN})_6$ .
4. L-lactate coupled activities were about  $10^3$  more sensitive to dicumarol than with other reducing substrates (Fig. 3.2.3.1). The very big difference in sensitivity to dicumarol of L-lactate-coupled dye reduction compared to that with other reducing substrates clearly indicates that electron transfer from L-lactate to these acceptors involves either distinctive dicumarol sensitive components or a distinctive location in the membrane to which dicumarol has ready access.

#### 4.4.3: NADH

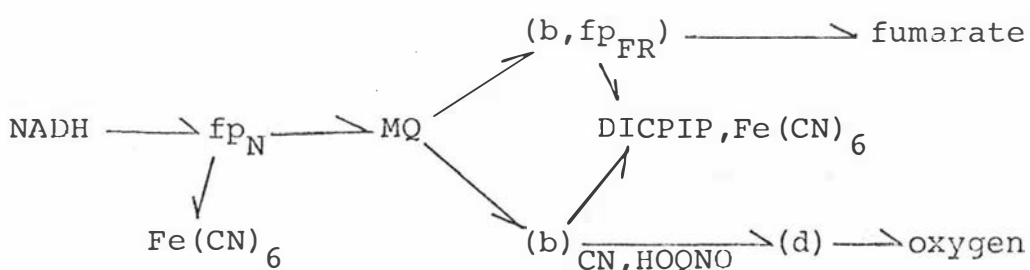
When NADH was used as the reducing substrate, the results were different from those obtained with either L-lactate or D-lactate. NADH-DICPIP oxidoreductase activity was much less than NADH-Fe(CN)<sub>6</sub> oxidoreductase activity and about the same as the activity of NADH oxidase and NADH-fumarate oxidoreductase. Oxygen inhibited NADH-Fe(CN)<sub>6</sub> oxidoreductase only slightly (by about 10%) whereas NADH-DICPIP oxidoreductase was inhibited by about 60%, indicating that the sites for DICPIP interaction are probably closer to the terminal oxidase than is the case for Fe(CN)<sub>6</sub> (Table 3.1.2.4).

In the presence of HOQNO and cyanide, NADH-DICPIP activity was stimulated in aerobic assays (Figs. 3.2.5.1, 3.2.5.1). The simplest interpretation of this is that the inhibitors block the pathway of electron flow to oxygen, thereby removing oxygen competition. This is supported by the finding that no such stimulation in the presence of HOQNO occurs in anaerobic assays (Table 3.2.5.1). Activity was stimulated similarly by a factor of 2.4 with both inhibitors, indicating that the same oxidative pathway is inhibited by both inhibitors.

In contrast to D-lactate-DICPIP oxidoreductase, NADH-DICPIP oxidoreductase is inactivated by UV light at a rate similar to that of NADH oxidase (Figure 3.2.2.1). This is consistent with the conclusion that the main site of interaction of DICPIP with the electron transport chain from NADH is on the oxidizing side of menaquinone. On the

other hand, only about 50% of the NADH- $\text{Fe}(\text{CN})_6$  oxidoreductase activity is sensitive to UV light, and this portion of the activity decays at a rate similar to that of the other UV sensitive activities, thus indicating a menaquinone requirement.

These results can be summarized in the following scheme:



The difference with respect to the points of interaction of DICPIP and  $\text{Fe}(\text{CN})_6$  indicated in the above scheme is consistent with the findings of Schwartz and Krause (1975) on the properties of a partially purified NADH dehydrogenase, which indicated that NADH-DICPIP oxidoreductase activity depended on a lipid component whereas NADH- $\text{Fe}(\text{CN})_6$  oxidoreductase did not.

#### 4.4.4: Succinate

Succinate-DICPIP oxidoreductase is present at lower activities than succinate- $\text{Fe}(\text{CN})_6$  oxidoreductase and neither activity is substantially inhibited by oxygen (Table 3.1.2.4). Neither of these activities were affected significantly by UV light or dicumarol, indicating that menaquinone is not involved.

Fumarate inhibits succinate-DICPIP oxidoreductase with a  $K_i(\text{fum})$  of 0.5 mM. The relationship between both succinate-DICPIP oxidoreductase and succinate- $\text{Fe}(\text{CN})_6$  oxidoreductase activity and succinate concentration suggests that there are two binding sites for succinate, (Fig. 3.1.2.1) which may indicate that there are two succinate dehydrogenase activities, one of which may be fumarate reductase. This parallels the observation in *E. coli* membrane preparations of two apparent  $K_m$  for succinate (Kasahara and Anraku, 1974).

