BIOCHEMICAL AND BIOPHYSICAL STUDIES ON MEMBRANE-BOUND NADH AND D-LACTATE DEHYDROGENASES OF ESCHERICHIA COLI K-12.

A Thesis presented by

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to

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in application for the

DEGREE OF MASTER OF SCIENCE

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February, 1984.

DECLARATION

I hereby declare that the following Thesis is based on work performed by me, that the Thesis is my own composition and that no part of it has been presented for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology of the University of St. Andrews under the direction of Dr. William John Ingledew.

F. A. ADENAIKE

CERTIFICATE

I hereby certify that Fidelis Adesegun Adenoike has spent seven terms as a matriculated post-graduate student under my direction and that he has fulfilled the conditions of Ordinance General No 12 and Resolution of the University Court 1967, No 1 and that he is qualified to submit the accompanying Thesis for the Degree of Master of Science.

W.J. Ingledew

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Dr. W.J. Ingledew for his assistance and constructive criticisms during the course of this work and to Dr. I.G. Young of the Department of Biochemistry, The John Curtin School of Medical Research, The Australian National University, Canberra, for the provision of the bacterial strains used in this study. My thanks also go to Mr. Alex Houston and Dr. Ann Lewis for their assistance with the electron paramagnetic resonance (e.p.r.) spectrophotometer. Last but not the least to Mr. C.O.J. Ikejiora for typing my Thesis.

DEDICATION

This Thesis is dedicated to my daughter

LILIAN DAMILOLA ADENAIKE.

CONTENTS

PROLOGUE

Young *et al.* (1982) reported the cloning of a plasmid (designated pIY2), lacking NADH dehydrogenase but possessing the gene of D-Lactate dehydrogenase. It was also reported that this plasmid was capable of complementing NADH oxidation when cloned with an *E.coli* strain with a mutation in NADH oxidation.

This strain (IY36) with the cloned gene of D-Lactate dehydrogenase and the NADH dehydrogenase amplified strain (IY35), in conjunction with some control strains (the wild type; *ndh* and D-Ldh$^-$ mutants) were studied. Electron paramagnetic resonance (e.p.r.) spectrophotometer was used to study their iron-sulphur centres and determine their redox chemistry.

Some of these studies were designed to unravel which part of the system in the E.coli electron transport chain was responsible for the complementation of NADH oxidation in the *ndh* mutant by the cloned gene of D-Lactate dehydrogena

ABSTRACT

Biochemical and biophysical studies have been carried out on the amplified NADH dehydrogenase and D-Lactate dehydrogenase of Escherichia coli. Strains of E. coli with amplified expression of these dehydrogenases were used. NADH and D-Lactate dehydrogenase mutants and the wild-type strains were also included in the studies.

These studies indicated that the strain with the amplified expression of the NADH dehydrogenase (IY35) did not have any alteration of some of its redox components compared to its isogenic strain. The strain with amplified expression of the lactate dehydrogenase (IY36) showed the enhanced presence of at least one iron-sulphur centre.

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ABBREVIATIONS

ADP	-	Adenosine diphosphate
ATP	-	Adenosine triphosphate
b_T	-	Cytochrome b_T
CR	-	Cohen & Rickenberg
CRTS	-	Cohen & Rickenberg trace elements plus supplements
dcip	-	dichlorophenolindophenol
EDTA	-	Ethylenediamine tetra acetic acid
Fo.Fi	-	ATP synthetase complex (Fo, hydrophobic portion; Fi, corresponds to the catalytic site)
FAD	-	Flavin adenine dinucleotide
FMN	-	Flavin mononucleotide
M/M^{2+}	-	Electron carrier
MTT	-	[3-(4,5-dimethylthiazolyl-)2,5 - diphenyl tetrazolium bromide]
NAD	-	Nicotinamide adenine dinucleotide (oxidised form)
NADH	-	Nicotinamide adenine dinucleotide (reduced form)
ndh	-	gene encoding NADH dehydrogenase
Pi	-	Phosphate
R/RH_2	-	Hydrogen carrier
S/SH_2	-	Succinate dehydrogenase (hydrogen carrier)
STM	-	Sucrose Tes Medium
Tes	-	N-Tris-(hydroxymethyl)-methyl-2-amino ethane sulphonic acid.

CHAPTER 1

1. INTRODUCTION

1.1 OVERVIEW OF STUDIES ON THE MECHANISM WHEREBY THE ELECTRON TRANSFER CHAIN CAUSES PROTON TRANSLOCATION

The mechanism whereby the electron transfer chain pumps protons with the ultimate synthesis of ATP has over the years generated much interest. In this respect, studies have been carried out by various workers on the mitochondrial membranes(see Tager et al., 1983), and the cytoplasmic membranes of various bacteria (Haddock & Jones, 1977). This has led to the proposition of various hypotheses (e.g., Mitchell, 1961; 1966; Boyer, 1977; Boyer et al., 1977; Williams, 1961; 1978) to explain this mechanism. The proposal of Boyer gave a scheme that could effect ATP synthesis by conformational coupling (Fig.1), the same way the presence of substrate induces conformational changes at the active sites of enzymes in enzyme-catalyzed reactions. The scheme of Williams (1961) on the other hand, is based on a diffusion mechanism. This diffusion mechanism later evolved into the localized model for ATP synthesis (Williams, 1978). However, of these hypotheses, the chemiosmotic hypothesis of Mitchell (1961),

which over the years was reiterated, or modified, (e.g., Mitchell, 1966; 1974; 1975a,b; 1977; 1979), appears to be the most popular.

The main tenet of this hypothesis resides in the fact that metabolic energy is conserved at the level of the membrane not in the form of a high energy intermediate (as proposed by Slater, 1953), but as a proton electrochemical gradient of hydrogen ions across the energy transducing membrane. In this mechanism, the proton electrochemical gradient generated by the electron transfer chain involving flavoprotein-containing dehydrogenases, quinones, cytochromes and iron-sulphur centers is used to drive ATP synthesis. The chemiosmotic proton circuit of Mitchell (Fig.2), depicts the electron transfer chain as being analogous to an electrical battery which creates an electrochemical gradient across the energy transducing membrane.

In describing the generation of the proton electrochemi gradient across the membrane, two kinds of mechanisms have been used over the years. They are the redox loop and the

proton pump. The redox loop (Fig.3), implies that protons and electrons flow across the membrane in opposite directions. The redox loop model was later embelished with a protonmotive ubiquinone (Q-cycle) by Mitchell (1977a,b). In the Q-cycle (Fig.4), ubiquinone (UQ) is said to play a central role in the proton and electron transfer within the bc_1 complex (e.g., Wikstrom, 1973; Rieske, 1976). Semiquinone ($QH^{\cdot}$) is the specific oxidant for the dehydrogenases. This is based on the assumption that the dehydrogenase reduction site for ubiquinone is located at the membrane site of the mitochondria and the cytochrome oxidation site at the cytoplasmic side. The proton pump (Fig.5), on the other hand has for many years been proposed as an alternative to the redox loop mechanism (Slater, 1967; Massari & Azzone, 1970; Chance et al., 1970; Papa, 1976).

However, despite all these investigations, the mechanism whereby the electron transfer chain pumps protons is still faced with some problems. These problems are: the lack of data on the organisation of the electron transfer chain components and the uncertainties on the number of H^+ translocated/O_2 (stoichiometry). Stoichiometry is important as the rate of respiration is controlled by the demand for ATP. Mitchell & Moyle (1967) by the O_2-pulse method reported that 4 protons were translocated per ATP synthesized for succinate respiration. Brand et al. (1976) found this to be underestimated and they reported a quotient of 6.0. On studies based on the rates of proton ejection and respiration, Reynafarge et al. (1976) reported a quotient of 8.0.

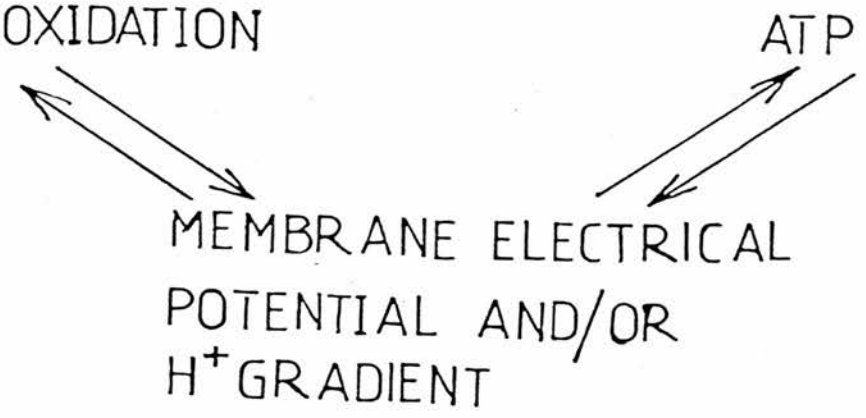
In conclusion, the controversy surrounding the mechanism of electron transfer as it affects stoichiometry is yet to be resolved. Recently, Wikstrom & Pentilla (1982) working on rat liver mitochondria, suggested the involvement of cytochrome c oxidase as a proton pump contrary to the earlier report of Mitchell & Moyle (1978). The solution to these problems can however be found with the detailed understanding of the redox chemistry and the structure of the electron transfer chain, and E. coli offers the best opportunity to attain this objective.

FIG. 1 Energy transmission in oxidative phosphorylation and photophosphorylation by

(a) membrane electrical potential and/or pH gradient

(b) membrane localized events (from Figs. 1 and 2 of Boyer, 1977).

a)



b)

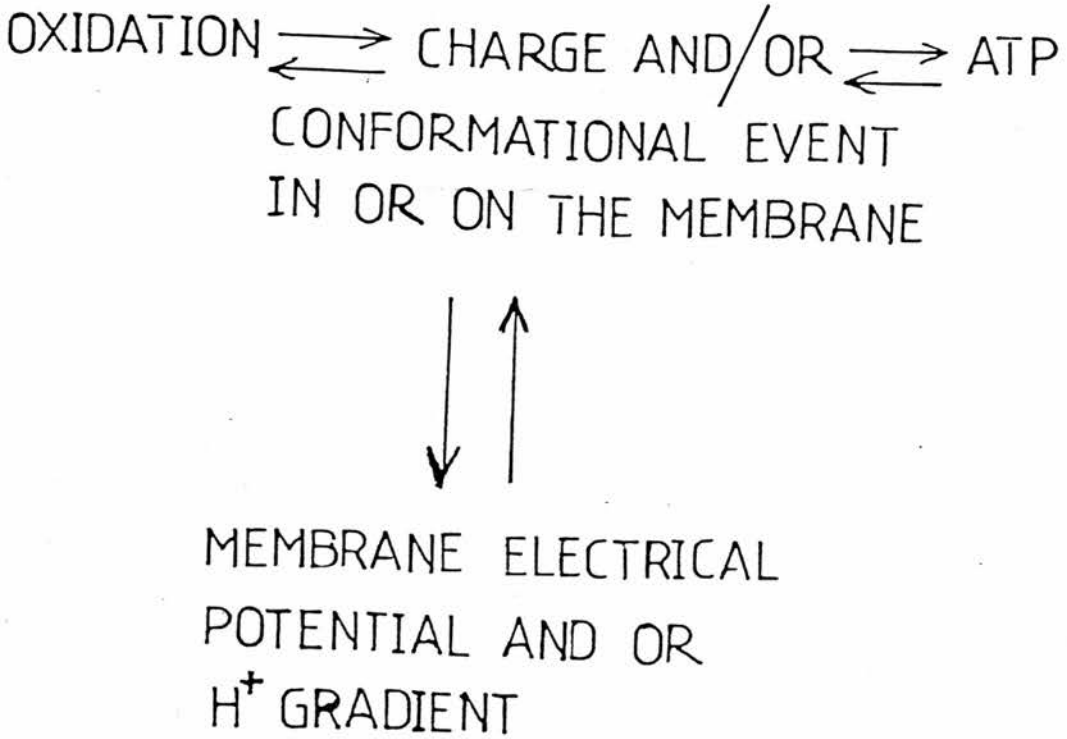


Fig. 2. The chemiosmotic proton current.

Abbreviations: M Side, membrane side; C Side, cytoplasmic side; ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; Fo.Fi, ATP synthetase complex.

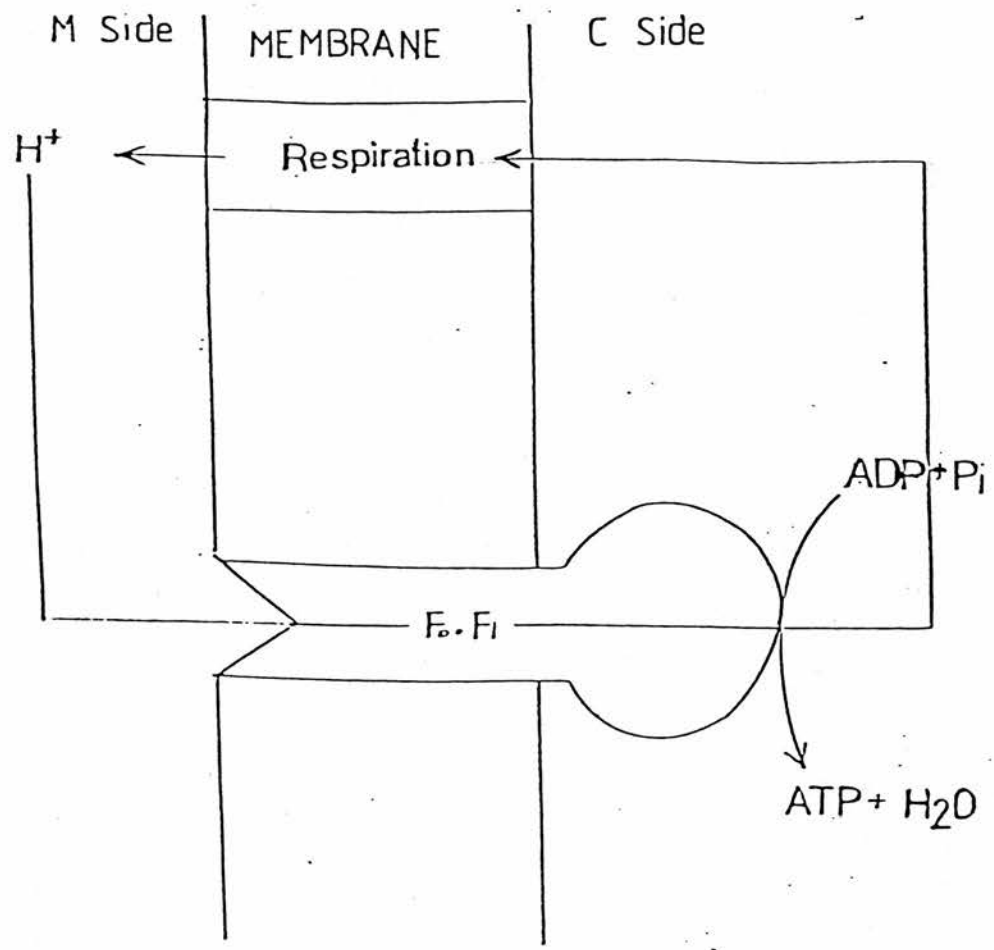


Fig. 3. Proton translocating oxido-reduction loop composed of a hydrogen carrier (R/RH_2) and an electron carrier (M/M^{2+}) - from Mitchell (1966).

Abbreviations: Phase L, Left Phase; Phase R, Right phase; SH_2, Succinate dehydrogenase.

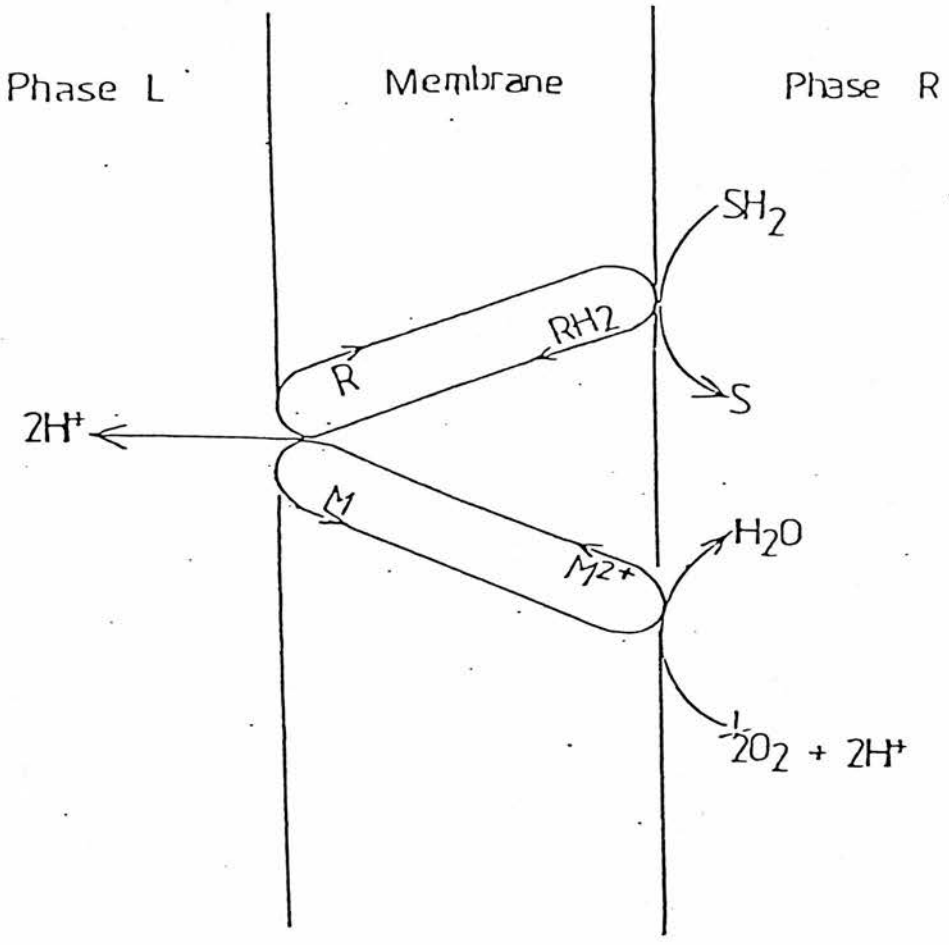


FIG.4 Proton translocating cyclic loop 2-3 system consisting of the Q-cycle system with cytochrome c and cytochrome oxidase ($a, 2Cu, a_3$) as oxidant for cytochrome c_1 (from Mitchell, 1975a).

Abbreviations; Q, Quinone; QH, Semiquinone; deH, dehydrogenase; M side, membrane side; c side, cytochrome side, b_T, cytochrome b_T.

FIG.4

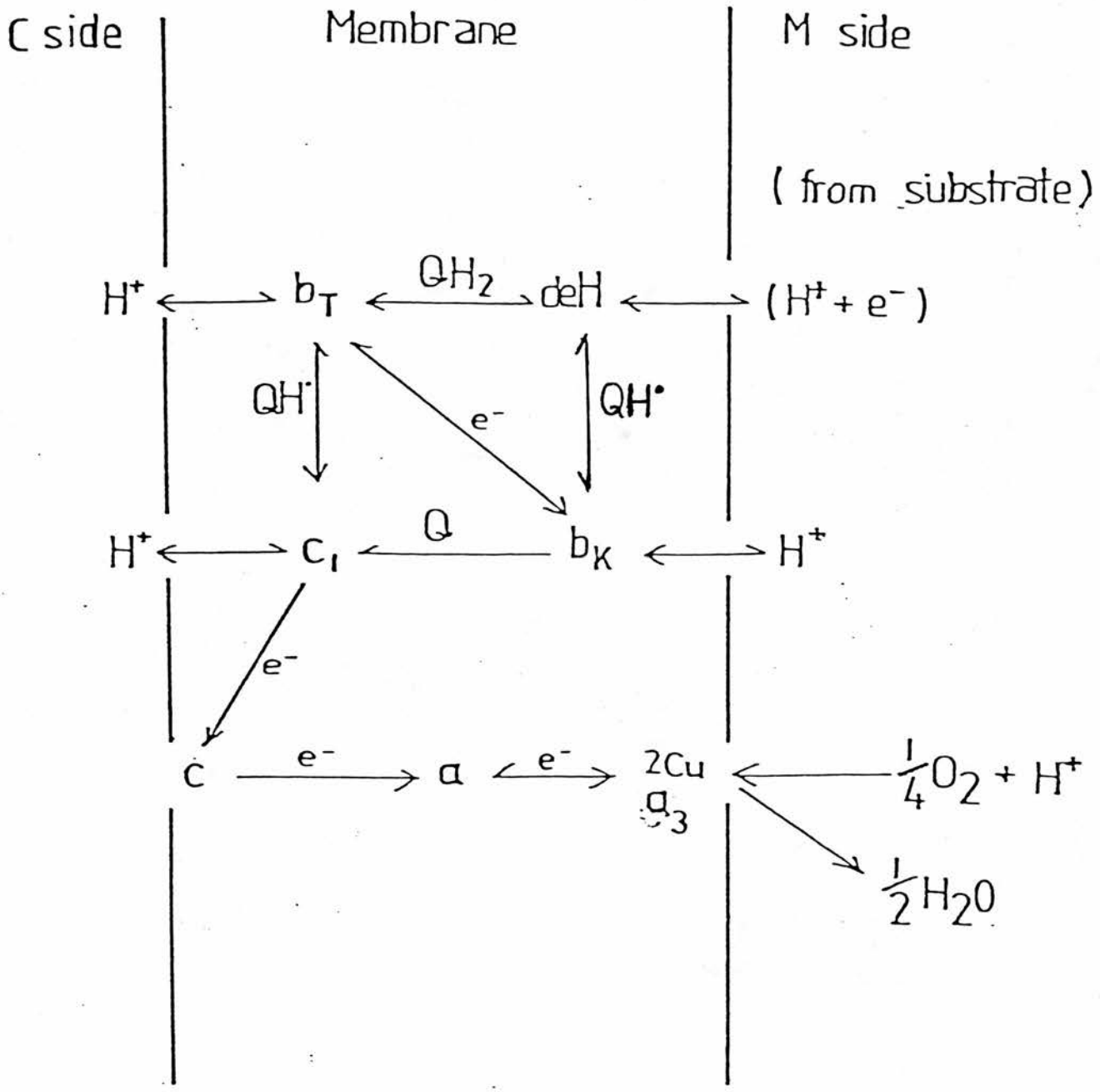
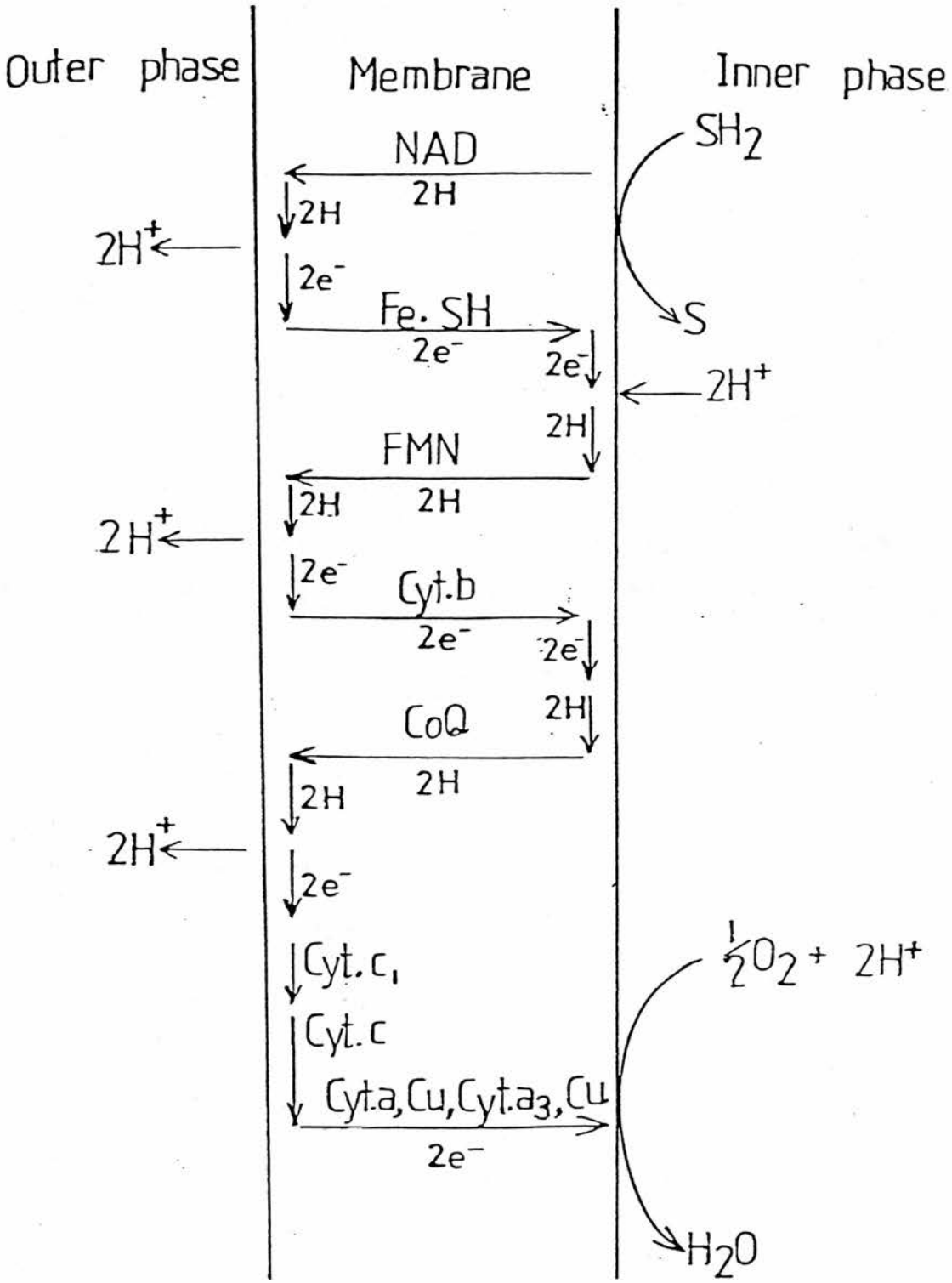

C side
Membrane
M side
(from substrate)
H+
bT
QH2
deH
(H+ + e-)
QH·
e-
QH·
H+
c1
Q
bK
H+
e-
c
e-
a
e-
2Cu
a3
1/4 O2 + H+
1/2 H2O

FIG.5 Proton pump mechanism (after Mitchell, 1966) for the oxidation of NAD-linked substrate of mitochondria.

Abbreviations: CoQ, Coenzyme Q; FMN, Flavin mononucleotide; Fe, SH, iron-sulphur proteins; Cyt, Cytochrome; Cu, Copper; NAD, Nicotinamide adenine dinuclotide; SH_2, succinate dehydrogenase.

FIG. 5


Outer phase
Membrane
Inner phase
NAD
2H
SH2
2H
2H+
2e-
Fe. SH
2e-
S
2e-
2H+
2H
FMN
2H
2H
2H+
2e-
Cyt.b
2e-
2e-
2H
CoQ
2H
2H
2H+
2e-
Cyt. c1
Cyt. c
Cyt.a, Cu, Cyt.a3, Cu
2e-
½O2 + 2H+
H2O

1.2 USE OF E.COLI FOR THE STUDY OF THE MECHANISM WHEREBY ELECTRON TRANSFER CHAIN PUMPS PROTONS.

In the study of the mechanism of proton translocation over the past 20 years, considerable attention has been devoted to the bacterial systems, and the bacterium - Escherichia coli appears to be the commonest organism used (see Bragg, 1979).

The simple nature of this microorganism - consisting of a cell wall with a plasma membrane which houses the electron transport components and the fact that this membrane contains a number of key enzymes similar to those found in the mitochondrial membrane offers it as a ready candidate for use in biochemical studies. In addition, compared to the mitochondrial counterparts, the E.coli cell offers an additional advantage in its being amenable to genotypic and phenotypic manipulations. In this direction, the E.coli cell has been genetically manipulated in the study of NADH dehydrogenase (Young et al., 1978; Poulis et al., 1981; Jaworowski et al., 1981a,b), and more recently in the cloning of the gene coding for the D-lactate dehydrogenase (Young et al., 1982), succinate dehydrogenase (Guest, 1981a), fumarate reductase (Cole & Guest, 1979; Lohmeier et al., 1980), and pyruvate dehydrogenase (Guest & Stephen, 1980). On the other hand, phenotypic manipulation

of the organism has been used for the study of the possible function of iron-sulphur components in energy coupling using iron-limited or sulphate-limited culture of this organism (Poole & Haddock, 1975). Hence the modification of or lack of the energy coupling site is being taken advantage of to investigate what impact such modifications or deficiency could have on the redox pump of the respiratory chain.

1.2.1 E.COLI REDOX COMPONENTS:

E.coli is a facultative anaerobe which changes its redox components in response to variation in growth conditions (Haddock & Jones, 1977; Reid & Ingledew, 1979). For example, aerobically grown E.coli cells beyond exponential phase produce two respiratory oxidases - cytochrome o and cytochrome d (Castor & Chance, 1959). When grown anaerobically with a non-fermentable carbon source (e.g., glycerol plus fumarate) the cells contain fumarate reductase as the terminal respiratory enzyme. These cells also contain a high level of cytochrome d while no cytochrome o is spectroscopically detectable. Cytochrome b_{555}, b_{558} and a_1 are found in addition to cytochrome d (Reid & Ingledew, 1979). The cells synthesize both a benzoquinone, ubiquinone-8 and a naphthoquinone, menaquinone-8 (Pandya & King, 1966) and the relative amounts of these

two quinones depend on the degree of aeration during growth. Lester & Crane (1959), Bishop *et al.* (1962), Pandya & King (1966), Polgase *et al.* (1966) and more recently Alexander & Young (1978b) showed that the principal quinone in aerobically grown *E.coli* cells is ubiquinone-8 while menaquinone-8 or its immediate precursor is the principal quinone in anaerobically grown cells. Cox *et al.* (1970) have suggested that ubiquinone is required for NADH and D-Lactate oxidation in *E.coli*. Wallace & Young (1977a) on the other hand suggested that while quinones link the individual dehydrogenases (Fig.6) to the respiratory chain, each dehydrogenase has specific structural requirements for quinone acceptors. Of all the quinones tested, only ubiquinone promoted the oxidation by succinate dehydrogenase while in contrast, electron transfer from quinones to cytochrome *b* of the terminal chain appears to be less sensitive to structural variations of quinone moiety (Wallace & Young, 1977a). The types of the dehydrogenases also vary with growth conditions and some of these dehydrogenases contain tightly bound prosthetic group - flavin which may be FMN or FAD. Some of these dehydrogenases contain iron-sulphur centres (Yoch & Carithers, 1979).

ATPase complexes are also essential components of E.coli energy coupling membranes and it has been shown by Abrahams et al.(1973) on the bacterial membranes and Senior (1973), on mitochondrial membranes that ATPase complexes from both sources by comparison have similar molecular features.

1.2.2. ARRANGEMENT OF E.COLI REDOX COMPONENTS:

The identification of the nature of redox components was made possible as a result of various studies which included the use of electrodes and spectroscopic techniques and specific inhibitors for the study of oxidative phosphorylation (Slater, 1967). This enabled the use of oxygen electrode to localize the relative position of electron entry and the site of inhibitor action. Redox potentiometric techniques of Wilson (1978) and Dutton (1978) are used to this end. The location of the relative positions of these redox components thus led to various proposals (e.g., Birdsell & Costa-Robles, 1970; Haddock & Schairer, 1973; Poole & Haddock, 1975; Haddock & Jones, 1977), for the sequence of the redox components of this organism. Fig.7 shows the proposed scheme of Haddock & Jones (1977) in which the E.coli aerobic redox carriers are arranged in

oxidoreduction loops or segments containing an alternating sequence of hydrogen and electron carriers.

1.3. MEMBRANE-BOUND NADH AND D-LACTATE DEHYDROGENASES OF ESCHERICHIA COLI

These two redox components of E.coli have been extensively studied (details later), and recently, Young et al. (1982) have a report on these enzymes on the ability of one complementing the effect of the deficiency of the other. It is therefore intended to study these two enzymes and possibly elucidate the mechanism of NADH oxidation in ndh mutants carrying a plasmid lacking the ndh gene but having the gene coding for the respiratory D-lactate dehydrogenase. Details of some studies on these two enzymes are given in the following subsections.

1.3.1 MEMBRANE-BOUND NADH DEHYDROGENASE OF E.COLI

E.coli membrane-bound NADH dehydrogenase is a relatively minor component of the cytoplasmic membrane. Although it is low in concentration, it has a high catalytic activity as it catalyzes the transfer of electrons from NADH generated by glycolysis to the respiratory chain. It is believed that its immediate electron acceptor is ubiquinone (Wallace & Young, 1977a) and that the electrons are transferred ultimately to molecular

oxygen with the eventual synthesis of ATP via oxidative phosphorylation. The enzyme therefore links the major catabolic and energy pathway of the cell. It performs a similar role to its mitochondrial counter part (Ragan, 1976).

There have been various attempts at its isolation and purification but this has created a lot of problems in view of its very low concentration. Brodie (1955) isolated a NADH-cytochrome c reductase from a heat-treated cell-free extract of this organism. Bragg & Hou (1967a) isolated a fraction which contained cytochrome b and cytochrome o from E. coli membrane particles that could oxidize NADH but would not oxidize succinate, formate, pyruvate or NADH. In the same year, Bragg & Hou (1967b) also isolated a menadione reductase and a soluble NADH oxidase partially purified from E. coli membrane particles. Gutman et al. (1968) reported a preparation which contained metalloproteins containing non-haem iron, labile sulphur, FMN and was capable of reducing ferricyanide, dichlorophenolindophenol (dcip) and menadione. Dancey et al. (1976) reported the isolation and purification of NADH dehydrogenase from osmotically lysed membrane vesicles active with a wide variety of electron acceptors including dcip and ferricyanide. Antibodies raised against the pure enzyme were used against the membrane-bound enzyme (Dancey & Shapiro, 1976). Both NADH dehydrogenase and NADH oxidase

activities decreased in parallel supporting the view that the purified enzyme is a component of the NADH oxidase pathway. More recently, this enzyme has been solubilized and purified by Jaworowski *et al.* (1981a) taking advantage of the cloning and amplification of this gene earlier reported by Young *et al.* (1978). The cloning was achieved by genetic recombination of an *E. coli* strain with a mutation in *ndh* activity (Young & Wallace, 1976) with a hybrid plasmid pIY1 (Young *et al.*, 1978) carrying the *ndh* gene. Amplification was achieved with either amino acid starvation or chloramphenicol addition.

The cloning and amplification of this enzyme has made possible its characterization (Jaworowski *et al.*, 1981b). The enzyme was shown to consist of 1 FAD per subunit, 70% (w/w) lipid predominantly phosphatidyl-ethanolamine, low levels of Fe and acid-labile sulphide suggesting the absence of iron-sulphur clusters (Jaworowski *et al.*, 1981b). SDS-gel electrophoresis shows the enzyme to consist of a single polypeptide species of M_r 47,000 (Jaworowski *et al.*, 1981a). Reconstitution studies showed that the pure enzyme is able to reconstitute membrane-bound cyanide sensitive NADH oxidase activity on membranes prepared from *ndh* mutant strains (Jaworowski *et al.*, 1981b). Wallace & Young (1977b) reported its reaction with ubiquinone in their studies with quinone

deficient mutants and concluded that quinones serve as a possible link of various dehydrogenases to the terminal transport pathway.

Inhibition studies (e.g., Bragg, 1965; Gutman et al., 1970) have shown the enzyme to be inhibited by some agents. Dicumarol, 2,4-dinitrophenol and pentachlorophenol have been shown by Bragg (1965) to inhibit NADH oxidase activity while Gutman et al. (1970) have shown that piericidin inhibits ubiquinone oxidoreductase activity.

1.3.2 MEMBRANE-BOUND D-LACTATE DEHYDROGENASE OF ESCHERICHIA COLI

D-Lactate dehydrogenase has been shown to be one of the membrane-bound primary dehydrogenases in the respiratory chain of E.coli (Cox et al., 1970). It is the first component of the membrane-bound D-lactate oxidase activity of this organism and plays a central role in the generation of energy for active transport of various sugars and amino acids (Kaback, 1974). It appears to be directed towards the production of lactate (Tarmy & Kaplan, 1968b). D-Lactate oxidation serves as a primary physiological electron donor for solute transport in E.coli (Kaback, 1974). The membrane-bound, flavin-linked

dehydrogenase converts D-Lactate to pyruvate and electrons derived from its oxidation process are passed into the cytochrome chain with oxygen as the terminal electron acceptor. Concomitant with electron flow, a proton electrochemical gradient is established and this provides an immediate driving force for electron transport (Ramos et al., 1976).

The enzyme has been extensively studied as shown by the reviews of Bragg (1979) and Garvie (1980); and studies show that the enzyme differs from the NADH dependent enzyme of E.coli (Tarmy & Kaplan, 1968a). It is functional in the membranes and vesicles from mutants deficient in active enzyme can be reconstituted by the addition of the purified enzyme (Futai, 1974; Short et al., 1974).

Like the respiratory NADH dehydrogenase, its low concentration has made several attempts for its solubilization and purification (e.g., Tarmy & Kaplan, 1968a,b; Futai, 1973; Kaczorowski et al., 1978; Pratt et al., 1979) difficult. However, Young et al. (1982) reported the cloning of a plasmid (designated pIY2) into a mutant lacking the ndh gene. This plasmid also lacks the ndh gene but possesses the gene coding for the respiratory D-Lactate dehydrogenase. This

plasmid was reported to be able to overcome the defect of NADH oxidation in ndh mutants. The cloning of the gene of this enzyme will no doubt remove the problems posed by its low concentration.

Studies (Futai, 1973; Kohn & Kaback, 1973; Pratt et al., 1979) have shown that the enzyme consists of a single subunit of $M_r \sim 74,000$ and contains 1 molecule of non-covalently bound FAD per molecule of the enzyme (Futai, 1973). Its optimum pH was given as ranging between 9.0 and 9.5 (Pratt et al., 1979) although assays were done routinely at pH 8.0 (Futai, 1973; Kohn & Kaback, 1973). Inhibition studies show that the enzyme was inhibited by oxamate and by 2,3-phosphoglyceric acid which are known to inhibit D-Lactate stimulated transport of membrane vesicles (Kaback, 1972). It is not inhibited by sulphydryl-reacting compounds such as iodoacetate, p-chloromecuribenzoate and N-ethyl maleimide at a concentration which inhibits the NAD^+ dependent D-Lactate dehydrogenase isolated from the cytoplasm (Tarmy & Kaplan, 1968b).

Immunological studies of Short et al.(1975) have shown the antiserum raised against the solubilized D-Lactate dehydrogenase as an effective inhibitor of lactate dehydrogenase activity and lactate dependent active transport.

The activity of the membrane-bound enzyme is enhanced by phospholipids (Kovatechev et al., 1981; Pratt et al., 1983) and the enzyme can be "delipidated" from its native state in the membrane by the action of detergents (Kohn & Kaback, 1973; Futai, 1973) or by "chaotropic" agents (Reeves et al., 1973).

FIG.6 **A simplified scheme showing quinones mediating redox reactions between the primary dehydrogenases and cytochrome b.**

Abbreviation: Cyt, Cytochrome.

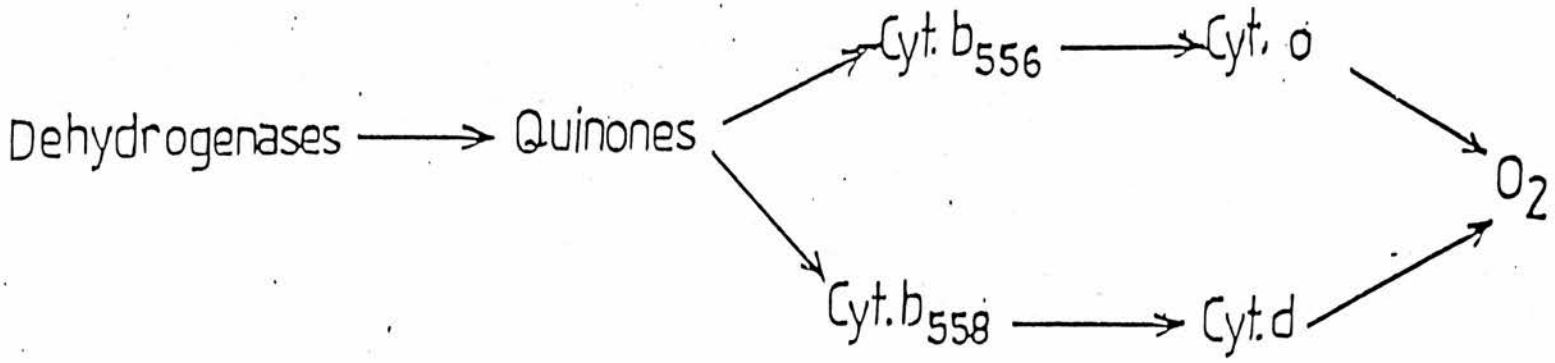
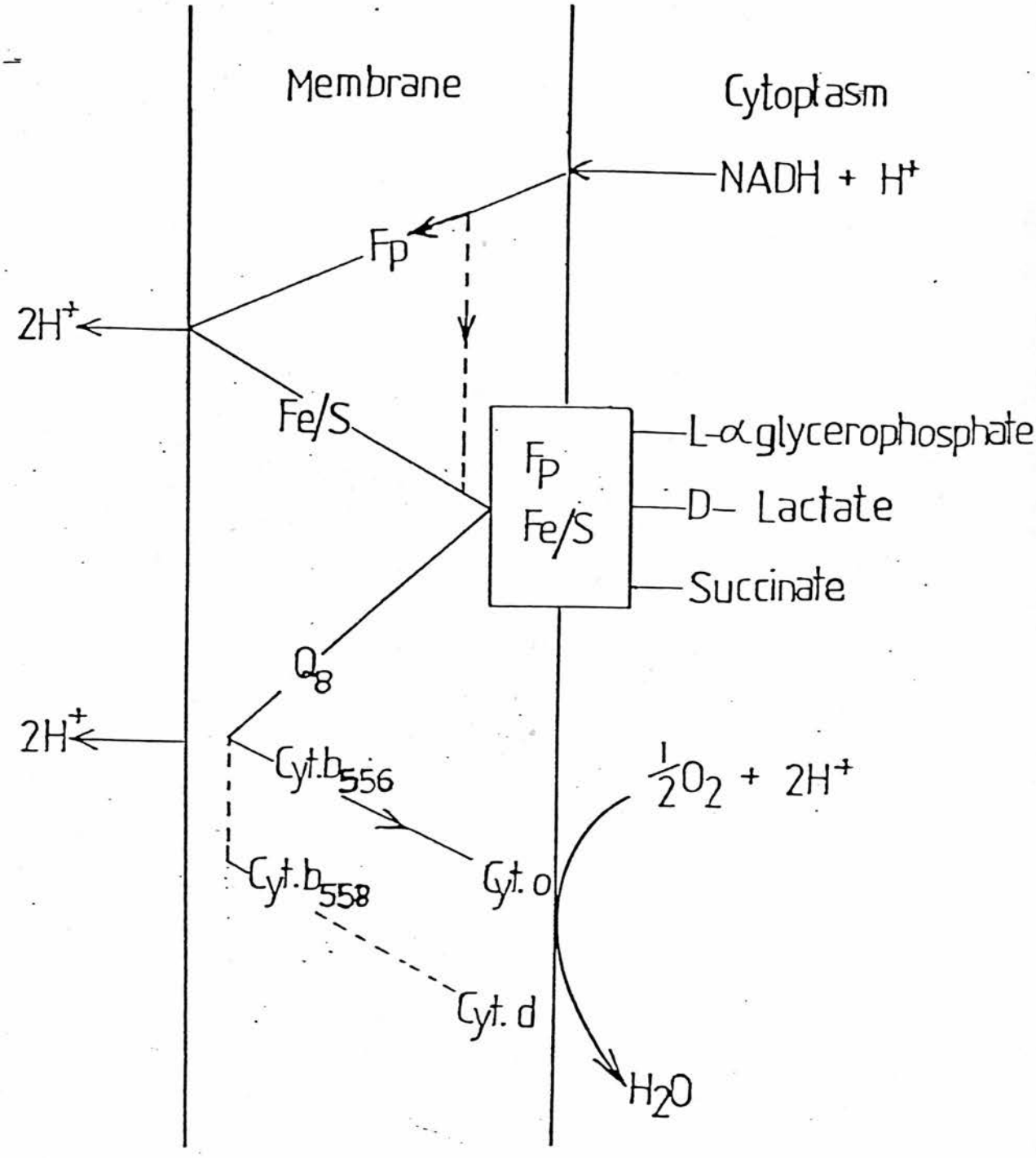


FIG.7 Proposed functional organization of the redox carriers responsible for aerobic electron transport in E.coli. The scheme includes the various routes for aerobic electron transport in E.coli with dashed lines indicating alternative pathways for reducing equivalents.

Abbreviations: Fp, flavoprotein; Fe/S, iron-sulphur proteins; Q, ubiquinone-8; Cyt, Cytochrome. (From Haddock & Jones, 1977).