#### 4.5: Differences Between Pathways Oxidizing Different Donors

Previously published studies on electron transport in propionibacteria have mostly involved the use of only one or two electron donors, such as NADH, lactate or glycerophosphate. It has not always been stated whether the D- or L-isomer or a D,L mixture of lactate has been used. In the work of Sone (1972, 1974) who did use a variety of donors, although not systematically so, some major differences between the pathways oxidizing different donors were reported. For instance, KCN (1 mM) was reported to inhibit NADH oxidase activity in membranes from *P. arabinosum* by 92%, whereas lactate (D or L?) oxidase was only inhibited by 3% (Sone, 1972). The sulfhydryl reagent, pCMB (0.08 mM) was reported to inhibit lactate-dependent fumarate reductase activity by 70% but glycerophosphate-dependent fumarate reductase was inhibited by only 24%. In a later paper, reduction of cytochrome b by lactate was



reported to be unaffected by UV irradiation, while that by NADH was strongly inhibited (Sone, 1974). These findings suggest that the pathways of electron transport for the oxidation of the different donors are quite distinct.

One of the aims of the present study was to make a systematic comparison of the effect of inhibitors on electron transport with NADH, D- and L-lactate and succinate as reductants, and thus to reinvestigate the extent to which the pathways from the different donors were similar or different. Many of the differences have already been presented and discussed in the preceding pages, but in the present section the data relevant to specific comparisons are collected together and discussed.

#### 4.5.1: Differences between pathways Oxidizing D-lactate and L-lactate

Although L-lactate supports a higher rate of oxygen consumption than D-lactate in intact cells, the activity with L-lactate observed in membrane preparations was at best equal to those of D-lactate and normally much less. This lower activity with L-lactate appeared to be due to instability of the dehydrogenase, since activities with electron acceptors other than oxygen also decreased when L-lactate was used as donor. Because of the instability of L-lactate dependent activities, the results obtained with this donor are less complete than for other donors.

A higher proportion of the L-lactate oxidase was insensitive to cyanide than the D-lactate and NADH oxidases although the specific activity of the insensitive portion was less than that with D-lactate or NADH. If direct electron transfer to oxygen from the dehydrogenase is the source of this cyanide resistant oxidase activity, then the higher proportion with L-lactate may be due to poorer coupling of the lactate dehydrogenase to the membrane electron transport system, thus allowing a greater proportion of peroxigenic oxidase activity at the dehydrogenase site.

The major differences between D-lactate and L-lactate dependent activities may thus be due to the manner in which the two dehydrogenases are integrated with the membrane. D-lactate dehydrogenase is apparently more tightly and stably integrated into the membrane than is L-lactate dehydrogenase. L-lactate dehydrogenase activity is apparently lost when it becomes uncoupled from the membrane. Whether or not the stability of the L-lactate dehydrogenase could be improved by modifications to the isolation procedure is a subject for further research. Another topic for further research is the role of pyrrolo-quinoline quinone reported to function in the lactate dehydrogenase of *P. pentosaceum* (Duine and Franks, 1981).

#### 4.5.2: Differences between pathways oxidizing NADH and D-lactate

NADH and D-lactate dependent activities were generally very similarly affected by inhibitors and by

UV treatment. The most notable difference between them was in their sensitivity to inhibition by pCMB. While D-lactate oxidase, D-lactate-DICPIP oxidoreductase and D-lactate- $\text{Fe}(\text{CN})_6$  oxidoreductase activities could be completely inhibited by pCMB at  $10^{-3}$  M, NADH oxidase, NADH-DICPIP oxidoreductase and NADH- $\text{Fe}(\text{CN})_6$  oxidoreductase activities could only be inhibited to about 50-60% at the highest concentrations of pCMB achievable in solution. The shape of the pCMB inhibition curve for NADH dependent activities suggested that only a discrete portion of these activities was inhibitable by pCMB and thus that there were two distinct pathways for oxidation of NADH. It is possible that there are two distinct NADH dehydrogenase systems by which electrons from NADH may be passed to the electron transport chain. Since pCMB completely inhibits NADH-fumarate oxidoreductase, it is suggested that one of these dehydrogenase systems, the pCMB insensitive one bypasses the branch point to fumarate. The inhibition of NADH oxidase activity by cyanide suggests that this is mediated by a single terminal oxidase (cytochrome d) so the pCMB-insensitive route must share common terminal steps with the pCMB sensitive route.

In the presence of pCMB, NADH caused a greater reduction of the b cytochromes in the substrate-reduced state and the anaerobic steady state than did D-lactate (Figure 3.2.4.2,A and B). This indicates that in pCMB inhibited membranes, NADH has a greater ability to reduce the b cytochromes than D-lactate and may indicate that the putative alternate dehydrogenase for NADH bypasses the menaquinone.

A difficulty with this scheme is that it also implies that a component of NADH oxidase activity should be less sensitive to UV than D-lactate oxidase, which was not observed (Figure 3.2.2.1). This may be due to the presence of separate pools of menaquinone associated with the pCMB insensitive dehydrogenase and the pCMB sensitive dehydrogenase. The existence of separate menaquinone pools (Houghton *et al*, 1976; John, 1980) or of alternate quinones (Duine and Frank, 1981) associated with different electron transport pathways in bacteria may explain these results. Alternatively, it may be that the rate of quinone destruction is less significant than is the destruction of cytochrome d for the decay of oxidase activity under UV light, since menaquinone appears to be not strictly required for oxidase activity.

#### 4.5.3: Distinctive features of succinate dependent electron transport activities

In previous studies on electron transport by membrane preparations from propionibacteria, only brief mention has been made of succinate-dependent electron transport activities. Lara (1959) published a brief report on a partially purified particulate succinate dehydrogenase from *P. pentosaceum*. The preparation retained cytochrome b throughout the purification procedure. The  $K_{M(\text{fum})}$  was about one third that for succinate, but the maximum activity with succinate was three times higher than that with fumarate. Lara concluded that this succinate dehydrogenase

was the enzyme responsible for fumarate reductase activity. Schwartz and Sporckenbach (1975) found that succinate reduced less of the cytochrome b than did other reductants and the absorption peak of the succinate reducible cytochrome b was at 562 nm rather than at 560 nm as with the other reducing substrates. They also found that succinate oxidase activity was much more sensitive to HOQNO inhibition than that of lactate and NADH oxidase activity. In order to account for this they include succinate dehydrogenase as a separate system from fumarate reductase in their scheme (Figure 11 in Schwartz and Sporckenbach, 1975). However, they conclude 'the relation between fumarate reductase and succinate dehydrogenase is an open question for *P. shermanii*'. Sone (1972) concluded that the succinate dehydrogenase of *P. arabinosum* acts as a fumarate reductase but gave no data to support this. Since, in *E. coli*, there are two quite distinct enzymes functioning in succinate oxidation and fumarate reduction (Hirsch *et al.*, 1963), it cannot be assumed that both activities are due to the same enzyme system in propionibacteria.

In the current study, succinate was systematically included as a reductant in all inhibitor studies in the hope that this might yield information on the question of whether succinate dehydrogenase activity and fumarate reductase activity were due to the same enzymes.

In the present study, succinate dependent electron transport activities show a number of major differences from those with other reducing substrates, as listed below:

1. As reported by Schwartz and Sporckenbach (1975), succinate reduces a smaller proportion of the total cytochrome b. This is to be expected since the redox couple succinate/fumarate has a redox potential of +33 mV, which places it between the two major groups of cytochromes b. Thus succinate would be expected to reduce fully only cytochrome  $b_{562-3}$ ,  $b_{556-7}^{HP}$  and cytochrome d. The succinate-reduced spectrum confirms this (Figure 3.2.1.1).
2. The activity of succinate oxidase is very low relative to that with other reducing substrates. Again this is not surprising since succinate oxidase activity is not required in normal fermentative metabolism in propionibacteria, i.e., succinate oxidation is not thought to have a physiological function such as that in more usual facultative anaerobes like *E. coli*. However, it raises the question of where the rate-limiting step in the succinate oxidase system occurs. Since PMS greatly stimulates succinate oxidation and succinate reduces DICPIP and  $Fe(CN)_6$  at quite high rates, it is clear that the rate-limiting step is not at the primary dehydrogenase.
3. The level of reduction of cytochrome b in the aerobic steady state is higher with succinate than with other reducing substrates (Figure 3.2.1.2) including D-lactate, which has a very high dehydrogenase activity. In particular, cytochrome  $b_{562-3}$  is more highly reduced by succinate in the aerobic steady state than by other substrates. This suggests that cytochrome  $b_{562-3}$  is a component in the pathway of oxidation of succinate and that this cytochrome

may be specifically associated with the succinate dehydrogenase. It also suggests that the rate limiting step in succinate oxidation may be between cytochrome  $b_{562-3}$  and cytochrome d or other terminal oxidase. Cytochrome d is fully reducible by succinate, but it is completely oxidized in the aerobic steady state, whereas cytochrome  $b_{562-3}$  is still quite highly reduced.