FIG. 7


Membrane
Cytoplasm
NADH + H+
Fp
2H+
Fe/S
Fp
Fe/S
L-α glycerophosphate
D- Lactate
Succinate
Q8
2H+
Cyt.b556
½O2 + 2H+
Cyt.b558
Cyt. o
Cyt. d
H2O

CHAPTER 2

2. MATERIALS

2.1 WATER

Glass distilled water was used for the preparation of all solutions. This water has a pH of approximately 6.0

2.2 pH METER

The pH of all media was checked using a Pye 'Dynacap' pH meter (W.G. Pye & Co. Ltd., Cambridge, U.K.). For medium to be standardized, the instrument was calibrated using standard reference buffer of appropriate pH range. The standard reference buffer solution was prepared by dissolving a tablet of the buffer (BDH Chemicals Ltd., Poole, England, U.K.) in the recommended amount of distilled water.

2.3 CHEMICALS

Wherever possible, analytical reagents were used. Chemicals for bacterial growth and general media were mostly from BDH Chemicals Ltd., Poole, England, U.K.). L-tryptophan, L-histidine, L-isoleucine, L-valine, chloramphenicol, ampicillin, β -nicotinamide adenine dinucleotide (reduced form, from yeast),

deoxyribonuclease II (from bovine spleen), N, N-dimethyl-p-phenylene-diamine HCl; sodium salts of α-glycerophosphate, β-hydroxyl butyrate; D(-)Lactate, L(+)Lactate (lithium salts), and [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide](MTT) were from Sigma Chemical Company U.S.A. Agar and casein acid hydrolysate were from London Analytical and Bacteriological Media Ltd., London, U.K. Oxidation-reduction mediators were from Aldrich, Koch-Light and Eastman Kodak, U.K.

2.4 SPECTROPHOTOMETER

The spectrophotometer used for the measurement of dehydrogenase activities was a C.E.272 linear readout ultraviolet spectrophotometer fitted with a water-jacketed cell carriage (Cecil Instruments Ltd., Cambridge, England, U.K.). Water maintained at a predetermined temperature flowed through the carriage from a thermostatically controlled water bath (Grant Instruments Ltd., Barrington, Cambridge, England, U.K.). The spectrophotometer was in turn connected to a Bryan's recorder

2.5 CLARK OXYGEN ELECTRODE

A Clark oxygen electrode (Rank Brothers, Bottisham, Cambridge, England, U.K.) was used for the determination of oxidase activities of the membrane fragments.

2.6 ELECTRON PARAMAGNETIC RESONNANCE (EPR) SPECTROPHOTOMETER

The EPR analysis of the membrane fragments were made using the Bruker ER 200D EPR spectrophotometer (Bruker Analytische Messtechnik, Silberstreiten, D-7512 Rheinstetten, F.R.G.). The instrument was fitted with a variable temperature cryostat and liquid helium transfer line (Oxford Instruments, Osney Mead, Oxford, U.K.).

2.7 KLETT COLORIMETER

The turbidity of the cells before harvesting was measured using a Klett Summerson colorimeter (Klett MFG Co. Inc., N.Y., U.S.A.).

2.8 WAVELENGTH SCANNER

Wavelength scanning for cytochromes were made using a split beam spectrophotometer consisting of a 150 watt xenon arc lamp, applied photophysics monochromator, Bulova oscillator and E.M.I. extended red photomultiplier (assembled at the department of biochemistry and microbiology, Universit of St. Andrews, St. Andrews, U.K.).

2.9 BACTERIAL STRAINS

Bacterial strains (Table 1) are derivatives of *Escherichia coli* K-12. They included the IY12 strain

(*ndh* mutant) and its isogenic Ndh^+ transductant, the IY13 strain (Young *et al.*, 1978, 1982). Strains IY35 and IY36 were derived from strain IY12. IY35 strain (Young *et al.*, 1978) was constructed from the IY12 strain with a hybrid plasmid (pIY1) possessing the gene coding for the respiratory NADH dehydrogenase (Young *et al.*, 1978; Poulis *et al.*, 1978). Strain IY36 (Young *et al.*, 1978) was constructed from IY12 strain with a hybrid plasmid (pIY2) lacking the gene of NADH dehydrogenase but possessing the gene coding for the respiratory D-Lactate dehydrogenase (Young *et al.*, 1982). The D-Lactate dehydrogenase-deficient strain ($D\text{-}Ldh^-$) was derived from the wild type strain (IY13) by treatment with NTG. Details of the procedure for the isolation of the $D\text{-}Ldh^-$ strain are given in section 3.3.

TABLE 1

STRAINS OF *ESCHERICHIA COLI* USED

Strains	Sex	Plasmid content	Relevant genetic loci*	Source or other information
IY12	F-	-	*thi*, *his*, *ilv*, *trp*, *rpsL*, *ndh*	Young *et al.*, 1978; 1982.
IY13	F-	-	*thi*, *his*, *ilv*, *trp*, *rpsL*,	Isogenic Ndh^+ transductant of IY12 (Young *et al.*, 1978; 1982).
IY35	F-	pIY1	*thi*, *his*, *ilv*, *trp*, *rpsL*,	Young *et al.*, 1978.
IY36	F-	pIY2	*thi*, *his*, *ilv*, *trp*, *rpsL*, *ndh*	Young *et al.*, 1978.
D-Ldh^-	F-	-	*thi*, *his*, *ilv*, *trp*, *rpsL*,	Derived by mutagenesis of strain IY13 with NTG treatment (see section 3.3).

* Genetic nomenclature used is that of Bachmann *et al.*, 1980.

CHAPTER 3

3. METHODS

3.1 PREPARATION OF GROWTH MEDIA

The constituents of the growth media were as g/l of solution except where otherwise stated. They consist essentially of Cohen & Rickenberg (CR) mineral salts medium, CR-trace elements and supplements. The CR medium, CR-trace elements and supplements will be referred to as CRTS-medium.

3.1.1 CR-MINERAL SALTS MEDIUM

This was prepared according to the method of Cohen & Rickenberg (1956) in a modified form and consists of K_2HPO_4 (3 g), KOH (0.9 g), and $(NH_4)_2SO_4$ (2 g).

3.1.2 CR-TRACE ELEMENTS MEDIUM

This was also prepared according to the method of Cohen & Rickenberg (1956) with some modifications and consists of $FeCl_3 . 6H_2O$ (0.48 g), $CaCl_2 . 2H_2O$ (0.36 g), $ZnCl_2$ (2 g), H_3BO_3 (0.29 g) and $CoSO_4 . 7H_2O$ (0.19 g).

3.1.3 SUPPLEMENTS

The following supplements (according to the method of Jaworowski et al., 1981a) were added. These consist of mannito

(30 mM), casein acid hydrolysate (0.1%, w/v), L-histidine (0.15 mM), L-isoleucine (0.30 mM), L-valine (0.30 mM), L-tryptophan (0.20 mM), ampicillin (25 µg/ml), thiamine (1.0 µM; Young & Wallace, 1976) and $MgCl_2$ (0.5 mM).

3.1.4 PREPARATION OF LIQUID GROWTH MEDIUM (PER LITRE)

The liquid growth medium consists of the CR-medium as in section 3.1.1. To this was added 1.0 ml of well shaken CR-trace elements medium (section 3.1.2). This was followed by the addition of supplements (except ampicillin, thiamine and $MgCl_2$). The pH of the medium was adjusted with KOH (6 M) to pH 7.2 and the medium was sterilized by autoclaving.

25 µg ampicillin (dissolved in 1.0 ml of obsolute ethanol), 1 µM thiamine (prepared from a 1 mM stock solution in absolute ethanol) and 0.5 mM $MgCl_2$ (prepared from a 0.5 M stock solution and separately sterilized by autoclaving) were added to the medium immediately before use.

3.1.5 PREPARATION OF SOLID GROWTH MEDIUM

The preparation of the solid growth medium was essentially as for the liquid growth medium except that the

supplements consist in addition 2% (w/v) agar. This was for plates used for the maintenance of the strains and other cultural methods.

3.1.6 MAINTENANCE CULTURE

All bacterial strains were maintained on 2% (w/v) agar plates containing CRTS-medium (section 3.1.4.2) and incubated at 30°C. They were subcultured on fresh plates weekly in order to maintain their viability.

3.2 GROWTH OF THE BACTERIAL STRAINS

About 2 loopfuls of culture from the maintenance culture were used to inoculate 500 ml of CRTS-medium (section 3.1.4.2) and was grown under vigorous aeration at 37°C in a Status rotary shaker (Northern Media, North Cave, North Humberside, England, U.K.) until the extinction has reached about 350 Klett units using the Klett colorimeter (section 2.7) with a blue filter.

The shake flask culture was then used to inoculate a fresh 20 litre CRTS-medium. It was grown at 37°C under vigorous aeration and mixing. Aeration was maintained by continuous passage of CO_2 - free oxygen (British Oxygen

Company, U.K.). Growth was allowed to continue for each strain until the turbidity of the medium has reached about 160 Klett units.

For the plasmid-amplified strains (IY35 and IY36 stains), when the Klett units has reached about 160, 50 $\mu g\ ml^{-1}$ chloramphenicol (prepared in a minimum volume of absolute ethanol for sterilization) was added to the medium. The cells were left for another 9 h at 37°C with continuous aeration and mixing. At exactly 9 h after chloramphenicol addition, the cells were harvested at 18,000 r.p.m. with a flow rate of 200 ml min^{-1} in a MSE superspeed 18 centrifuge (Measuring & Scientific Equipment, London, U.K.) with a continous rotor. The cells were washed once with mannitol-free CR-medium (section 3.1.1) by resuspension and centrifugation in a Sorvall RG-5B refrigerated superspeed centrifuge (Du Pont Instruments Co. Inc., Delaware, U.S.A.) at 10,000 g for 10 min to remove the chloramphenicol. The cell paste was resuspended in CR-medium and was used to inoculate a fresh 10 litre CRTS-medium. The cells were again left to grow for 2 h with continuous aeration and mixing at 37°C. The cells were once again harvested (as previously described) and washed twice with mannitol-free CR-medium. The pellet was frozen in pea-form by dropping in small volumes with a pipette into liquid

nitrogen and refrigerated at -20°C until required for the membrane fragments preparation.

The growth of the plasmid-free strains were identical to the plasmid-amplified strains except that there was no addition of chloramphenicol. Hence the cells were harvested when the turbidity has reached about 160 Klett units, washed twice with mannitol-free CR-medium and pellet stored as described for the plasmid-amplified strains.

3.3 ISOLATION OF MUTANT LACKING D-LACTATE DEHYDROGENASE ACTIVITY (D-Ldh$^-$)

In order to have a control for the IY36 (the strain with the plasmid carrying the gene of D-Lactate dehydrogenase, mutant lacking D-Lactate dehydrogenase was isolated. Mutants of *Escherichia coli* have been useful in the study of the mechanism whereby electron transfer chain translocate protons (Cox & Gibson, 1974), as the absence of a particular gene could help to aid in studying the effect or modification on the organism as a result of this deficiency.

3.3.1 PROCEDURE

A Luria culture of the wild type (strain IY13) was diluted 1:20 with Luria broth and grown at 37°C with shaking to a Klett of 100 units (blue filter). The cells were harvested and the pellet resuspended in the same volume of 50 mM potassium phosphate buffer (pH 7.0). 9 ml of the cell suspension was then mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NTG; Adelberg et al., 1965). 1 ml of 500 μg ml^{-1} NTG (in 1 M citrate buffer; pH 5.5) giving the final concentration of 50 μg ml^{-1} NTG was used. It was then incubated at 37°C in a standing water bath for 1 h. Cells were washed with the 50 mM phosphate buffer and the pellet resuspended in a minimum volume of the same buffer. The cells were diluted with fresh broth and distributed into smaller volumes and were left in a shaker for 10 h at 37°C to allow for the expression of the mutants.

The cells were then washed twice with enrichment medium consisting of g/litre of citric acid powder (2 g), K_2HPO_4 (10 g), and 16.7 ml of 1 M NaH_2PO_4 neutralized to pH 7.0 with concentrated ammonia solution. The cells were then diluted to 10^6 cells ml^{-1} with CRTS-medium plus

1% (w/v) D-Lactate (instead of mannitol) and the culture was incubated at 37°C for 3 h.

200 $\mu g\ ml^{-1}$ ampicillin was added to the culture and it was returned to a shaker until lysis became apparent. Examination for lysis was carried out by taking 1.0 ml of the culture every 30 min and reading the absorbance at 610 nm. The cells were harvested, washed with phosphate buffer and the pellet resuspended in sterile solution (3%) of dimethyl sulphoxide (dmso)/glycerol (v/v). This was then distributed into small volumes (2 ml quantities) and frozen at -20°C.

3.3.1.1 SELECTION FOR D-LACTATE DEHYDROGENASE MUTANT (D-Ldh$^-$)

0.5 ml of the cell suspension in dmso/glycerol was added to 4.5 ml of sterile 0.9% NaCl solution. It was mixed thoroughly and 0.5 ml of this dilution was taken and added to the next tube (also containing 4.5 ml sterile 0.9% NaCl solution). This process was repeated with subsequent tubes until the final dilution reached 10^{-10}. 0.1 ml of each dilution was used to inoculate plates of CRTS-medium containing 1% (w/v) succinate (instead of mannitol). A

sterile spreader was used to spread the inoculum and the plates were incubated overnight at 37°C.

Discrete colonies were selected (with the aid of sterile tooth picks) on fresh plates of mannitol-free CRTS-medium containing 1% (w/v) succinate. The plates were again incubated at 37°C overnight.

The plates were then replicated according to the method of Lederberg & Lederberg (1952) on plates of CRTS-medium (with mannitol, section 3.1), CRTS (mannitol-free) but containing 1% (w/v) succinate and also CRTS (mannitol-free) medium containing 1% (w/v) D-Lactate.

Colonies which showed poor growth on D-Lactate plates were then selected, grown in 1 litre CRTS-liquid medium in a shaker at 37°C overnight. Cells were harvested and tested for D-Lactate oxidase activity. The above procedure was repeated several times until the desired result was obtained.

3.4 PREPARATION OF ELECTRON TRANSPORT PARTICLES (MEMBRANE FRAGMENTS).

The membrane fragments were prepared in the manner similar to that of Reid & Ingledew (1979) with some

modifications. The procedure was carried through at 4°C.

The cells were resuspended in EDTA/Tris buffer (pH 7.5) consisting of 5 mM EDTA and 10 mM Tris to give a protein concentration of ~10 mg ml^{-1}. The cells were well homogenized and 1 mg DNase (per 300 ml volume of the homogenized cells) was added to help render the cells free in solution. The DNase was allowed to act for about 15 min and the cells were then French pressed (American Instrument Co., Silver Springe, U.S.A.) at 120 MPa.

Unbroken cells were removed by centrifugation at 10,000 g for 15 min at 4°C in a Sorvall RG-5B refrigerated superspeed centrifuge (Du Pont Instruments Co. Inc., Delaware, U.S.A.). The decanted supernatant was centrifuged at 100,000 g for 1 h at 4°C using the MSE prespin 50 ultracentrifuge (MSE Fison Ltd., U.K.) to pellet the membrane fragments fractio

The pellet was well homogenized in a minimum volume (1:4) of Tes buffer (pH 7.5) consisting of 20 mM Tes. Aliquots of the homogenized membrane fragments were frozen in pea-form by dropping in small volumes (~0.3 ml) from a pipette into liquid nitrogen and stored at -20°C.

3.5 PROTEIN DETERMINATION

Protein determinations for all samples were performed according to the method of Lowry *et al.* (1951). The method was however modified as assays were performed in the presence of 1% SDS (Wang & Smith, 1975).

3.5.1 PRINCIPLE OF PROTEIN DETERMINATION

Proteins react with Folin and Ciocalteau reagent (a complex phosphotungstic molybdic acid reagent) to give a coloured complex. The colour formed is due to the reduction of the alkaline copper by the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the sample. The intensity of the colour which changes from original golden yellow to a deep blue depends on these aromatic amino acids present and thus varies with different proteins.

3.5.2 REAGENTS

3.5.2.1 LOWRY REAGENT - SOLUTION A

This consists of sodium carbonate (20 g), sodium potassium tartrate (0.5 g) and sodium hydroxide (4.0 g) dissolved in a litre of distilled water.

3.5.2.2 LOWRY REAGENT - SOLUTION B

0.5 g copper sulphate ($CuSO_4.5H_2O$) was dissolved in 500 ml of distilled water.

3.5.2.3 10% SODIUM DODECYL SULPHATE (SDS) SOLUTION

10 g of SDS was dissolved in 100 ml of distilled water.

3.5.2.4 WORKING LOWRY REAGENT

This was prepared immediately before use by mixing 9 parts of Lowry solution A with 1 part of Lowry solution B. Appropriate volume of SDS solution was added to give a final concentration of 1% SDS in the working solution.

3.5.2.5 WORKING FOLIN AND CIOCALTEAU REAGENT

This was prepared by diluting the stock solution of Folin and Ciocalteau reagent (BDH Chemicals Company, Poole, England, U.K.) 1:1 (v/v) with distilled water immediately before use.

3.5.2.6 STOCK PROTEIN STANDARD SOLUTION

The stock protein standard solution was prepared by dissolving 2 mg of defatted bovine serum albumin in 1 ml

of distilled water.

3.5.3 PROCEDURE

3.3 ml of working Lowry reagent was added to all tubes (in triplicate) and to each was added 2,5 and 10 µl of the sample. A protein calibration curve was constructed using 10,20,30,40,50 and 60 µl of 2 mg ml^{-1} stock standard protein solution corresponding respectively to 20,40,60,80,100 and 120 µg of protein. The tubes were well mixed as soon as samples and standards were added to the Lowry reagent and allowed to stand in a 37°C water bath for 10 min. This was immediately followed by addition of 0.3 ml of working Folin and Ciocalteau reagent and then mixed once again. All tubes were then allowed to remain in a 37°C water bath for 1 h. Extinction of each tube was read at 790 nm using a reagent blank to zero the instrument.

3.6 DETERMINATION OF OXIDASE ACTIVITIES FOR THE MEMBRANE FRAGMENTS

Oxidase activities for the membrane fragments were determined as described by Wallace & Young (1977b) except that 15 mM of substrates; succinate, formate,

β-hydroxybutyrate, malate, DL-Lactate, α-glycerophosphate (sodium salts); D-Lactate, L-Lactate (lithium salts) and 2.0 mg of NADH were used in a 3.0 ml sucrose/Tes/Mg^{2+} (S.T.M.) buffer pH 7.5 (consisting of 0.25 M sucrose, 0.1 M Tes and 0.02 M magnesium acetate) using a Clark oxygen electrode to monitor the rate of oxygen uptake.

3.6.1 PROCEDURE

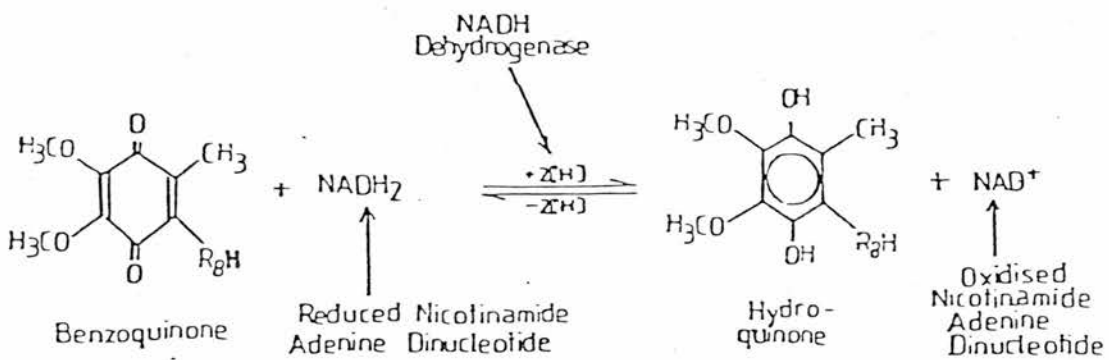
Through a suction pump, the distilled water used to set the sensitivity of the Clark electrode (maintained at 30°C) was withdrawn from the reaction vessel. 3 ml of S.T.M. buffer was added and the stopper was replaced in such a way that no air bubble was entrapped. With the aid of a Hamilton microsyringe, the desired volume of the membrane fragments (containing $\sim$ 0.3 - 1.0 mg protein) was added and the rates due to endogenous activity were determined. 15 mM of each substrate (section 3.6) was added to the reaction mixture. For the determination of the NADH oxidase activity, 2.0 mg NADH was added directly to the reaction mixture.

The Bryan's recorder which was connected to the Clark oxygen electrode allowed a continuous trace of the rate of oxygen consumption to be determined. Specific activities were expressed as μg-atom O/min/mg protein.

3.7 DETERMINATION OF BENZOQUINONE-DEPENDENT NADH DEHYDROGENASE (NADH: UBIQUINONE OXIDOREDUCTASE) ACTIVITY FOR THE MEMBRANE FRAGMENTS

3.7.1 PRINCIPLE OF ASSAY

NADH: ubiquinone oxidoreductase of the respiratory chain mediates the transfer of hydride ions from NADH to benzoquinone reducing the latter to hydroquinone while the former is oxidised thus:



R_8H = octaprenyl side chain

NADH absorbs at 340 nm while the oxidised form does not. Advantage of this characteristic is taken by monitoring the rate of oxidation (decrease in absorbance) at that wavelength. By knowing the rate of oxidation of NADH, the level of enzyme activity could be determined.

3.7.2 PROCEDURE

This was essentially as described by Jaworowski et al. (1981a) with some modifications which included the alteration of the concentration of the NADH in the final reaction mixture and the complete omission of exogenous flavin adenine dinucleotide (FAD).

The NADH dehydrogenase activity of the membrane fragments was determined at 30°C by measuring the ubiquinone-dependent oxidation of NADH at 340 nm in a 1 ml reaction (1 cm light path) cuvette containing 20 mM Tes, (pH 7.5) 50 mM benzoquinone, 3 mM KCN and 315 μM (freshly prepared) NADH.

The reaction was commenced immediately by the addition of the sample (containing ~0.3 - 1.0 mg protein) and the rate of oxidation (decrease in absorbance per minute) were measured under anaerobic conditions with a C.E. linear readout ultraviolet spectrophotometer fitted with a water jacketted cell carriage. The rate of oxidation per minute was measured using a Bryan's recorder connected to the spectrophotometer.

1 unit of enzyme activity was expressed as μmol of NADH oxidised per mg protein at 30°C under the assay

conditions. Oxidation of 1 μmol NADH in a 1 ml reaction mixture causes a decrease of 6.22 in extinction at 340 nm.

3.8 DETERMINATION OF LACTATE DEHYDROGENASE ACTIVITY FOR THE MEMBRANE FRAGMENTS

Lactate dehydrogenase activity for the membrane fragments was determined as described by Futai (1973), the difference being that assays were done at 30°C as against 23°C.