The level of reduction of cytochrome  $b_{562-3}$  is further increased by cyanide which acts, at concentrations below 10 mM, primarily by binding to cytochrome d. However, since cyanide has very little inhibitory effect (10%) on succinate oxidase activity, the major 'oxidase' for succinate must be some component other than cytochrome d. The present study does not give an indication of what this might be, unless reduced cytochrome  $b_{562-3}$  is itself capable of slow reduction of oxygen, or the majority of the succinate 'oxidase' is due to reaction of the dehydrogenase with oxygen (possibly peroxigenic).

4. In contrast to D-lactate, L-lactate and NADH, UV irradiation of membranes does not greatly inhibit reduction of cytochrome b by succinate (Figure 3.2.2.2), thus indicating that there is no quinone functioning between succinate and cytochrome b. Likewise, pCMB had little effect on the substrate-reduced and aerobic steady state cytochrome b spectra with succinate (Figure 3.2.4.2, A & C) which agreed with the finding that maximum inhibition of oxidase activity by pCMB is only about 20%. This indicated that there was no significant involvement of a pCMB sensitive site in cytochrome b reduction or oxidation with succinate

Succinate dehydrogenase and fumarate reductase activities are performed by a single enzyme in beef heart mitochondria (Massey and Singer, 1957) and in baker's yeast (Singer, Massey and Kearney, 1957). In the latter case, a Lineweaver-Burke plot for succinate is linear (as opposed to the results shown in Figure 3.1.2.1) indicating a single binding site constant for succinate. As mentioned previously, there are two distinct enzymes for these activities in *E. coli* (Hirsch *et al*, 1963).

#### 4.6: The Role of Cytochromes in Electron Transport

The discussion of the functional role of the different cytochromes has largely been covered in previous sections. This section gathers together and summarizes the information relevant to the role of the cytochromes.

In respect to its cytochrome content, *P. shermanii* clearly belongs with those anaerobic to microaerophilic heterotrophic bacteria in which the reduced minus oxidized difference spectra are dominated by b and d peaks with the possible presence of  $a_1$  (Meyer and Jones, 1973). There appear to be minor a and c components, but little can be concluded regarding the functional role of these cytochromes. A broad peak at 585-595 nm is present in the substrate-reduced and anaerobic steady state spectra but is absent in aerobic steady state spectra. This broad peak appears to remain reduced in the cyanide-inhibited aerobic steady state (Figure 3.2.6.2,C) so that it may constitute a minor oxidase, but, like the cytochrome  $a_1$  of *E. coli*, it may be kinetically



inadequate to make a significant contribution to total oxygen uptake (Haddock, Downie and Garland, 1976).

A very small but distinct shoulder at 548 nm in some difference spectra is the only indication that a c-type cytochrome is present. A similar small shoulder was observed by Schwartz and Sporckenbach (1975). This component was most evident in the succinate-reduced spectra (Figure 3.2.1.2,D), probably because it was not masked by cytochrome  $b_{553-4}$ , which, because of its low redox potential, is not substantially reduced by succinate. It is also evident in some uninhibited minus inhibited difference spectra (Figures 3.2.3.2 and 3.2.4.2) again due to its unmasking by the decreased reduction level of  $b_{553-4}$ . Cytochrome c was not observed in the pyridine hemochromogen spectrum of the ether insoluble acid acetone extract of membrane particles, but this may be due to the small amount present. A much larger scale preparation may be necessary to characterize this minor cytochrome component if present.

A functional role for cytochrome d ( $E_{m7} = +150$  mV) as the cytochrome of the major terminal oxidase seems to be fairly clear from the present study. This oxidase is known to be the main terminal oxidase in *E. coli* grown at low partial pressures of oxygen where its high affinity for oxygen enables *E. coli* to maintain high rates of aerobic electron transport under these conditions (Rice and Hempfling, 1978). A similar high affinity terminal oxidase in *P. shermanii* membrane particles is indicated by the linearity of oxygen uptake rates until a low

concentration of oxygen is reached. Its function *in vivo* may well be as an oxygen scavenger, since free oxygen is known to be inhibitory to growth of propionibacteria (Schwartz, 1973; deVries *et al*, 1972; Pritchard *et al*, 1977).

Cytochrome d is fully oxidized in the aerobic steady state but not in the anaerobic steady state. Cyanide binds to the d cytochrome forming a complex which lacks both the reduced absorption peak at 627 nm and the

oxidized peak at 650 nm (seen as a trough when oxidized membranes are in the reference beam) (Kaufmann and van Gelder, 1973; Pudek and Bragg, 1974). The high  $I_{50}$  concentration of cyanide (about 2 mM) for inhibition of oxygen consumption is consistent with cytochrome d being the major terminal oxidase since cytochrome d is known to have the highest  $K_i$  for cyanide of the known cytochrome oxidases (Jones, 1973; Oka and Arima, 1965) the value obtained in this study is of a comparable order to that of *E. coli* cytochrome d (7.4 mM, (Pudek and Bragg, 1974)).

The function of the b-type cytochromes has already been discussed in earlier sections (4.1, 4.2, 4.5). Room temperature redox titrations of the b peak indicated the presence of two groups of b-type cytochromes with  $E_{m7}$  of about -20 mV and about +100 mV. Low temperature difference spectra of redox poised membranes revealed at least four b-type cytochromes, two with low  $E_{m7}$  of about -20 mV ( $b_{553-4}$  and  $b_{556-7}^{LP}$ ), one with an approximate  $E_{m7}$  of +90 mV ( $b_{556-7}^{HP}$ ) and one with an approximate  $E_{m7}$  of +120 mV ( $b_{562-3}$ ). The two low potential b-type cytochromes,  $b_{553-4}$  and  $b_{556-7}^{LP}$ , both

have  $E_{m7}$  values which would permit a function role in electron transport to fumarate. As discussed in sections 4.2 and 4.3, some results indicate that cytochrome  $b_{556-7}^{LP}$  is the most likely candidate for a function in this role, whereas low temperature spectra in the presence of pCMB suggest that  $b_{553-4}$  is not substantially oxidized by fumarate and is therefore more likely to function on a pathway to oxygen.

As discussed in section 4.4.3, the high potential b cytochrome,  $b_{562-3}$ , appeared to be more rapidly reduced by succinate than by D-lactate or NADH, hence it was postulated that it is closely associated with succinate dehydrogenase. It is presumedly not a component of a terminal oxidase since it was at least partially reduced in the aerobic steady state (with succinate especially, but to a lesser extent with D-lactate and NADH (Figure 3.2.1.2)). The function of this cytochrome is thus obscure.

The other high potential b type cytochrome,  $b_{556-7}^{HP}$ , was not distinguishable from  $b_{556-7}^{LP}$ , except in the redox titrations, so that little can be deduced concerning its function. It may be the CO-binding cytochrome (Figure 3.1.5.2) and hence an o-type cytochrome. If this is the case, however, it has a very low oxidase activity, since no CO inhibition of oxygen consumption was observed. This result would have to be verified since CO inhibition of oxygen uptake has been reported in *P. pentosaceum* (Chaix and Fromageot, 1942). While the role of this b cytochrome thus remains obscure, it is tentatively placed as an element

of an aerobic pathway between  $b_{553-4}$  and cytochrome d.