3.8.1 PRINCIPLE OF ASSAY

The NADH-independent lactate dehydrogenase has a physiological function of catalyzing the oxidation of lactate with the subsequent formation of pyruvic acid.

In a reaction involving phenazine methosulphate coupled reduction of [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide] (MTT), the two protons from lactate oxidation are taken up by the phenazine methosulphate/MTT complex. As the complex is being reduced the absorbance increases. Hence by monitoring the rate of increase in absorbance at 570 nm, the lactate dehydrogenase activity of the sample could be determined.

3.8.2 PROCEDURE

The assays were done in a 1 ml (1 cm light path) cuvette containing 0.08 M Tris-HCl buffer pH 8; 60 μg ml^{-1} MTT; 120 μg ml^{-1} phenazine methosulphate and sample (containing $\sim$ 0.6 - 1 mg protein). The contents of the cuvettes were well mixed and 2 - 3 min were allowed for endogenous activity due to the utilization of endogenous substrate (if any).

From a steady state on the recorder, 10 mM of substrate (D-Lactate, L-Lactate or DL-Lactate) was added to the cuvette to initiate the reaction and the increase in absorbance was measured at 570 nm. 1 unit of enzyme activity was taken as 1 μmol of MTT reduced per minute taking the extinction coefficient of MTT at 570 mM as 17 mM^{-1} cm^{-1} (Kistler & Lin, 1971).

As in the NADH dehydrogenase determination, all assays were done under strict anaerobiosis, more so that both phenazine methosulphate and MTT are autooxidizable. The reagents were also wrapped with tin-foil to exclude direct contact with light.

3.9 DETERMINATION OF ACID LABILE SULPHIDE CONTENTS FOR THE MEMBRANE FRAGMENTS

The acid labile sulphide or "acid labile sulphur" (Hall et al., 1975) contents for the membrane fragments were determined as described by King & Morris (1967) except that the volumes of the samples were halved to enable the use of Eppendorf tubes for the subsequent centrifugation. Also, since high protein in the sample resulted in turbid supernatant, samples with a protein concentration of $\sim$ 1.5 - 3 mg per assay were used for the determinations.

3.10 DETERMINATION OF THE CYTOCHROME CONTENTS FOR THE MEMBRANE FRAGMENTS

Cytochrome contents for the membrane fragments were determined from the reduced minus oxidised difference spectra at 25^{o}C (for molar extinction coefficients used - see Table 9) using a split beam spectrophotometer. The samples were reduced with dithionite ($\sim$ 0.5 mg ml^{-1}) while oxidation was effected with 3 µM hydrogen peroxide, Samples with a protein concentration ranging from 3.0 - 10.5 mg ml^{-1} were used for the determinations.

3.11 DETERMINATION OF THE PEPTIDE-BOUND FLAVIN CONTENTS FOR THE MEMBRANE FRAGMENTS.

The peptide-bound flavin contents for the membrane fragments were determined as described by Rao *et al.* (1967) for mitochondrial preparations, with some modifications.

3.11.1 PRINCIPLE:

The membrane fragments were treated with trypsin to release the flavins bound to proteins, followed by heat and acid denaturation. The total flavins obtained under this condition were estimated by decrease in absorbance at 450 nm on reduction with sodium dithionite. The difference between the values of the trypsin treated and the untreated sample gave the peptide-bound flavin contents for the membrane fragments.

3.11.2 PROCEDURE:

The procedure was as described by Rao *et al.* (1967), with the following modifications: Membrane fragments containing 20 - 30 mg ml^{-1} protein were used for the determinations. 15 mg trypsin was used to digest 2.0 ml of membrane preparations while 0.75 ml of 50 % (v/v) perchloric acid was used for the acid denaturation.

3.12 PREPARATION OF SAMPLES FOR LOW TEMPERATURE EPR SPECTROSCOPY

3.12.1 PREPARATION OF SUBSTRATE REDUCED SAMPLES

This was essentially as described by Beinert et al. (1978). With the aid of a narrow rubber tubing attached to the tip of a pasteur pipette, about 0.5 ml of well homogenized membrane fragments was sucked and gently lowered to the bottom of an e.p.r. tube (consisting of a 3 mm diameter quartz tube) and the pipette was gently withdrawn to prevent the sample from being dragged up again. The required amount of substrate was added and was well stirred by means of a long piece of wire whose end going into the tube was specially designed to fit the inner wall of the tube but at the same time allowed the free movement up and down the length of the tube. The tube was then shaken to bring its contents down the tube and also to prevent entrapment of air. The tube was then rapidly cooled in isopentane/cyclohexane (4:1, v/v) freezing mixture. The cooling mixture itself was contained in a dewar into which was suspended a glass cup containing liquid nitrogen. The tube was then removed and placed in a dewar under liquid nitrogen pending e.p.r. spectroscopy.

3.12.2 REDOX TITRATIONS OF THE REDOX COMPONENTS FOR THE MEMBRANE FRAGMENTS

Titrations were performed as described by Dutton (1978), using a vesel which was continuously flushed with white spot nitrogen (British Oxygen Company) which has been passed through a nil-ox apparatus (Jencon's Scientific, Mark Rd., Hemel Hempstead, U.K.) to remove residual oxygen and thus maintained a state of anaerobiosis throughout the determinations. This is very important because most of the redox dyes used in the experiment are autooxidizable and hence could destabilize the equilibrium of the system. Also, the presence of oxygen in the system could also interact with individual components of the biological electron transfer system at widely differing rates.

The membrane fragments to be titrated was made up to a protein concentration of approximately 20 - 30 mg ml^{-1} in 50 mM Tes/2 mM EDTA buffer (pH 7) and was added to the titration vessel. Since redox centers are often encased in protein and do not have direct contact with the electrodes, redox mediators (which act as a go-between the biological redox couples and electrodes), were added. These consisted of 50 μl of each of the dyes listed in Table 2.

The stirrer was switched on and the contents of the reaction chamber were left for sufficient time to allow for endogenous reduction. With the aid of a Hamilton pipette, freshly prepared dithionite solution ($\sim$ 0.5 g ml^{-1}) was added in a stepwise manner until a steady E_h was reached, and samples were taken at appropriate intervals. The measurement of the ambient E_h was done with a reference electrode which is a combination ombination of platinum-calomel (Russell pH, Auchtermuchty, Fife, U.K.). The samples were immediately cooled in iso-pentane/cyclohexane mixture and rapidly frozen in liquid nitrogen (section 3.12.1.1) pending e.p.r. spectroscopy. The electrode was calibrated with 50 mM phosphate buffer (pH 7) by adding few microlitres of 5 mg/ml quinhydrone in ethanol to the phosphate buffer in a stepwise manner with mixing until a steady E_h was reached. This reading was subtracted from those obtained for the redox titrations to give the true E_h values of the redox samples.

TABLE 2

LIST OF THE REDOX MEDIATORS USED FOR THE REDOX TITRATIONS.

DYES	CONCENTRATION (mg ml^{-1})
Anthraquinone-2,6-disulphonate	10.0
Anthraquinone-2-sulphonate	10.0
Cysteine	10.0
Pyocyanine	10.0
Methyl viologen	10.0
2-hydroxyl-1, 4-naphthoquinone	10.0
1,4-methyl naphthoquinone	5.0
Anthraquinone-1, 5-disulphonate	10.0
5 hydroxyl-1, 4-naphthoquinone	10.0
Benzyl viologen	10.0
Indigo tetrasulphonate	10.0
N-methyl phenazonium sulphate	10.0
Indigodisulfonic acid	10.0
Duroquinone	5.0
Phenosafranine	10.0
Resorufin	10.0

CHAPTER 4

4. STUDIES ON ENZYME ACTIVITIES FOR THE MEMBRANE FRAGMENTS

4.1 INTRODUCTION

Studies were carried out on the membrane fragments from the plasmid-amplified strains (IY35 and IY36) and where necessary, on the membrane fragments from the wild type (IY13) and the mutant strains (IY12 and D-Ldh$^-$). The inclusion of the membrane fragments from the wild type and the mutant strains enabled comparative analysis to be made. Studies on the mechanism whereby electron transfer chain pumps protons have been simplified with the use of mutants (Cox & Gibson, 1974). These studies include the determination of the effect of storage at -20^{o}C on the activities of the membrane-bound NADH and D-Lactate dehydrogenases; NADH dehydrogenase, NADH oxidase, lactate dehydrogenase and lactate oxidase D-lactate, L-lactate and DL-lactate as substrates); pH profiles of the membrane-bound NADH and D-Lactate dehydrogenases and effect of some additions on the

activities of both NADH and D-Lactate dehydrogenases. Other oxidase activities (formate, α-glycerophosphate, malate, succinate and β-hydroxybutyrate) were also determined. All these determinations (except the effect of storage or some additions on the activities of NADH and D-Lactate dehydrogenase and their pH profiles) were designed to see the effect of the amplication of the gene of NADH or D-Lactate dehydrogenase on other oxidase activities.

4.2. EFFECT OF STORAGE AT -20°C ON THE ACTIVITIES OF MEMBRANE-BOUND NADH AND D-LACTATE DEHYDROGENASES

The activities of some respiratory oxidases have been shown by Jones & Redfearn (1966) on Azotobacter vinelandii membrane fragments to decrease on storage at 5°C and -15°C with time. Succinate and lactate oxidase activities were shown to decrease slowly during storage (37 % and 33 % respectively) while NADH oxidase activity on the other hand, slowly increased over the period of the experiment. Hence the effect of storage at -20°C on NADH and D-Lactate dehydrogenase activities over a given period was determined.

4.2.1 RESULTS AND DISCUSSION OF THE EFFECT OF STORAGE AT -20°C ON THE MEMBRANE-BOUND NADH AND D-LACTATE DEHYDROGENASE ACTIVITIES

The results of the effect of storage at -20°C on the activities of NADH and D-Lactate dehydrogenases are presented in Table 3. The results show that the activity of the NADH dehydrogenase appears to decrease slowly while the activity of the D-Lactate dehydrogenase decreases rapidly. This thus indicates that the activity of the NADH dehydrogenase is more stable than that of the D-Lactate dehydrogenase. The results are in agreement with the results of NADH and lactate (DL-Lactate) oxidase activities of the membrane fragments from Azotobacter vinelandii (Jones & Redfearn, 1966).

4.3 NADH DEHYDROGENASE AND NADH OXIDASE ACTIVITIES FOR THE MEMBRANE FRAGMENTS

The membrane-bound NADH dehydrogenase or NADH oxidase catdlyses the entry of NADH into the respiratory chain and commits the electrons of NADH to the production of an electrochemical gradient or ATP.

TABLE 3

Effect of storage at -20°C on the activities of the membrane-bound NADH and D-lactate dehydrogenase. Enzyme assays were performed as described under methods. Specific activities are expressed as μmol/ min/ mg protein and as the mean ± standard deviation of 3 determinations. Percentage activities are expressed as percentage activities for day 1 determinations. The NADH and D-Lactate dehydrogenase activities were determined with membrane fragments from the IY35 and IY36 strains respectively.

DAYS	NADH DEHYDROGENASE		D-LACTATE DEHYDROGENASE	
	Specific activity	Percentage activity	Specific activity	Percentage activity
1	25.147 ± 1.166	100.0	5.98 ± 0.243	100.0
14	24.120 ± 1.222	95.9	4.82 ± 0.336	82.0
20	23.120 ± 1.166	91.9	2.90 ± 0.200	49.3
25	22.280 ± 1.083	88.6	2.72 ± 0.190	48.5
30	21.830 ± 1.123	86.8	1.536 ± 0.176	26.1

The determinations of the activities of the NADH dehydrogenase and the NADH oxidase were intended to study the effect of the amplification of both components in the plasmid-amplified strains and the plasmid-free strains.

4.3.1. RESULTS AND DISCUSSION OF NADH DEHYDROGENASE AND NADH OXIDASE ACTIVITIES FOR THE MEMBRANE FRAGMENTS DERIVED FROM THE BACTERIAL STRAINS

Results of the NADH dehydrogenase and NADH oxidase activities for the membrane fragments from the bacterial strains are presented in Table 4. The results show that the membrane fragments derived from the IY35 strain (ndh amplified strain) have the highest NADH dehydrogenase and NADH oxidase activities. This is consistent with the values of 31.1 μmol / min/ mg protein and 3.23 μg-atom O/ min/ mg protein for NADH dehydrogenase and NADH oxidase activities obtained by Young et al. (1978) for membrane fragments from IY35 strains under similar conditions.

The values obtained for the membrane fragments from the IY13 strains are in agreement with the values of 0.70 μmol / min/ mg protein and 0.550 μg-atom O/ min/ mg protein for NADH dehydrogenase and NADH oxidase activities respectively obtained by Jaworowski et al. (1981a) for membrane fragments from this strain under similar conditions.

TABLE 4

NADH dehydrogenase (NADH: ubiquinone-dependent) and NADH oxidase activities for the membrane fragments derived from the bacterial strains. Fresh membrane fragments were used for each determination and assays were performed as described under methods. Results are expressed as the mean ± standard deviation of 3 determinations.

MEMBRANE FRAGMENTS FROM	NADH DEHYDROGENASE ACTIVITY (μmol/ min/ mg protein)	NADH OXIDASE ACTIVITY (μg-atom O/ min/ mg protein)
IY12	0.062 ± 0.005	0.100 ± 0.009
IY13	0.639 ± 0.060	0.515 ± 0.031
IY35	36.280 ± 2.842	1.476 ± 0.239
IY36	0.183 ± 0.016	0.215 ± 0.030
D-Ldh$^-$	0.774 ± 0.109	0.695 ± 0.032

The values obtained for these components from the IY12 (ndh mutant) strain are higher than the values expected for a ndh mutant. Similar inconsistency was shown by the values obtained for these components by Young et al. (1978) and Jaworowski et al. (1981b). While Young et al. (1978) obtained a value of 0.02 μg-atom O/ min/ mg protein for NADH oxidase activity for the membrane fragments from the IY12 strain, Jaworowski et al. (1981b) obtained a value of 0.115 μg-atom O/ min/ mg protein for the NADH oxidase activity from this strain. Also, a value of 0.03 μmol / min/ mg protein was obtained for IY12 NADH dehydrogenase activity by Young et al. (1978) while Jaworowski et al. (1981b) obtained a value of 0.426 μmol / min/ mg protein for this component for membrane fragments from the IY12 strain. In both cases mentioned above and for the values obtained in this study, the IY12 NADH dehydrogenase and NADH oxidase activities appear to increase with time. These higher values for the IY12 NADH dehydrogenase and NADH oxidase activities may well be due to the presence of some NADH revertants in the culture of this strain.

The results obtained for the membrane fragments from the IY36 strain (derived from the IY12 strain) are elevated

compared to the values obtained for the IY12 strain. This raises the question of whether or not the amplification procedure for the IY36 strain affects the levels of NADH dehydrogenase and NADH oxidase activities for membrane fragments from these strains.

The values of 0.774 μmol / min/ mg protein and 0.695 μg-atom O/ min/ mg protein respectively for NADH dehydrogenase and NADH oxidase activities for the D-**L**dh$^-$ membrane fragments are in agreement with the values obtained for these components from the IY13 strain (from which the D-**L**dh$^-$ strain was derived).

In conclusion, the results obtained for NADH dehydrogenase and NADH oxidase activities for the membrane fragments from the bacterial strains confirm the amplificatio of the gene of NADH dehydrogenase in the IY35 strain.

4.4 LACTATE DEHYDROGENASE AND LACTATE OXIDASE ACTIVITIES FOR THE MEMBRANE FRAGMENTS

4.4.1. INTRODUCTION

D-Lactate serves as a primary physiological donor in E.coli (Kaback, 1974). The membrane-bound, flavin-linked D-lactate dehydrogenase converts D-Lactate to pyruvate and the electrons derived from its oxidative process are passed to the cytochrome oxidation chain with oxygen as the terminal electron acceptor. This causes the production of electrochemical gradient which provides a driving force for active transport (Ramos et al., 1973).

In addition to the determinations of the D-Lactate dehydrogenase and D-Lactate oxidase activities; the L-Lactate dehydrogenase and L-Lactate oxidase activities for the membrane fragments were also determined. The determinations of the latter activities were designed to show whether or not the amplification of the gene of D-Lactate dehydrogenase in the IY36 strain affected the activities of these components. The determinations are of interest as it had been shown by Short et al., 1975a; (by preparing an antiserum specific against the membrane-bound D-Lactate dehydrogenase solubilised and

purified from E.coli strain ML 308 - 225) that the membrane-bound D-Lactate dehydrogenase does not share the same antigenic determinants with the membrane-bound flavin-linked L-Lactate dehydrogenase.

4.4.2 RESULTS AND DISCUSSION OF LACTATE DEHYDROGENASE AND LACTATE OXIDASE ACTIVITIES FOR THE MEMBRANE FRAGMENTS

D-Lactate and L-Lactate dehydrogenase activities; D-Lactate and L-Lactate oxidase activities were determined using lithium salts of D-Lactate and L-Lactate. The DL-Lactate dehydrogenase and DL-lactate oxidase activities were determined with the sodium salts of D and L-Lactate prepared from sodium Lactate syrup (containing a mixture of 1.375 to 1.385 g ml^{-1} of D-Lactate and L-Lactate respectively).

Results obtained for the lactate dehydrogenase activities are presented in Table 5a while the results for the lactate oxidase activities are presented in Table 5b. The values obtained for the lactate dehydrogenase and lactate oxidase activities for the IY36 fragments were the highest compared to the values obtained for these activities for membrane

fragments from other bacterial strains. However, the value of 4.708 μmol/ min/ mg protein obtained for the D-Lactate dehydrogenase activity for the IY36 membrane fragments is higher than the value of 2.33 μmol/ min/ mg protein. obtained by Young et al.(1982). The higher value obtained could be explained by the variations in the assay conditions. For example, Young et al. (1982) determined the lactate dehydrogenase activities at 23 °C while in this study, assays were performed at 30 °C. The value of 1.03 μg-atom O/ min/ mg protein for the IY36 D-Lactate oxidase activity obtained by Young et al. (1982) compared to the value of 0.185 μg-atom O/min/ mg protein obtained in this study could also be due to the differences in the assay conditions.

The generally low levels of the L-Lactate dehydrogenase and L-Lactate oxidase activities are consistent with the suggestion that the L-Lactate dehydrogenase and L-Lactate oxidase activities are induced under aerobic condition in the presence of lactate (Kline & Mahler, 1965; Pascal & Pichinoty, 1965); or under nitrate respiration, fumarate reduction and trimethylamine-N-oxide reduction (Nishimura et al., 1983). Membrane fragments used for these determinations were derived from E.coli cells grown under oxygen respiration but in the presence of mannitol.

TABLE 5a

Lactate dehydrogenase activities for the membrane fragments derived from the bacterial strains. Assays were performed (on fresh membrane fragments) as described under methods. Specific activities are presented as μmol/ min/ mg protein and as the mean ± standard deviation of 3 determinations.

MEMBRANE FRAGMENTS FROM	LACTATE DEHYDROGENASE ACTIVITIES		
	D-Lactate	L-Lactate	DL-Lactate
IY12	3.212 ± 0.318	0.008 ± 0.0013	1.606 ± 0.168
IY13	2.128 ± 0.111	0.017 ± 0.001	1.064 ± 0.0118
IY35	2.952 ± 0.236	0.009 ± 0.001	1.476 ± 0.165
IY36	4.708 ± 0.371	0.022 ± 0.002	2.354 ± 0.220
D-Ldh$^-$	0.192 ± 0.011	0.008 ± 0.001	0.123 ± 0.010

TABLE 5b

Lactate oxidase activities for the membrane fragments from the bacterial strains. Assays were performed (on fresh membrane fragments) as described under methods. Enzyme activities are presented as μg-atom O/ min/ mg protein and as the mean ± standard deviation of 3 determinations.

MEMBRANE FRAGMENTS FROM	LACTATE OXIDASE ACTIVITIES		
	D-Lactate	L-Lactate	DL-Lactate
IY12	0.140 ± 0.017	0.010 ± 0.001	0.070 ± 0.007
IY13	0.092 ± 0.008	0.012 ± 0.001	0.050 ± 0.006
IY35	0.115 ± 0.015	0.011 ± 0.001	0.068 ± 0.003
IY36	0.185 ± 0.015	0.016 ± 0.002	0.096 ± 0.007
D-Ldh$^-$	0.027 ± 0.003	0.007 ± 0.001	0.017 ± 0.0017

4.5 DETERMINATION OF OTHER OXIDASE ACTIVITIES FOR THE MEMBRANE FRAGMENTS

4.5.1 INTRODUCTION

In addition to the NADH and lactate dehydrogenases there are other dehydrogenases, (e.g., formate, malate, α-glycerophosphate, succinate and β-hydroxybutyrate) with corresponding oxidase activities in E.coli electron transport chain. Of these oxidase activities, NADH, D-Lactate and succinate oxidase activities have been determined for the membrane fragments from some of the bacterial strains. For example, Young et al. (1978) determined the NADH oxidase activities for the membrane fragments from the IY12, IY13 and IY35 strains; while Jaworowski et al. (1981b) determined the oxidase activities for the IY12 membrane fragments, the reconstituted particles and the purified enzyme. The NADH, D-Lactate and succinate oxidase activities for the IY12, IY13 and IY35 strains were determined by Jaworowski et al. (1981a); while Young et al. (1982) determined similar activities for the IY13 and IY36 strains. Some of these studies were designed to study the effect of the amplification of the gene of NADH dehydrogenase (Young et al., 1978; Jaworowski et al., 1981a) and D-Lactate dehydrogenase (Young et al., 1982) on the levels

of the NADH and D-Lactate activities and also whether or not the amplification procedure affected the levels of other oxidase activities. Succinate, formate, malate, α-glycerophosphate and β-hydroxybutyrate oxidase activities for the membrane fragments were included in this study.

4.5.2 RESULTS AND DISCUSSION OF FORMATE, MALATE, α-GLYCEROPHOSPHATE, β-HYDROXYBUTYRATE AND SUCCINATE OXIDASE ACTIVITIES FOR THE MEMBRANE FRAGMENTS

Shown with Table 6 are the results for formate, α-glycerophosphate, malate, succinate and β-hydroxybutyrate oxidase activities for the membrane fragments. Formate is the most rapidly oxidised by all the membrane fragments followed by α-glycerophosphate, succinate, malate and β-hydroxybutyrate. Of these oxidase activities only succinate oxidase activity have been determined for the membrane fragments from some of the bacterial strains (Jaworowski et al., 1981a; Young et al., 1982). The results of the succinate oxidase activities for the IY13 and IY36 membrane fragments are consistent with the values of 0.09 and 0.063 μg-atom O/ min/ mg protein respectively obtained by Young et al., (1982). The value of 0.097 μg-atom O min/ mg protein obtained for the IY13 membrane fragments is

lower than the value of 0.367 µg-atom O/ min/ mg protein obtained by Jaworowski et al., (1981a) for the same strain. This disparity could be due to variations in the growth conditions as E.coli is capable of altering the composition of its redox carriers in response to the growth conditions (Haddock & Jones, 1977).