#### 4.7: Conclusion

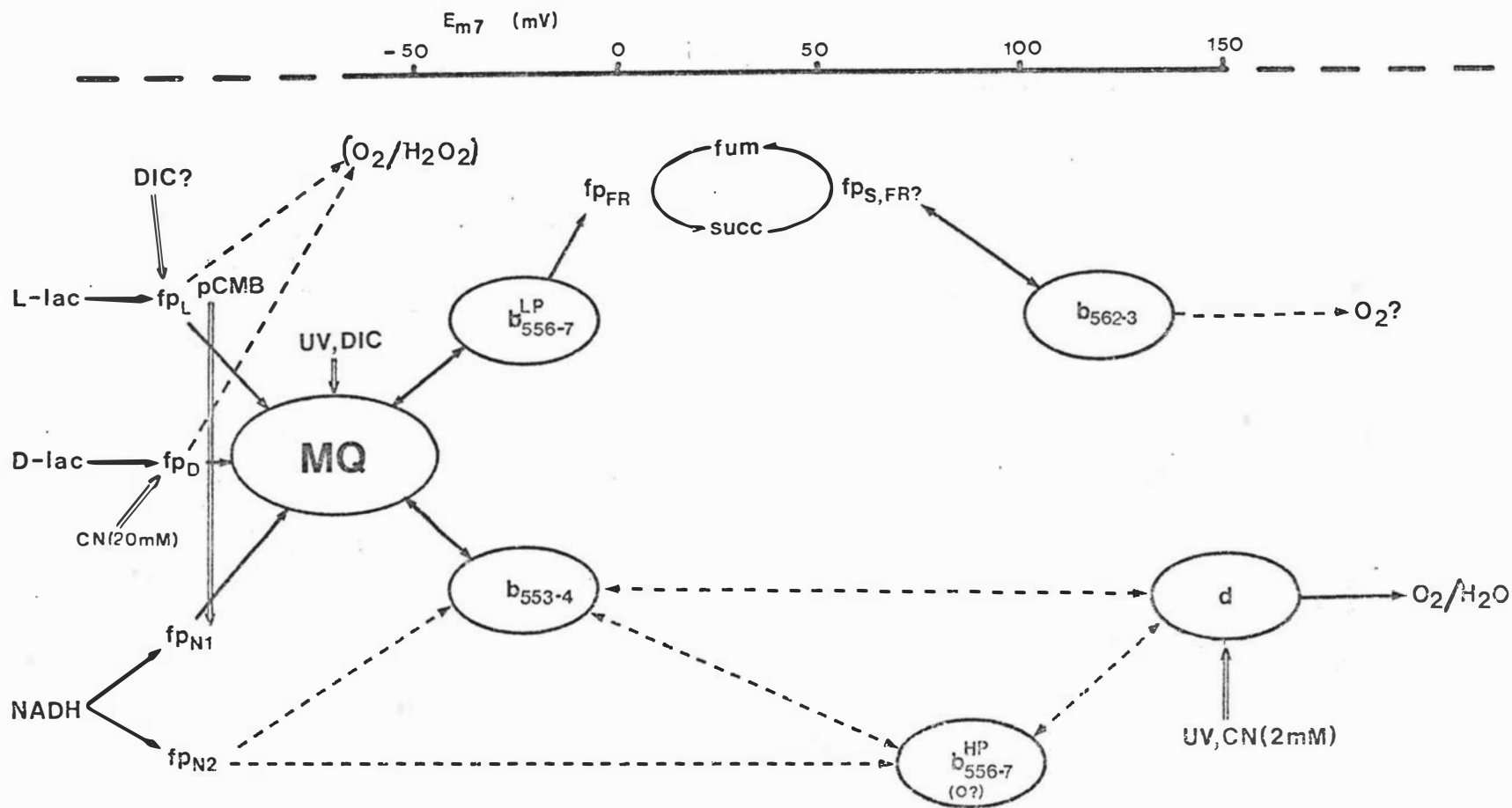
In conclusion, a scheme for electron transport in *P. shermanii* is presented in Figure 4.1. This scheme embodies the well established findings as well as the many tentative suggestions described in the Discussion. Several of the relationships suggested in this scheme are speculative and would require confirmation by more systematic analysis using a further extension of the approaches employed in work for this thesis or by other means. Further work on electron transport in *P. shermanii* should probably include isolation and characterization of the dehydrogenases of lactate, NADH and succinate, the reductase of fumarate and the terminal oxidase, by use of detergent fractionation or amphipathic column chromatography. Complexities which should be considered during such work are the presence and functions of quinones other than the characterized menaquinone (e. g., ubiquinone and pyrroloquinoline quinone), the presence and function of the four b type cytochromes, and the possibility that multiple dehydrogenases for NADH and succinate occur.

Another important consideration is the extent to which the electron transport processes studied in isolated membrane fractions reflect the processes occurring in the intact cell. For example, it was shown that L-lactate coupled activities were lower relative to D-lactate coupled activities in membranes than in whole cell suspensions due to instability of the L-lactate dehydrogenase system in the membrane preparation. Other less

readily detected changes may accompany membrane isolation, especially in the presence of oxygen.

The involvement of the systems studied in this work in the generation of a proton gradient and membrane potential and their coupling to ATP synthesis and active transport in propionibacteria are major areas for future investigation. No work has been done on the membrane bound ATPases of propionibacteria. The important role of inorganic pyrophosphate in carbohydrate catabolism in propionibacteria and the possibility that the generation of this pyrophosphate may be coupled to electron transport as suggested by Wood *et al* (1977) raise intriguing areas for future research on the bioenergetic mechanisms of propionibacteria.

Figure 4.1: Tentative Scheme for Electron Transport in *P. shermanii*



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