TABLE 6

Formate, α-glycerophosphate, malate, succinate and β-hydroxybutyrate oxidase activities for the membrane fragments. Assays were performed (on fresh membrane fragments) as described under methods. Results are expressed as μg-atom O/ min/ mg protein and as the mean ± standard deviation of 3 determinations.

	SUBSTRATES				
Membrane fragments from	Formate	α-glycero-phosphate	Malate	Succinate	β-hydroxy-butyrate
IY12	0.222 ± 0.032	0.038 ± 0.006	0.013 ± 0.002	0.091 ± 0.003	0.016 ± 0.003
IY13	0.185 ± 0.025	0.046 ± 0.006	0.010 ± 0.001	0.097 ± 0.004	0.008 ± 0.001
IY35	0.146 ± 0.017	0.053 ± 0.006	0.011 ± 0.002	0.060 ± 0.005	0.014 ± 0.001
IY36	0.215 ± 0.020	0.055 ± 0.002	0.010 ± 0.001	0.063 ± 0.008	0.011 ± 0.001
D-Ldh$^-$	0.081 ± 0.006	0.015 ± 0.002	0.008 ± 0.001	0.064 ± 0.003	0.006 ± 0.001

4.6 STUDIES ON THE EFFECTS OF SOME INHIBITORS AND SOME QUINONE ANALOGUES ON THE ACTIVITIES OF THE MEMBRANE-BOUND NADH AND D-LACTATE DEHYDROGENASES

4.6.1 INTRODUCTION

Inhibitors and "uncouplers" have been used for the study of oxidative phosphorylation (e.g., Slater, 1967), and since the behaviour of the isolated enzymes of electron transfer chain towards inhibitors resembles that observed with whole particles, the activities of the segments therefore appear to be a reliable index of the reaction occuring in the intact electron transfer chain (Kashket & Brodie, 1963).

Bathophenanthroline, a lipophilic iron chelator (Crane et al., 1975) has been shown to inhibit to a varying degree, the NADH and D-Lactate oxidase activities of E.coli membrane vesicles. 2,4-dinitrophenol (DNP) is a commonly used uncoupler of oxidative phosphorylation while cyanide (used as KCN) is a powerful inhibitor of NADH oxidase activity (Slater, 1967); and uncoupler of D-Lactate transport (Kaback, 1974). Ethylenediamine tetra acetic acid (EDTA) was employed at varying concentrations at various stages in

this study. Hence it was decided to study its effect at a 4 mM concentration on the activities of these enzymes. This is significant in view of the chelating effect of EDTA on metal ions. Diuron 3-(3-4-dichlorophenyl)-1, 1-dimethyl urea was included to determine its effect on these enzymes at the concentration used.

Finally, various forms of quinone analogues were used to determine their effects on the activities of these enzymes. Similar studies on the effects of quinone analogues on NADH, D-Lactate, succinate and α-glycerophosphate oxidase activities of E.coli have been carried out by Wallace & Young.,(1977) using a ubi A$^-$men A$^-$ double quinone mutant. Kashket & Brodie (1963) through a similar experiment showed that K_2C_{45} (a naphthoquinone) stimulated NADH oxidation.

4.6.2 RESULTS AND DISCUSSION ON THE EFFECTS OF SOME INHIBITORS AND SOME QUINONE ANALOGUES ON THE ACTIVITIES OF NADH AND D-LACTATE DEHYDROGENASES

The results obtained for the effects of some inhibitors and some quinone analogues on the activities of the membrane-bound NADH and D-Lactate dehydrogenases are presented in Table 7. A control sample to which no addition was made

was included in the assay and the percentage activities were expressed as the percentage activities obtained for the control sample.

2,Methyl, 1,4-naphthoquinone

The results obtained from the quinone additions show that 2,methyl, 1,4-naphthoquinone (0.058 mM) enhanced the activity of NADH dehydrogenase by 30 % while the same concentration inhibited the activities of the D-lactate dehydrogenase by 40 %. A similar enhancement of the NADH oxidase activity was observed by Brodie & Ballantine (1960) on the addition of Vitamin K (a naphthoquinone) on extracts from Mycobacterium phlei ATCC-354. Also, Kashket & Brodie (1963) showed that K_2C_{45} (a naphthoquinone) stimulated NADH oxidation while Stroobant & Kaback (1975) showed that the addition of ubiquinone stimulated NADH, D-lactate, L-lactate, NADPH and succinate oxidase activities at between 17-29 %.

Quinhydrone

Quinhydrone at 0.03 mM concentration enhanced the activity of the NADH dehydrogenase by 13 % while the same concentration inhibited the activity of the D-Lactate dehydrogenase by 39 %.

Duroquinone

Duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone) at 0.06 mM concentration did not alter significantly the activity of the NADH dehydrogenase. However, the same concentration of duroquinone inhibited the D-Lactate dehydrogenase activity by 20 %.

Tetrachlorohydroquinone

Tetrachlorohydroquinone at 0.04 mM concentration inhibited the activities of the NADH and the D-Lactate dehydrogenases by 56 % and 95 % respectively.

These variations in the effects of these quinones of the activities of these two enzymes is consistent with the suggestion that certain substituents of the benzoquinone (of Q-8) are important in determining its reactivity with each of the various dehydrogenases (Stroobant & Kaback, 1975).

2,4-Dinitrophenol

The results of other additions showed that 2,4-dinitrophenol (50 mM) reduced the activity of NADH dehydrogenase by 62 % but the same concentration enhanced the D-Lactate activity by 20 %. The increase in the D-lactate activity might be due to the dinitrophenol (at this concentration) acting as an "uncoupler" (e.g., Slater, 1967) of the D-Lactate oxidation.

KCN

KCN at 5 mM concentration showed 92 % inhibition of NADH dehydrogenase activity. The same concentration of KCN on the other hand, enhanced the activity of the D-Lactate dehydrogenase by 22 %. This is consistent with the view that KCN is a powerful inhibitor of NADH oxidase activity and an "uncoupler" of the D-Lactate dependent transport (Kaback, 1974).

Diuron

Diuron at 0.064 mM concentration inhibited the activities of NADH and D-Lactate dehydrogenases by 60 % and 40 % respectively. The loss in both activities could be due to some changes induced by the diuron addition in both enzymes as suggested by Watari et al., 1963; on NADH dehydrogenase exposed to it and other agents (heat and ethanol).

EDTA

EDTA at 4 mM concentration reduced the D-Lactate dehydrogenase activity by 11 % while the same concentration of EDTA has no effect on the NADH dehydrogenase activity. It has however been shown by Taniguchi & Kamen (1965) that a 3×10^{-3}M concentration of EDTA inhibited the NADH oxidase activity of Rhodospirillum rubrum by 25 %.

Bathophenanthroline

Bathophenanthroline at 0.15 mM concentration inhibited the activities of the NADH and D-Lactate dehydrogenases by 74 % and 39 % respectively. The effect of bathophenanthroline on the NADH and D-Lactate oxidase activities have been reported by Crane et al. (1975). Their results gave a 50 % inhibition of NADH oxidase activity at a bathophenanthroline concentration of 5×10^{-5}M while lactate oxidase activity gave a 50 % inhibition at a concentration of 1.3×10^{-4}M.

TABLE 7: Effects of some inhibitors and some quinone analogues on the activities of the membrane-bound NADH and D-Lactate dehydrogenases using fresh membrane fragments from IY35 and IY36 respectively. Enzyme assays were performed as described under methods. Specific activities are presented as the mean ± standard deviation of 3 determinations and percentage activities are expressed as the percentage of activities obtained with control samples.

TABLE 7

INHIBITORS/ QUINONE ANALOGUES	CONCENTRATION ADDED	NADH DEHYDROGENASE ACTIVITY		D-LACTATE DEHYDROGENASE ACTIVITY	
		Specific activity (μmol/min/mg protein)	Percentage activity	Specific activity (μmol/min/mg protein)	Percentage activity
Control	No addition	21.32 ± 1.15	100.0	2.13 ± 0.210	100.0
KCN	5 mM	1.62 ± 0.09	7.6	2.59 ± 0.216	121.6
EDTA	4 mM	21.32 ± 1.20	100.0	1.90 ± 0.106	89.4
Tetrachloro-hydroquinone	0.04 mM	9.37 ± 0.80	43.9	0.11 ± 0.009	4.98
Bathophenan-throline	0.15 mM	6.13 ± 0.08	28.8	1.30 ± 0.105	61.0
Diuron	0.064 mM	8.19 ± 0.56	38.8	1.29 ± 0.087	60.8
2,4-dinitro-phenol	50 mM	8.0 ± 0.770	37.5	2.55 ± 0.206	119.7
Duroquinone	0.06 mM	21.03 ± 1.92	98.6	1.70 ± 0.09	79.6
2,methyl,1,4-naphthoquinone	0.058 mM	29.40 ± 2.50	137.9	1.29 ± 0.082	60.6
Quinhydrone	0.030 mM	24.02 ± 1.80	112.6	1.29 ± 0.095	60.6

4.7 DETERMINATION OF THE OPTIMUM pH FOR THE MEMBRANE-BOUND NADH AND D-LACTATE DEHYDROGENASES

4.7.1 INTRODUCTION

Although the determinations of the activities of the membrane-bound NADH and D-Lactate dehydrogenases were carried out at pH of 7.5 and 8.0 respectively, attempts were made to determine the optimum pH for each enzyme. This was found necessary in view of the different pH optimum already reported for each enzyme. For example, Dancey & Shapiro (1976) reported that the membrane-bound NADH dehydrogenase is maximally active at a pH of between 6.5 - 7.0 when dichloroindophenol (dcip) is used as the electron donor and that the activity of the enzyme was the same when measured at pH 7.4 in either potassium phosphate or Tris/chloride buffer. Jaworowski et al. (1981b) used a pH of 7.5 for the determination of NADH: ubiquinone dependent dehydrogenase activity.

Unlike the NADH dehydrogenase, the optimum pH for the determination of the D-Lactate dehydrogenase activity has been controversial. For example, Futai (1973) gave a range of 8.0 - 9.0 which is outside the range 9.0 - 9.5 quoted by Pratt (1979) on the membrane-bound enzyme. Kaczorowski et al. (1978) reported a pH of 8.5 for maximal activity for the

purified enzyme and an optimum pH of 6.6 for the membrane-bound enzyme. Kaczorowski et al., 1978; added that Tris/chloride buffer interferes with the membrane-bound enzyme while it has no such effect on the purified enzyme.

Since Futai (1973) and Pratt (1979) employed the use of Tris/chloride buffer as in this study, it was found necessary to determine the pH optimum of the D-Lactate dehydrogenase to resolve these differences. The determination of the pH optimum for the membrane-bound NADH:ubiquinone dependent dehydrogenase was to establish whether or not the pH optimum obtained for this enzyme agrees with the pH of 7.5 at which the activity of the enzyme was determined.

4.7.2 RESULTS AND DISCUSSION OF OPTIMUM pH DETERMINATIONS FOR THE MEMBRANE-BOUND NADH AND D-LACTATE DEHYDROGENASES

The results obtained for the determinations of the optimum pH for the NADH and D-Lactate dehydrogenase activities are presented with Table 8. The pH profiles for the NADH and D-Lactate dehydrogenases are shown with Figs. 8 and 9 respectively.

The results show that the pH for maximal activity for NADH dehydrogenase is pH 7.8. It also shows that the activity of NADH dehydrogenase increases steadily from pH 7.1 to 7.8. The activity fell sharply from pH 8.0 reaching its minimum activity at pH 9.2. The results in the case of D-Lactate dehydrogenase activity show that the enzyme has a pH of 8.8 for maximal activity. The pH of 8.8 for the D-Lactate dehydrogenase activity falls within the range of 8.0 - 9.0 quoted by Futai (1973) but contrasts with the pH optimum of between 9.0 - 9.5 quoted by Pratt (1979) and pH of 6.6 quoted by Kaczorowski et al. (1978) for the membrane-bound enzyme.

In conclusion, the value obtained at pH of 7.5 in this study and by Jaworowski et al. (1981) for the membrane-bound NADH dehydrogenase activity does represents 88.5% of actual activity when compared to the pH of 7.8 for maximal activity for this enzyme. Also, the pH of 8 used in this study and routinely (Futai, 1973; Kohn & Kaback, 1973; Pratt et al., 1979) for the determination of the activity of the membrane-bound D-Lactate dehydrogenase represents about 62% of actual activity for this enzyme when compared to its activity at the optimum pH of 8.8.

TABLE 8: Determination of the pH optimum for the membrane-bound NADH and D-Lactate dehydrogenases using membrane fragments from the IY35 and IY36 strains respectively. Enzyme assays were performed as described under methods. Specific activities are presented as μmol/min/mg protein and as the mean ± standard deviation of 3 determinations. Percentage activities are expressed as the percentage of the values obtained at the optimum pH.

TABLE 8

PH	NADH DEHYDROGENASE		D-LACTATE DEHYDROGENASE	
	Specific activity	Percentage activity	Specific activity	Percentage activity
7.1	24.03 ± 1.56	69.8	2.123 ± 0.121	34.0
7.2	-	-	2.147 ± 0.162	38.7
7.3	28.75 ± 1.44	83.6	-	-
7.4	29.10 ± 1.73	84.5	-	-
7.5	30.45 ± 1.95	88.5	2.961 ± 0.125	47.4
7.7	33.70 ± 1.28	97.9	-	-
7.8	34.40 ± 2.108	100.0	3.785 ± 0.295	60.6
8.0	33.00 ± 1.70	95.9	3.898 ± 0.315	62.4
8.1	-	-	4.246 ± 0.469	68.0
8.3	-	-	4.706 ± 0.815	75.4
8.4	18.65 ± 0.915	54.2	-	-
8.6	-	-	5.071 ± 0.367	81.2
8.7	9.45 ± 0.83	27.5	-	-
8.8	-	-	6.245 ± 0.815	100.0
9.0	-	-	4.525 ± 0.258	72.5
9.2	1.80 ± 0.105	52.0	3.087 ± 0. 47	49.4
9.4	-	-	2.053 ± 0.147	32.9

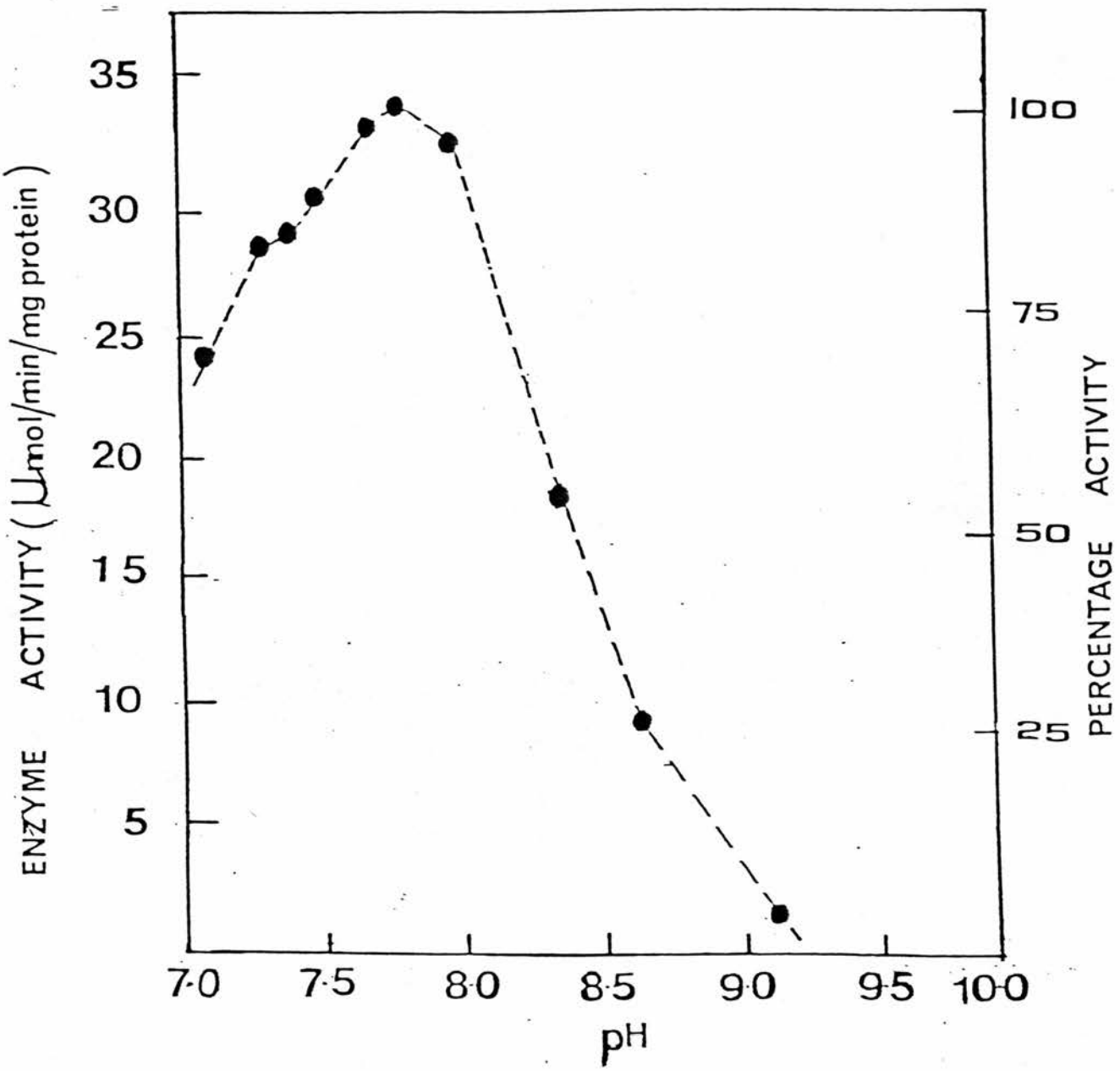
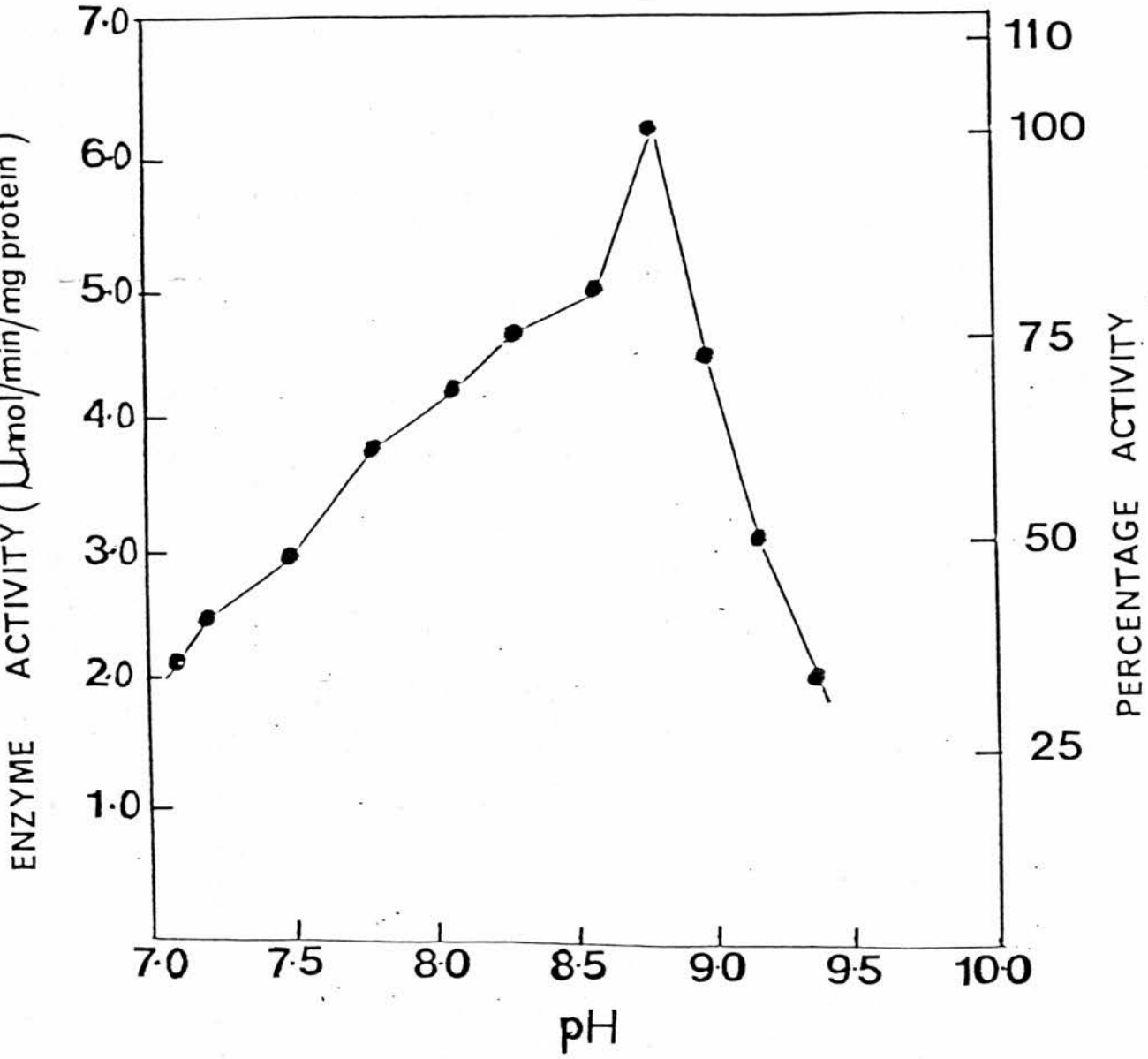


FIG. 8 - pH profile for NADH dehydrogenase activity. Details for the determination are as presented in Table 8.



FIG·9 - pH profile for D-Lactate dehydrogenase activity. Details for the determination are as presented in Table 8.

CHAPTER 5

5. STUDIES ON THE CYTOCHROME, "ACID LABILE" SULPHIDE AND PEPTIDE-BOUND FLAVIN CONTENTS FOR THE MEMBRANE FRAGMENTS.

5.1 INTRODUCTION

5.1.1 Cytochrome contents

E. coli is capable of synthesizing at least nine different cytochromes (Haddock & Jones, 1977), and growth conditions greatly modify the qualitative and quantitative cytochrome contents of the *E.coli* respiratory chain (Poole *et al.*, 1980). A major alteration to the aerobic respiratory chain of *E.coli* grown on non-fermentative carbon source involves the addition of cytochrome b_{558} and cytochrome *d* (Shipp, 1972). Different spectra can be used to probe the nature and quantities of cytochrome contents present in bacteria (e.g., Castor & Chance 1959; Poole *et al.*, 1979b); and comparison of the wavelength of the observed bands with the wavelengths of alpha bands of cytochromes in other systems indicates that the cytochromes of *E.coli* are spectroscopically similar to those found in many microorganisms and mitochondria (Shipp, 1972).

In the reduced minus oxidised spectra, cytochrome $\underline{a}_1$ appears as a minor peak at 594 nm in the difference spectra of late exponential and stationary phase and cytochrome d shows a complex band consisting of a peak at 628 nm and a trough at 650 nm in the reduced minus oxidised spectra. Cytochrome o is however detectable by the characteristic spectrum of its compound with carbon monoxide. The results of various experiments (see Bragg, 1979 for a review) on cytochrome d and o suggested that both cytochromes function as terminal oxidases where both are present.

5.1.2 "Acid labile" sulphide contents

The presence of "acid labile" sulphide (Hall et al., 1975) characteristic of non-haem iron-containing proteins has been found to be associated with the respiratory chain of E.coli. (Poole & Haddock, 1975). The acid labile sulphide is unique to iron-sulphur centres and occurs in either 2Fe-2S or 4Fe-4S clusters (Orme-Johnson & Orme-Johnson, 1978). The presence of iron not bound to haem and an equivalent amount of "acid labile" sulphur is prima facie evidence for the presence of iron sulphur centres (Rabinowitz, 1978).

"Acid labile" sulphide contents can be determined by various methods (e.g., King & Morris, 1967; Rabinowitz, 1978).

5.1.3 Flavin contents

Some of the E.coli respiratory chain dehydrogenases contain flavins which may be FMN or FAD. The pyridine-linked dehydrogenases transfer of electrons from substrates. The presence of flavins have been demonstrated in the E.coli membrane-bound NADH dehydrogenase (e.g., Jaworowski et al., 1981b) and D-Lactate dehydrogenase (Futai, 1973). These flavins consist of two forms - the peptide-bound flavins and the peptide-free flavins. The peptide-bound flavins can be estimated by trypsin digestion of the membrane fragments followed by acid treatment (Rao et al., 1967).

5.1.4 Rationale for these studies

Studies on the cytochrome, acid labile sulphide and peptide-bound flavin contents of the membrane fragments were to determine whether or not the amplification of the gene of NADH dehydrogenase (Jaworowski et al., 1981b) or the gene of D-Lactate dehydrogenase (Young et al., 1982) affected the levels of these components. For example, similar studies on the D-Lactate dehydrogenase amplified strain (IY36) and the wild type strain (IY13) by Young et al. 1982; showed that the cytochrome b and o contents in both

strains were similar while the cytochrome d level was found to be elevated in the plasmid amplified strains. In this study, membrane fragments from the NADH and D-lactate dehydrogenase mutant strains were included to determine whether or not their respective mutations affected the levels of their cytochrome contents.

5.2 RESULTS AND DISCUSSION FOR THE CYTOCHROME, "ACID LABILE" SULPHIDE AND PEPTIDE-BOUND FLAVIN CONTENTS FOR THE MEMBRANE FRAGMENTS.

Presented with Table 9 are the results for the cytochrome "acid labile" sulphide and peptide-bound flavin contents for the membrane fragments.

5.2.1 Cytochrome contents

From the results obtained, the levels of cytochrome b_{560} do not appear to be affected by the amplification procedure. The levels of cytochrome d on the other hand, appear to be increased in membrane fragments from the plasmid-amplified strains (IY35 and IY36 strains) compared to the level of cytochrome d from the wild type (IY13) strain. Also, both mutant strains show low levels of cytochrome d. Although the levels of cytochrome a_1 were not quantitated, from the

peaks obtained (having corrected from the protein concentration), the levels of this component do not appear to vary significantly for the membrane fragments from all the bacterial strains. The insignificant alterations in the levels of cytochrome $\underline{b}_{560}$ are in agreement with the findings of Jaworowski *et al.* (1981b) on amplified NADH dehydrogenase and Young *et al.* (1982) on amplified D-Lactate dehydrogenase. Low but similar levels of the cytochrome *d* contents in membrane fragments from both *ndh*⁻ mutant and the D-Ldh⁻ strains raise the question of whether the mutation probably affected this terminal oxidase as well. The cytochrome contents were calculated from the reduced -oxidised spectra (Fig.10).

5.2.2 "Acid labile" sulphide content

Except for the membrane fragments from the IY36 strain, there is no significant alteration in the level of the "acid labile" sulphide from the membrane fragments. The level for the membrane fragments from the IY36 strain is increased when compared to the level from the IY12 strain (the strain from which IY36 was derived). The elevation of the "acid labile" sulphide in the IY36 strain may be due to the cloned gene of D-Lactate dehydrogenase it carries or other co-factors. On the other hand, the IY35 strain (also derived from the IY12 strain), carrying the gene of NADH dehydrogenase has a similar level of "acid labile" sulphide

with the IY12 strain. This appears to indicate that the amplification of the gene of NADH dehydrogenase in the IY35 strain does not affect the level of its "acid labile" sulphide content.

5.2.3 Peptide-bound flavin contents

Although the levels obtained for the peptide-bound flavin contents in all membrane fragments were generally low, there was no significant variation in all the values. The results however show that the amplification of the gene of NADH dehydrogenase or the gene of D-Lactate dehydrogenase has not affected this component.

TABLE 9: Cytochrome, acid-labile sulphide and peptide-bound flavin contents for the membrane fragments. Experiments were performed as described under methods. Results are expressed as nmol/mg protein and as the mean ± standard deviation of 3 determinations for cytochromes and peptide-bound flavins and of 4 determinations for acid-labile sulphide. Fresh membrane fragments were used for each determination.

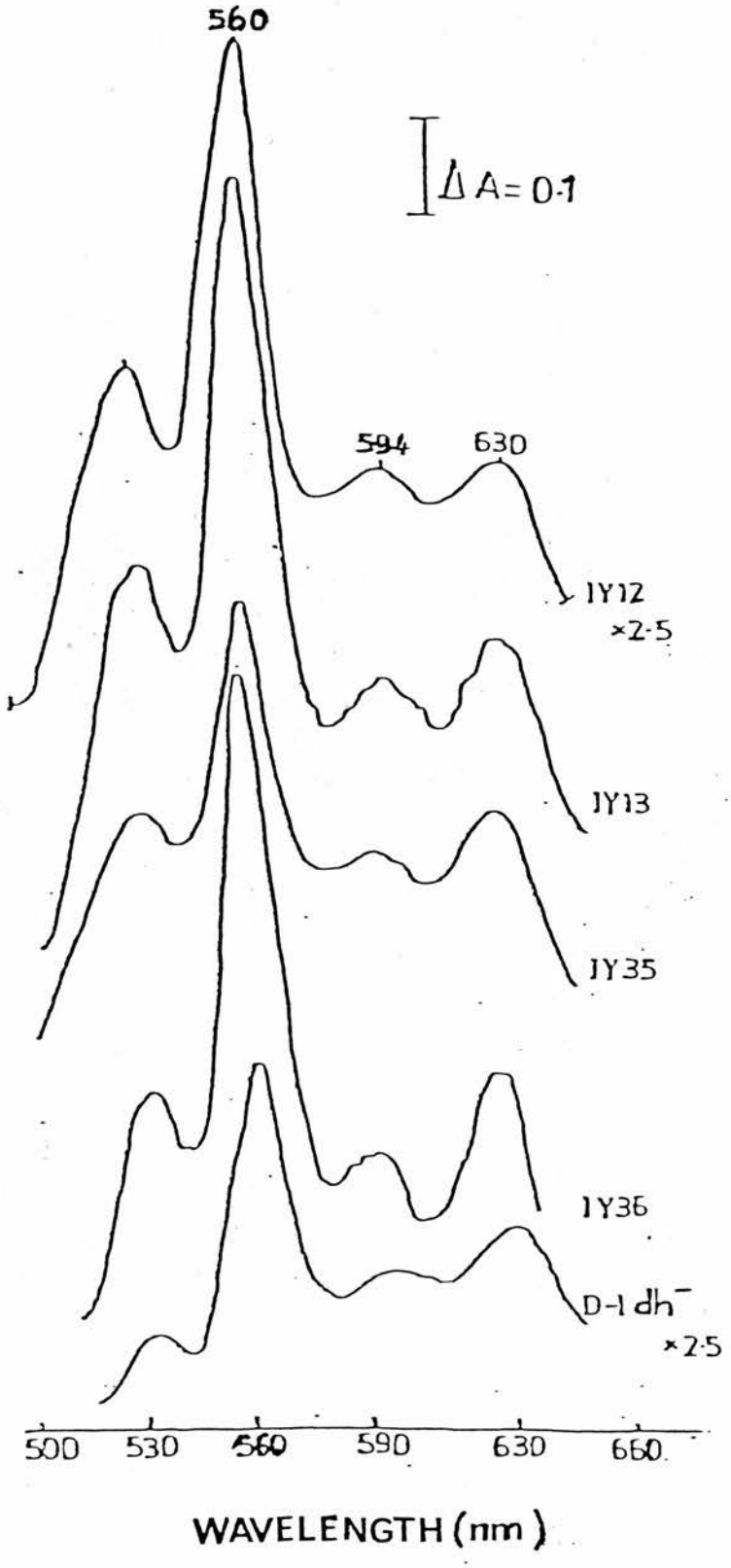
TABLE 9

MEMBRANE FRAGMENTS FROM	CYTOCHROMES[a]		ACID-LABILE SULPHIDE	PEPTIDE-BOUND FLAVINS
	b_{560}	d		
IY12	0.45 ± 0.023	0.08 ± 0.007	5.8 ± 0.350	0.049 ± 0.005
IY13	0.37 ± 0.035	0.12 ± 0.105	6.3 ± 0.512	0.055 ± 0.006
IY35	0.35 ± 0.030	0.20 ± 0.013	5.8 ± 0.450	0.055 ± 0.006
IY36	0.37 ± 0.036	0.19 ± 0.0125	7.8 ± 0.605	0.053 ± 0.005
D-Ldh$^-$	0.35 ± 0.032	0.07 ± 0.005	5.4 ± 0.260	0.050 ± 0.005

[a] The following wavelength pairs and molar extinction coefficients were used: cytochrome b_1, $\Delta_{E560-575m\mu}$ (E, 17 500 calculated from the data of Deeb and Hager (1964) for E. coli cytochrome b; cytochrome d, $\Delta_{E630-615m\mu}$ (E, 8 500 for Pseudomonas aeruginosa Cytochrome d (Horio et al., 1961; White, 1965).

Fig. 10 - Typical difference spectra (25°C) for IY12, IY13, IY35, IY36, and D-Ldh$^-$ membrane fragments. Procedure for the determination is as described under methods. The samples contained 10.2, 6.3, 3.0, 6.0 and 7.2 mg protein ml^{-1} respectively.

FIG·10


560
ΔA=0·1
594
630
IY12
×2·5
IY13
IY35
IY36
D-1 dh⁻
×2·5
500
530
560
590
630
660
WAVELENGTH (nm)

CHAPTER 6

6. ELECTRON PARAMAGNETIC RESONANCE (E.P.R.) STUDIES ON THE MEMBRANE FRAGMENTS

6.1 INTRODUCTION

E.p.r. has been used for the detection of iron-sulphur centres in membrane fragments of E. coli which have been reduced either with dithionte or with other substrates (Poole & Haddock, 1975). The method has also been used by the same authors to confirm the role of iron in iron-sulphur proteins. Low temperature electron paramagnetic resonance spectra of these E. coli membrane fragments (grown in glycerol and sulphate limited media) at 12 K showed these to be an e.p.r. signal present. By virtue of its g value this signal was assigned to be that of an iron-sulphur centre. The presence of non-haem iron in these fragments were confirmed by chemical analysis. It was also noted that when there was a decrease in the intensity of the iron-sulphur e.p.r. signal there was a loss in the amount of non-haem iron present as measured by chemical analysis.

Blum et al. (1980) have shown that when dithionite or succinate reduced oriented multilayers of E. coli membranes are rotated in the magnetic field of an e.p.r. spectrophotometer about an axis lying in the membrane plane and perpendicular to the magnetic field, an angular dependence of a signal from iron-sulphur clusters of $g_x = 1.92$, $g_y = 1.93$ and $g_z = 2.02$ is seen. Iron-sulphur clusters (e.g., Salerno et al., 1979) appear to be fixed at definite angles relative

to the membrane plane.

In unoriented E. coli membrane particles derived from aerobically grown E. coli cells at least four iron-sulphur centres have been detected (Ingledew et al., 1980). Two of these have been resolved potentiometrically into components having midpoint potentials at pH 7 (Em_7) of -20mV and -220mV. More recently, Ingledew, 1983; using an e.p.r. spectrophotometer further characterized the iron-sulphur centres from membrane fragments derived from anaerobically grown E. coli cells.

Iron-sulphur proteins have been implicated in redox reactions based on their reactivity with chelating agents (Bragg, 1974; Crane et al., 1975). The e.p.r. resonance at g = 1.94 obtained in sulphate-limited E. coli cells (Poole & Haddock, 1975) were shown to have markedly reduced amplitude confirming the role of iron in iron-sulphur proteins. Also, cells of Paracoccus denitrificans from sulphate-limited medium (Meijer et al., 1977) have been shown to have decreased oxidase and NADH dehydrogenase activities concomitant with decreased e.p.r. detection of iron-sulphur centres.

An e.p.r. spectrophotometer was thus used to study the composition of the iron-sulphur centres of membrane fragments derived from the bacterial strains used in this study. Sodium dithionite was used to reduce the iron-sulphur centres as the ferredoxin-type iron-sulphur clusters are paramagnetic in their

reduced states. The effect of substrates (NADH and D-Lactate) on these signals was also examined.

6.2. RESULTS AND DISCUSSION OF E.P.R. STUDIES

6.2.1 E.p.r. spectra of dithionite-reduced membrane fragments from the bacterial strains

Shown with Fig. 11 are typical spectra of dithionite-reduced samples from the bacterial strains analysed at a temperature of 8 K. The spectra obtained for the IY12, IY35 and IY36 membrane fragments superficially resemble each other. Those from the IY13 membrane fragments on the other hand, present a different picture with the absence of the resonance at g = 1.883 with a trough at g = 1.857. All the bacterial strains show resonances corresponding to g = 2.01, 2.033 and 2.10. The spectra from the D-Ldh$^-$ membrane fragments have a broad resonance centred at g = 1.902 and like the membrane fragments from the IY13 strain (from which D-Ldh$^-$ strain was derived), lack the resonance at g = 1.857. The amplitude of the g = 1.930 resonance of the 1Y36 membrane fragments (having corrected for protein concentration, is the most enhanced.

Fig. 11 Typical e.p.r. spectra of dithionite reduced IY12, IY13, IY35, IY36 and D-Ldh$^-$ membrane fragments at a temperature of 8 K. E.p.r. conditions: Microwave frequency, 9.47GHz; Receiver gain, 10×10^4; Microwave power, 10 dB; Modulation frequency, 100 Hz; Field modulation intensity, 100 Gpp; Time constant, 500 secs; Mid range, 3,400 G; Scan range, 1000 G; Scan time, 1000 secs. Protein concentration was 43.8, 33.5, 51.8, 42.0 and 35.0 mg ml^{-1} respectively.

FIG.11

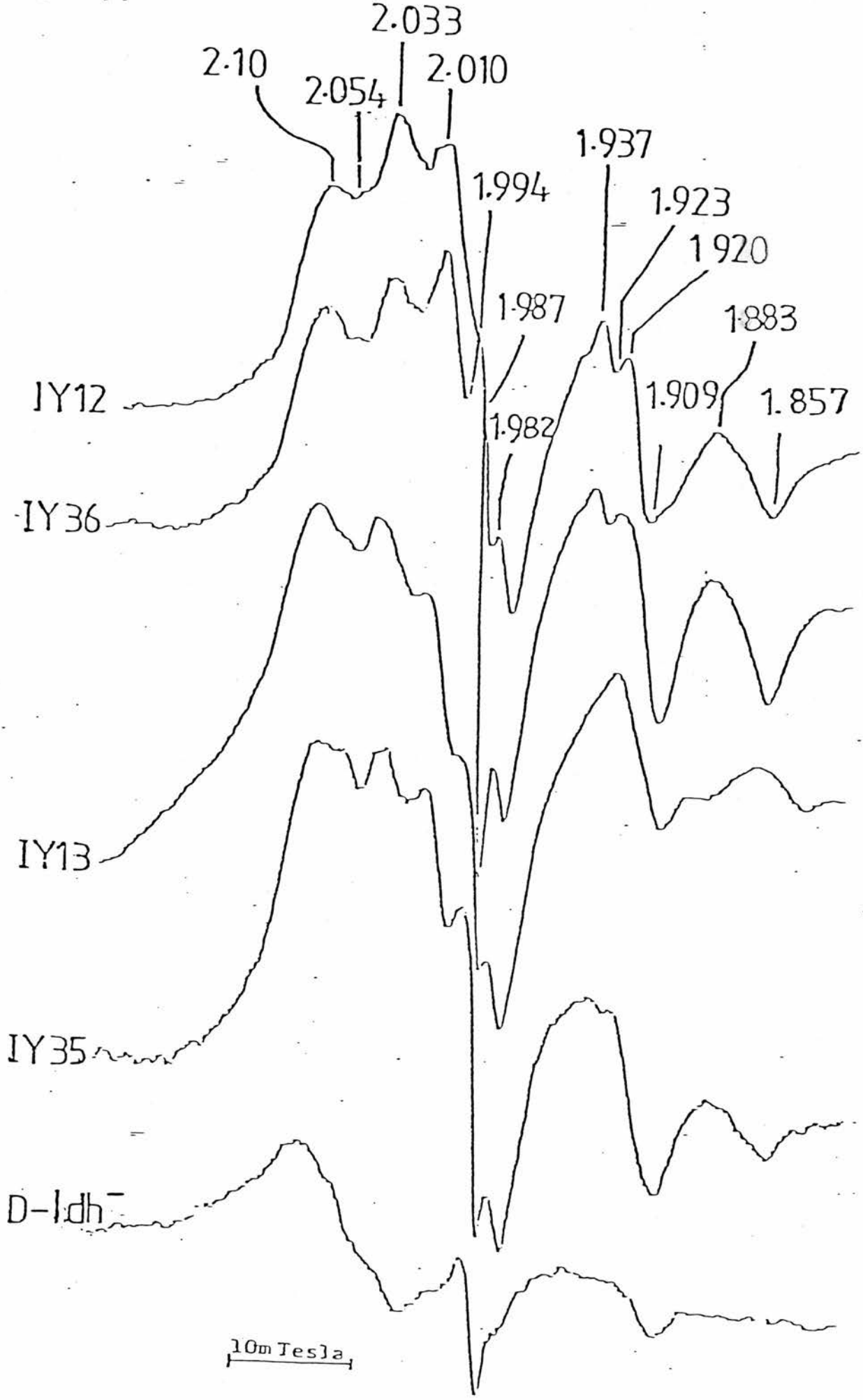

2.033
2.10
2.054
2.010
1.937
1.994
1.923
1920
1.987
1.883
IY12
1.909
1.857
1.982
IY36
IY13
IY35
D-Idh⁻
10m Tesla

Fig. 12 Typical spectra of dithionite-reduced IY36 membrane fragments at different temperatures. The number by each spectrum indicates the temperature (K). The number on top and below the fig. indicates the g values belonging to a single centre. E.p.r. conditions were as in fig. 11. Protein concentration was 42.0 mg ml^{-1}.

FIG. 12

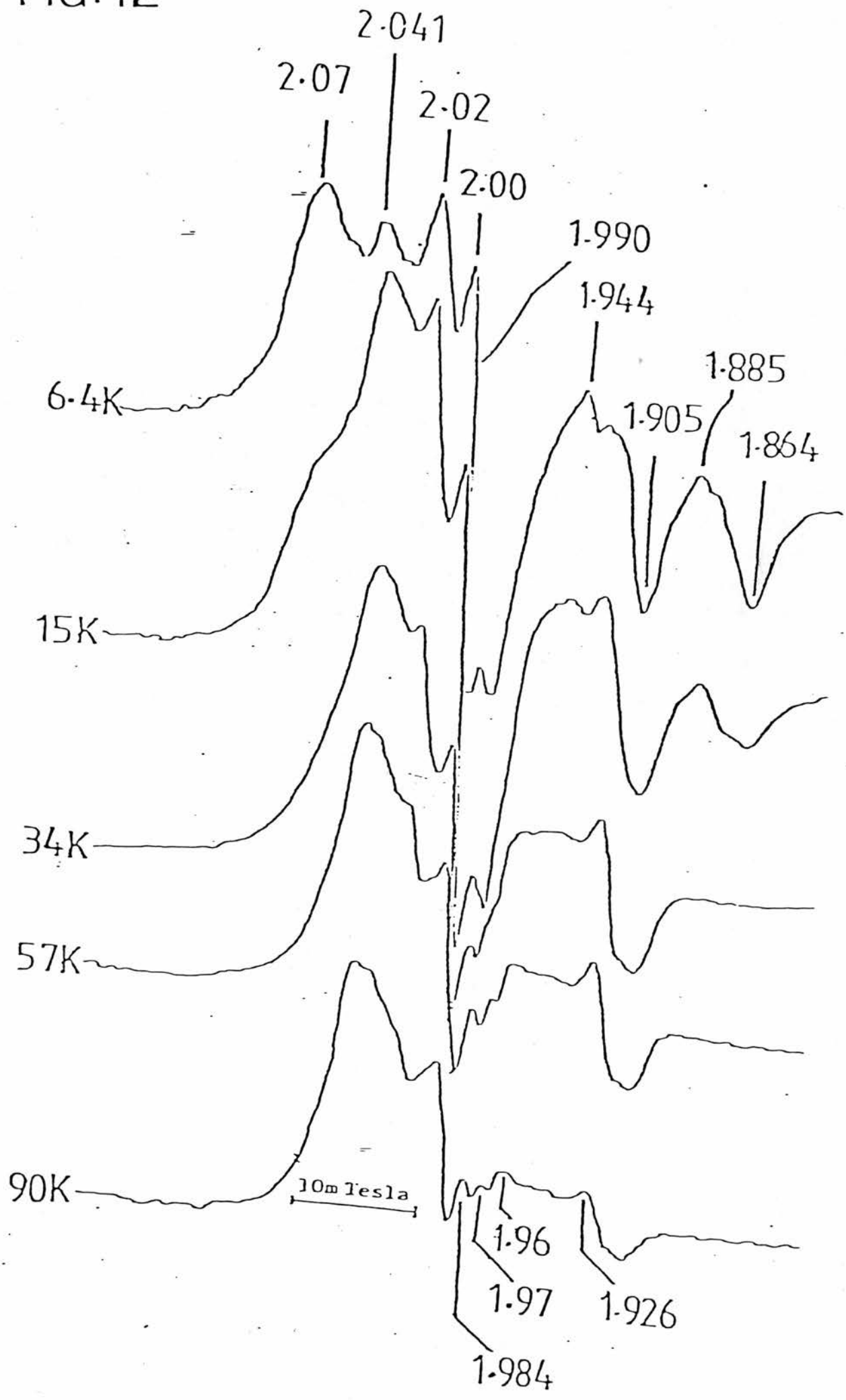
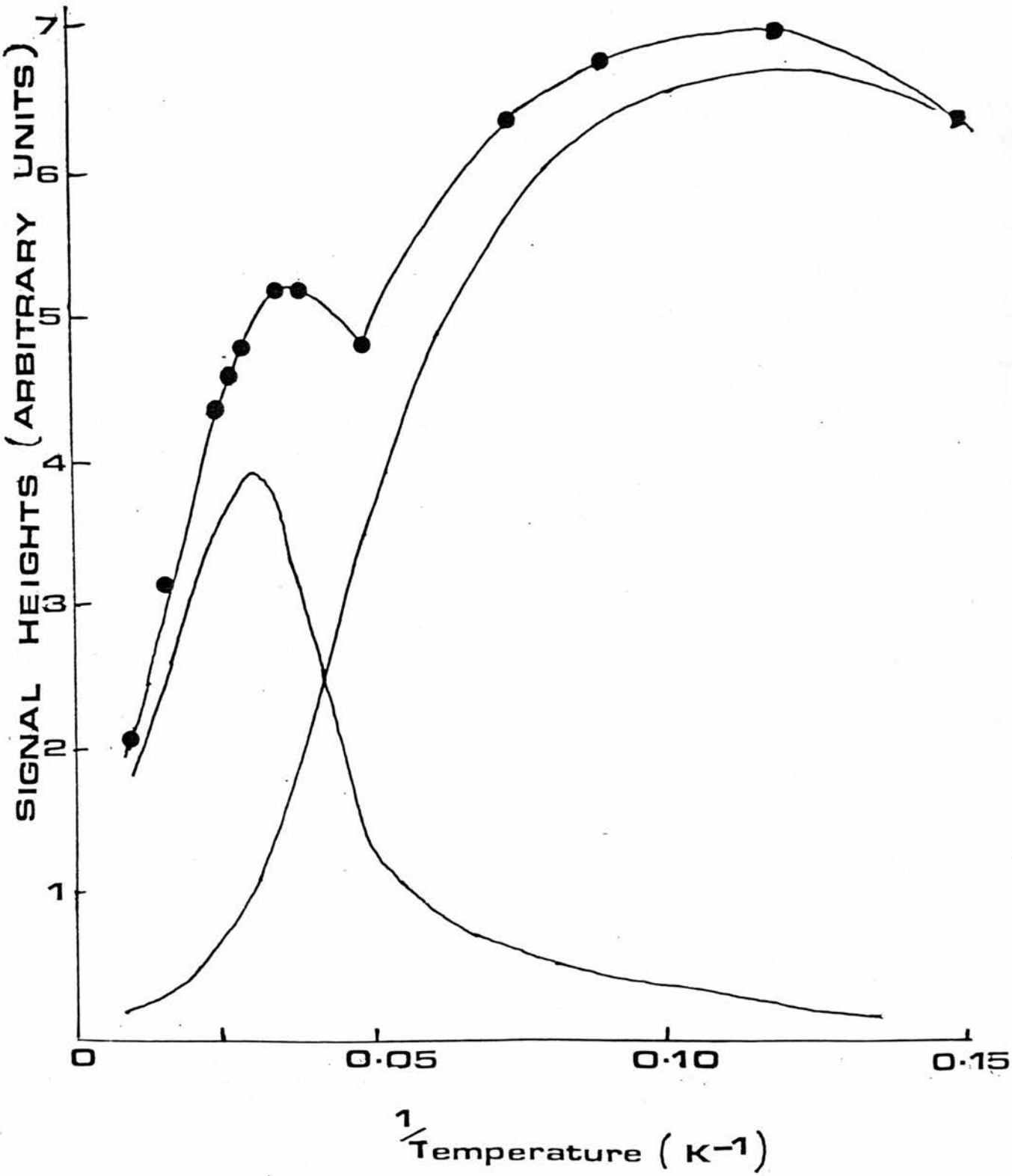

2·041
2·07
2·02
2·00
1·990
1·944
1·885
1·905
1·864
6·4K
15K
34K
57K
90K
10m Tesla
1·96
1·97
1·926
1·984

Fig.13 Typical temperature dependence of the g = 1.93 signal heights (measured from the peak to trough) for IY36 membrane fragments reduced with dithionite. E.p.r. conditions were as for fig. 11.

FIG·13


SIGNAL HEIGHTS (ARBITRARY UNITS)
1
2
3
4
5
6
7
0
0·05
0·10
0·15
1/Temperature (K⁻¹)

6.2.2 E.p.r. detectable components in the membrane fragments

Shown in Fig. 12 are paramagnetic components and a number of lines attributable to iron-sulphur centres seen after reduction with dithionite. The resonance at g = 1.93 and 1.864 are observed at 6.4 K and 15 K. Both resonances decreased in amplitude as the temperature was increased from 6.4 K to 15 K and at 34 K the resonance at g = 1.864 is not seen. The g = 1.93 signal was seen to further decrease in amplitude with increase in temperature reaching its lowest level at 90 K (the highest temperature used). Minor resonances at g = 1.96 and 1.97 could be seen at 57 K and these resonances appear to increase in amplitude with decrease in temperature. Other resonances observed include that at g = 2.07 which is prominent at 6.4 K but was not seen at higher temperature; the resonance at g = 2.02 which decreased with increase in temperature and the resonance at g = 2.041 which seemed to decrease with increase in temperature. This confirms that each centre has resonances which have their temperature profiles at which it would be well resolved for study.

The data obtained from the above were used to plot a graph showing the temperature dependence of the g = 1.93 resonance (Fig. 13) and the results from the curve indicated the presence of more than one component.

Fig.14 - Typical e.p.r. spectra of IY35 membrane fragments at a temperature of 10 K. Alphabets besides each spectrum indicates reduction with a) dithionite b) 2.5 mM NADH and c) 10 mM D-Lactate. Other e.p.r. conditions are as in fig. 11 except that the gain was 1.25×10^5. Protein concentration was 51.8 mg ml^{-1}.

FIG. 14

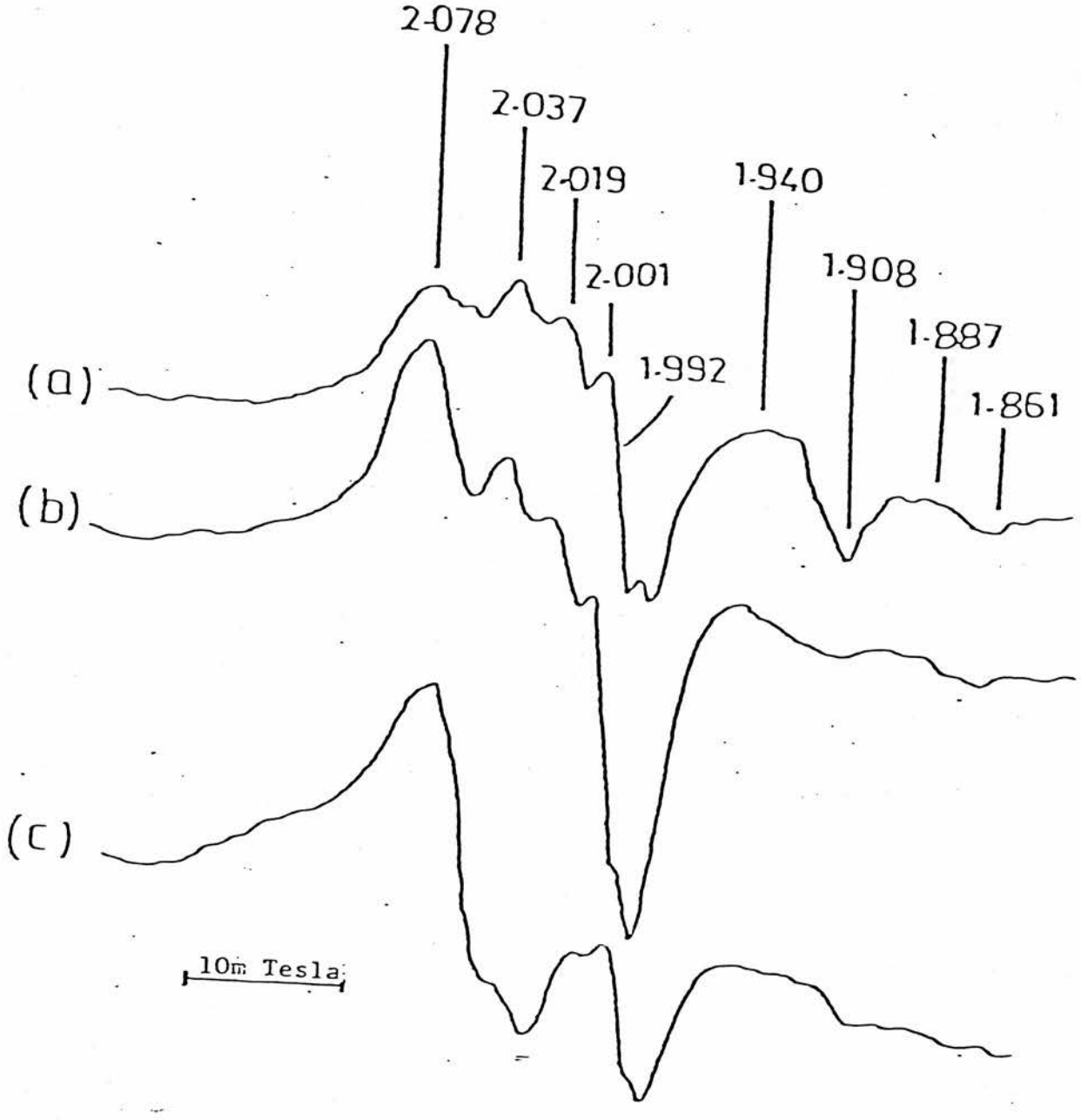
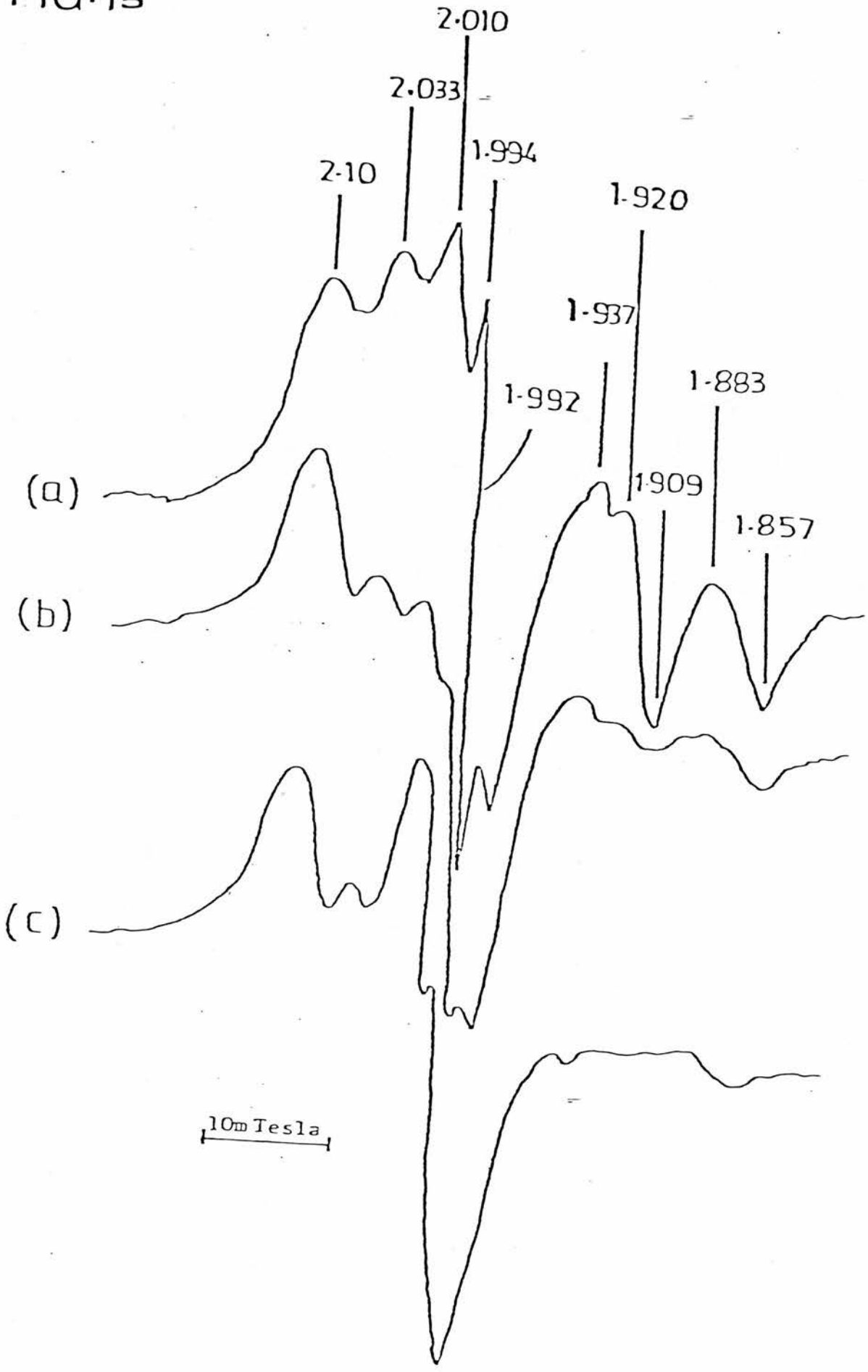


Fig.15 - Typical e.p.r. spectra of reduced IY36 membrane fragments. Reductions were as in fig. 16. E.p.r. conditions were as in fig. 11 except that the gain was 8×10^4. Protein concentration was 42.0 mg ml^{-1}

FIG. 15


2·010
2·033
1·994
2·10
1·920
1·937
1·992
1·883
1·909
1·857
(a)
(b)
(c)
10m Tesla

6.2.3 Reduction of 1Y35 and 1Y36 membrane fragments with dithionite and substrates (NADH and D-lactate)

6.2.3.1 1Y35 membrane fragments reduced with dithionite and substrates

Shown in Fig. 14 are the typical spectra for the IY35 membrane fragments reduced with (a) dithionite; (b) 2.5 mM NADH and (c) 10 mM D-Lactate. Seen with the dithionite-reduced (a), the substrate reduced (b) and (c) are a number of lines attributable to iron-sulphur centres. The signal with a trough at g = 1.908 was seen to have the highest amplitude in the dithionite reduced sample. The signal with a trough at g = 1.861 had similar amplitude in the dithionite and substrate reduced samples.

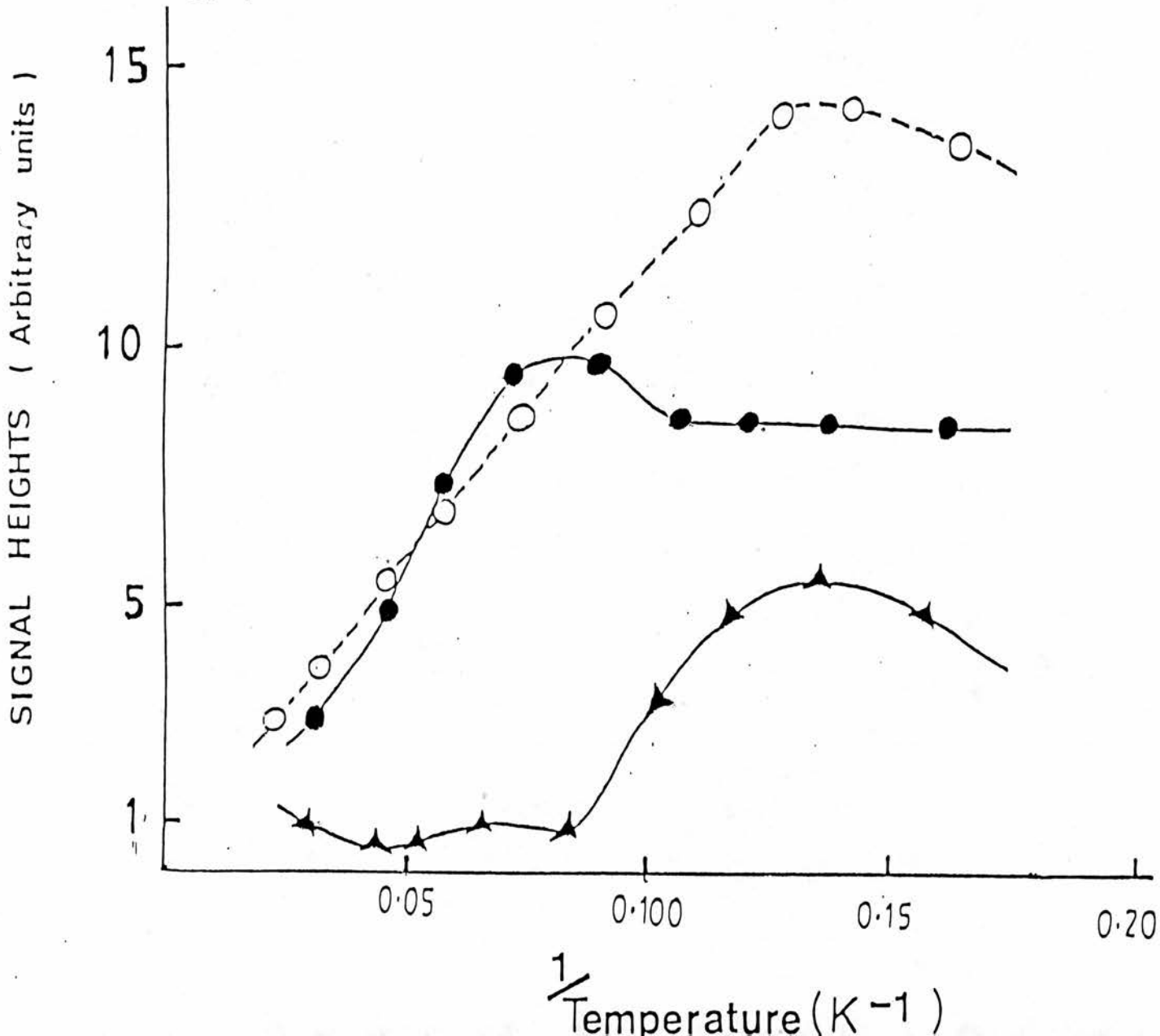
6.2.3.2 1Y36 membrane fragments reduced with dithionite and substrates

Shown in Fig. 15 are typical spectra of IY36 membrane fragments reduced with dithionite and substrates. The membrane fragments were reduced as discussed under section 6.2.3.1.

Like the 1Y35 membrane fragments the signals with troughs at g = 1.909 and 1.857 were seen to have the highest amplitude compared to the amplitude of these signals in the substrate reduced samples. The signal with a trough at g = 1.857 was seen to be partially reducible by NADH and D-Lactate.

Fig.16 - Typical temperature dependence spectra of g = 1.93 signal for IY12 (—●—●—) and IY36 (-O--O-) membrane fragments; Spectral difference (—▲—▲—). E.p.r. conditions were as in fig. 11 except that the microwave power was 15 dB; Microwave frequency, 9.48 GHz; Scan time, 500 secs. Protein concentration was 39.4 and 68.0 mg ml^{-1} respectively.

FIG·16


SIGNAL HEIGHTS (Arbitrary units)
15
10
5
1
0·05
0·100
0·15
0·20
1/Temperature (K⁻¹)

6.2.4 Temperature dependence profiles of the g = 1.93 signal for the 1Y12 and 1Y36 membrane fragments

Fig. 16 shows the temperature dependence profiles of the g = 1.93 ferredoxin-type signal for the IY12 and IY36 membrane fragments. It appears that an extra iron-sulphur centre was developed or rather a bit of it is present in the IY36 membrane fragments than in the control sample (IY12 membrane fragments) and the spectra confirmed this. The temperature dependence profiles were plotted and the spectral difference (Fig. 16) corrected for the protein concentrations for both samples indicated the presence of an extra iron-sulphur centre.

6.2.5 Quantitation of the total ferredoxin-type signals from the 1Y36 membrane fragments

The total ferredoxin-type signals from the 1Y36 membrane fragments were quantitated using an EDTA/$CuSO_4$ standard (consisting of 4 mM EDTA and 0.5 mM $CuSO_4$). This was done by integrating the spectra of dithionite-reduced sample with the EDTA/$CuSo_4$ standard. Signals that were not attributable to iron-sulphur centres were substracted from the spectra obtained with the dithionite-reduced sample. Total ferredoxin-type signals were found to be equivalent to 0.702 µmol/spins/mg protein.

Fig. 17 - Analysis of typical redox titrations for the IY35 membrane fragments. The redox titrations were carried out as described under methods. The signal heights of the absorbance g = 1.93 were plotted as a function of ambient redox (E_h) at a temperature of 15 K. E.p.r. conditions were as in fig. 11 except the gain which was 5×10^5. Protein concentration was 20.3 mg ml^{-1}.

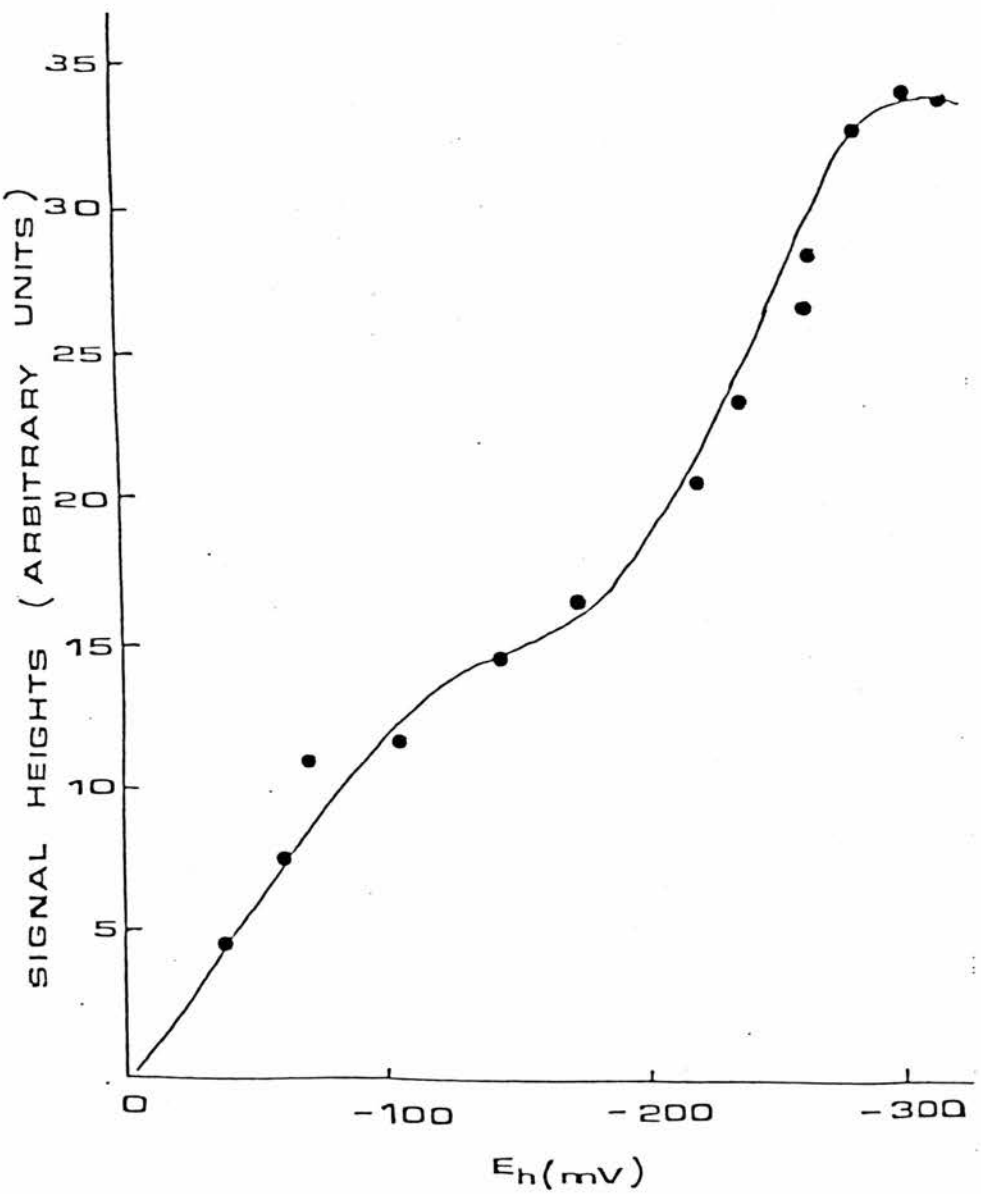


Fig. 18 - Resolution of the ferredoxin-type components of g = 1.93 from the IY35 membrane fragments. E.p.r. conditions were as in fig. 17

FIG·18

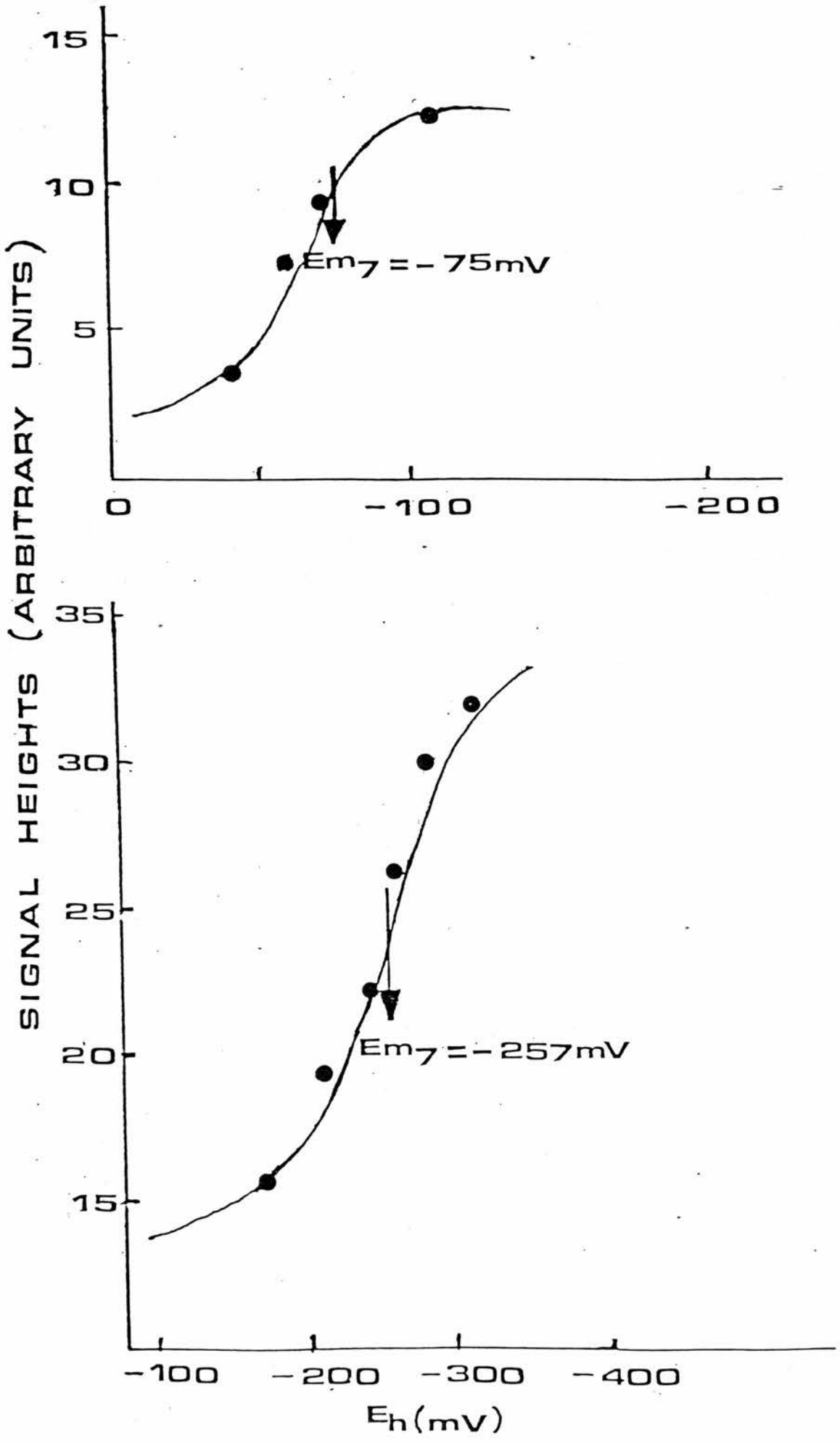
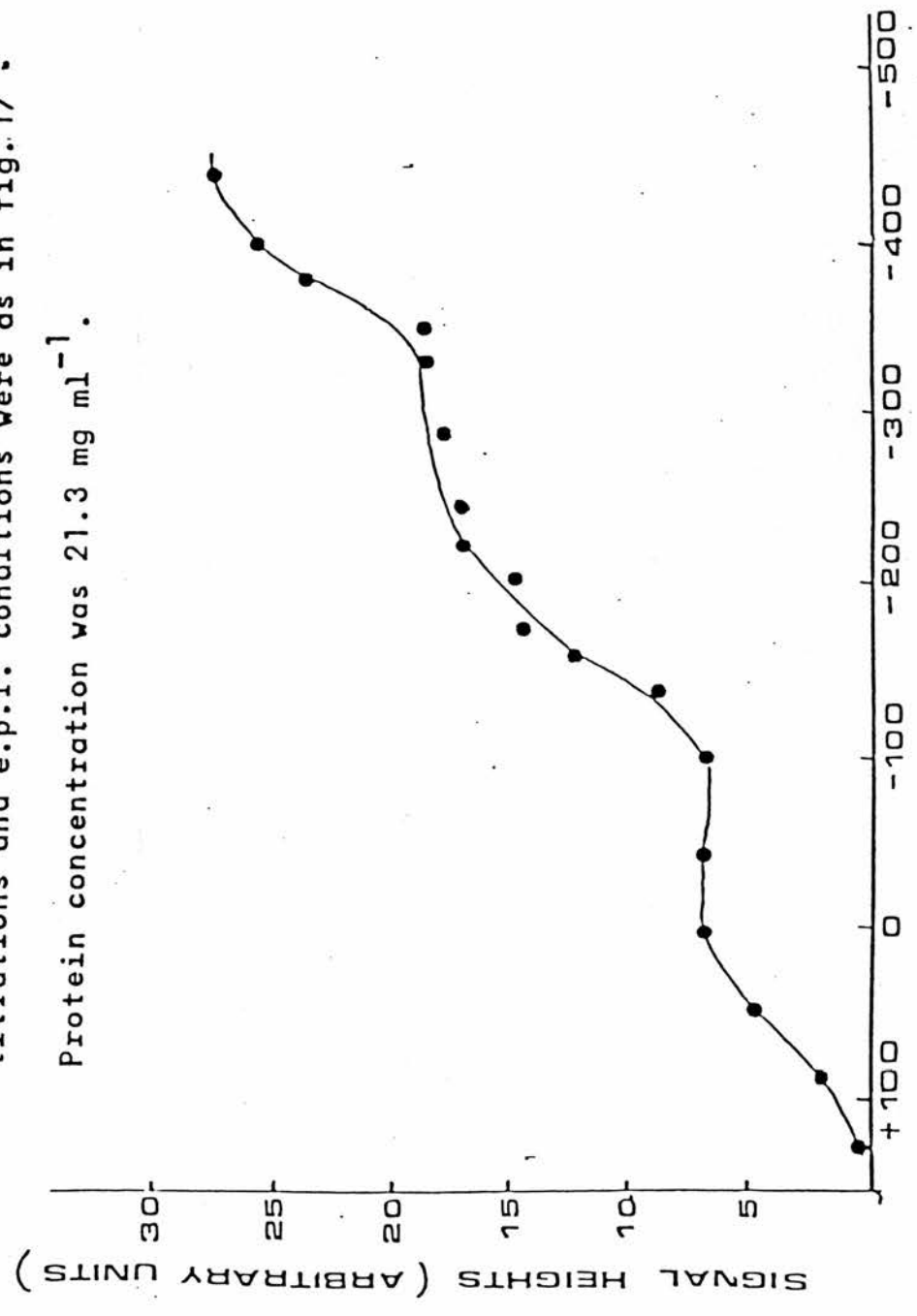

15
10
5
0
−100
−200
$Em_7 = -75mV$
35
30
25
20
15
SIGNAL HEIGHTS (ARBITRARY UNITS)
$Em_7 = -257mV$
−100
−200
−300
−400
E_h (mV)

Fig. 19 - Analysis of typical redox titrations for the IY36 membrane fragments. Details of redox titrations and e.p.r. conditions were as in fig. 17. Protein concentration was 21.3 mg ml^{-1}.



6.2.6 Redox titrations of the g = 1.93 signal from the 1Y35 membrane fragments

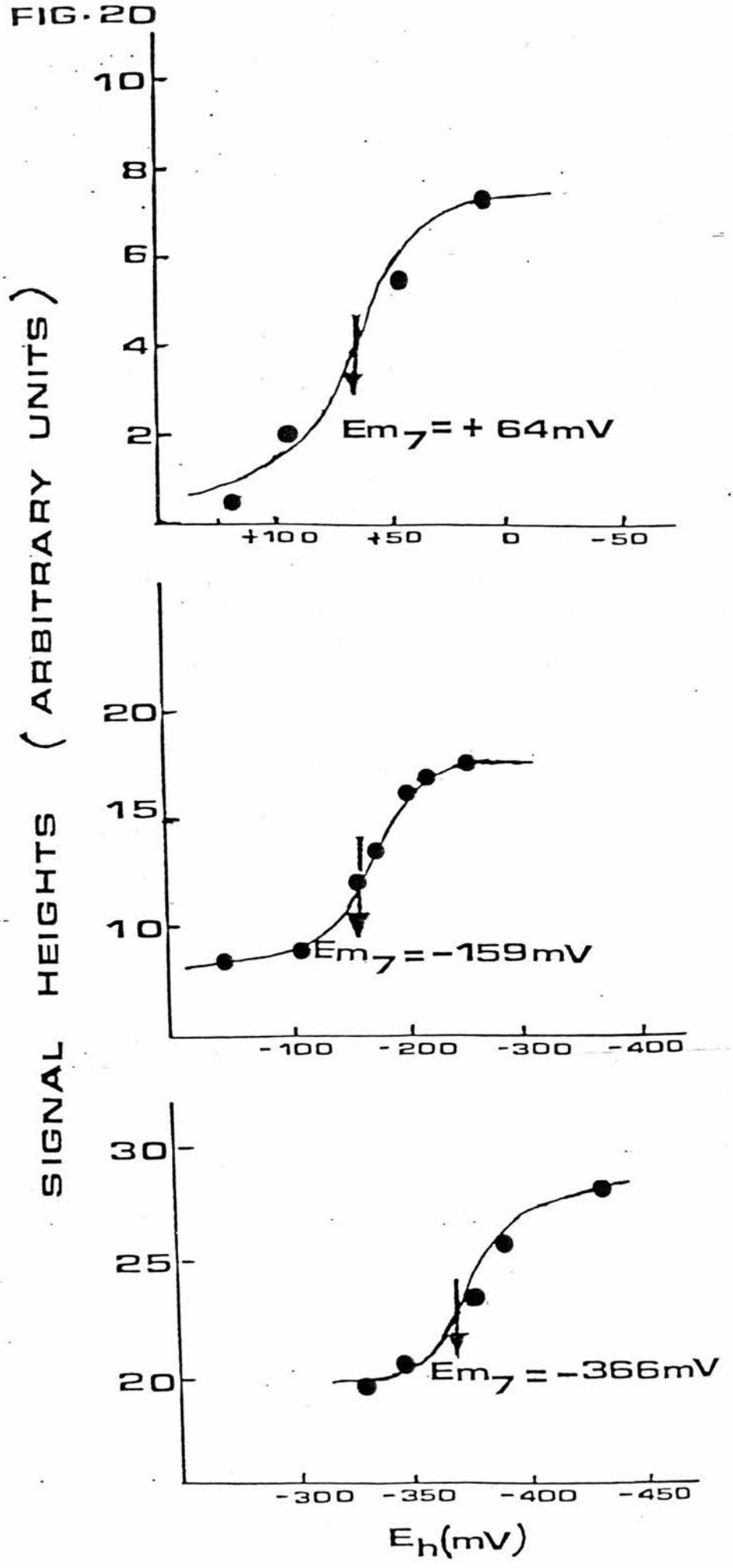
The redox titration of the g = 1.93 ferredoxin-type signal from the 1Y35 membrane fragments (Fig. 17) indicated the presence of at least two components. Data obtained from the redox titration were analysed with a computer and the theoretical n = 1 curve was drawn. The best fit theoretical curve gave $Em_{7.0}$ of -75 mV and -257 mV for these components respectively (Fig. 18). The low potential component may be from centre-2 of succinate dehydrogenase (Ohnishi et al., 1976a); this remains to be studied more quantitatively.

6.2.7 Redox titrations of the g = 1.93 signal from the 1Y36 membrane fragments

The redox titrations for the g = 1.93 ferredoxin-type signal for the 1Y36 membrane fragments indicated the presence of at least three components (Fig. 19). The data from the redox titrations were analysed with a computer and the theoretical n = 1 curve was drawn. The best fit theoretical curve gave $Em_{7.0}$ of +64 mV while the low potential components gave midpoint potential values of -159 mV and -366 mV respectively (Fig. 20). Two of these centres are similar to those of the well characterized mammalian succinate dehydrogenase (Ohnishi et al., 1976a). Since the 1Y36 strain was derived from a ndh mutant strain (1Y12) these low potential components may come from centre S-2 of succinate dehydrogenase or from the cloned gene of D-Lactate dehydrogenase.

Fig. 20 - Resolution of the ferredoxin-type components of g = 1.93 from the IY36 membrane fragments. E.p.r. conditions were as in fig. 19.

FIG·20


SIGNAL HEIGHTS (ARBITRARY UNITS)
10
8
6
4
2
Em7 = + 64mV
+100
+50
0
-50
20
15
10
Em7 = -159mV
-100
-200
-300
-400
30
25
20
Em7 = -366mV
-300
-350
-400
-450
Eh(mV)

CHAPTER 7

7. CONCLUSIONS

The results obtained for the pH profiles of the NADH dehydrogenase and D-Lactate dehydrogenase activities have shown that the membrane-bound enzymes are maximally active at pH of 7.8 and 8.8 respectively.

Studies on the effect of some additions on the activities of NADH and D-Lactate dehydrogenases showed that 4 mM EDTA had no effect on the activity of NADH dehydrogenase while the activity of the D-Lactate dehydrogenase was reduced by ~11%. The effect of 4 mM EDTA on the D-Lactate dehydrogenase activity should be significant in view of the fact that EDTA is usually employed in the preparation of electron transport fragments and also in the preparation of sample for e.p.r. studies. 0.064 mM diuron resulted in the reduction of the activities of both enzymes although the reduction in activity was more on the NADH dehydrogenase

activity. It is possible that diuron acts like "chaotrophic" agents (Reeves et al., 1973), causing dislodgement or "delipidation" of the enzymes from the membrane with concomitant decrease in activities. The results obtained for the KCN (5 mM) addition on the activities of both enzymes confirmed earlier reports on this substance as a potent inhibitor of oxidative phosphorylation (Slater, 1967). The effect of this concentration on the D-Lactate dehydrogenase showed KCN at that concentration as uncoupler of D-Lactate-dependent transport (Kaback, 1974). However, the effect of KCN on D-Lactate dehydrogenase activity has earlier been shown to be pH-dependent. For example, 10 mM KCN caused 0% inhibition on solubilised D-Lactate phenazine methosulphate reductase activity at pH 6.6 but resulted in an inhibition of 25% at pH 7.5 (Reeves et al., 1973). While 50 mM 2,4-dinitrophenol inhibited the activity of the NADH dehydrogenase, the same concentration of this substance enhanced the activity of the D-Lactate dehydrogenase. It is possible that this concentration of dinitrophenol acted as an "uncoupler" (e.g., Slater, 1967) of the D-Lactate oxidation. Bathophenanthroline (0.15 mM) inhibited the activities of both enzymes; although the inhibitory effect was more on the NADH dehydrogenase activity. Crane et al., 1975; have shown that

the D-Lactate dehydrogenase activity was inhibited by 50% at 1.3×10^{-4} M bathophenanthroline concentration but saturated above that concentration.

The results obtained for the quinone additions showed that both enzymes were affected at varying degrees by these additions. These confirmed that both enzymes have specific structural requirements for quinone acceptors (e.g., Stroobant & Kaback, 1975; Wallace & Young, 1977a).

Studies on the NADH oxidase, NADH dehydrogenase, lactate oxidase, lactate dehydrogenase and other oxidase activities for the membrane fragments have shown that the levels of other oxidase activities (except the amplified ones - NADH and D-Lactate oxidases and dehydrogenases) were unaffected as a result of the amplification procedure. Similar results were obtained with respect to the NADH and D-Lactate dehydrogenase activities by Jaworowski *et al*, (1981a) and Young *et al*. (1982) respectively.

The results obtained for the cytochrome contents showed that while the cytochrome b_{560} level for all membrane fragments appeared unaltered, the cytochrome *d* levels from both amplified strains were increased compared to the level obtained for the IY12 strain from which they were both derived.

A similar increase in cytochrome d level was obtained by Young et al. (1982) for the IY36 strain (containing the amplified gene of D-Lactate dehydrogenase) when compared to the level from wild-type (IY13) membrane fragments. Both mutant strains (IY12 and D-Ldh$^-$) have the lowest but similar level of cytochrome d when compared to the level of cytochrome d from other strains. This raises the question of whether or not their respective mutations affected the level of this terminal oxidase. This requires to be further investigated.

The levels of the peptide-bound flavins for the membrane fragments were also unaffected as a result of the amplification of the gene of the NADH and D-Lactate dehydrogenases in the IY35 and IY36 strains respectively.

The results obtained for the acid-labile sulphide showed that while the level for this component remained unaltered in other strains, it was found to be increased in the strain with the amplified gene of D-Lactate dehydrogenase (IY36 strain). This increase in the acid-labile sulphide might thus be due to the presence of additional iron-sulphur cluster from the cloned gene of

D-Lactate dehydrogenase. The presence of additional iron-sulphur cluster in the IY36 strain was confirmed by the results of the e.p.r. studies.

This additional iron-sulphur cluster was apparently absent or possibly present in low concentration in the NADH amplified (IY35) and the ndh mutant (IY12) strains. Hence it is possible that the cloned gene of D-Lactate dehydrogenase might be responsible for this additional iron-sulphur cluster. It is therefore possible that this cloned gene of D-Lactate dehydrogenase with this additional iron-sulphur cluster might help in the conversion of lactate (which accumulates in ndh mutants as a result of the reduction of pyruvate by the pyridine-nucleotide linked D-Lactate dehydrogenase - Tarmy & Kaplan, 1968; Kaback, 1974) back to pyruvate; thus providing a cyclic system for the reoxidation of NADH independently of the NADH dehydrogenase. It might also mean that the loss of the iron-sulphur protein in the NADH dehydrogenase region of the chain of the ndh mutant (IY12 strain) led to a "short circuit" of the oxidoreduction segment normally associated with this region by the presence of the gene of the D-Lactate dehydrogenase, similar to the proposed scheme of Poole & Haddock (1975).

The e.p.r. studies showed that a ferredoxin-type cluster at g = 1.93 from the 1Y36 membrane fragments has the greatest amplitude compared to the amplitude of this g value from other membrane fragments and particularly from the membrane fragments for the 1Y12 strain (from which it was derived). The temperature dependence of this signal indicated the presence of more than one component. This signal was analysed by potentiometric titrations and was resolved into three ferredoxin-type clusters with $Em_{7.0}$ values of +64 mV, -159 mV and -366 mV. Two of these centres are similar to those of the well chracterized mammalian succinate dehydrogenase (Ohnishi et al., 1976 a,b).

The e.p.r. signal at g = 1.93 from the NADH dehydrogenase amplified strain (1Y35) was analysed by potentiometric titrations and was resolved into two ferredoxin-type clusters with $Em_{7.0}$ values of -70 mV and -257 mV. The low potential component may be from centre S-2 of succinate dehydrogenase (Ohnishi et al., 1976a); this remains to be studied more quantitatively.

It is however recommended that this additional iron-sulphur clusters from the lY36 strain be further characterized as results obtained from this study have only indicated that these additional iron-sulphur clusters have been contributed by the cloned gene of D-Lactate dehydrogenase. It might however be that the presence of this gene in the ndh mutant, in addition to being able to convert accumulated lactate back to pyruvate, might in addition induce some conformational changes in the iron-sulphur clusters of other primary respiratory chain dehydrogenases that are known to contain iron-sulphur centres (Yoch & Carithers, 1979).

CHAPTER 8

8 REFERENCES

Abrahams, A., Nolan, E.A., Jensen, C. & Smith, J.B. (1973). Tightly-bound adenine nucleotide in bacterial membrane ATPase. Biochem. Biophys. Res. Commun. 55, 22 - 29.

Adelberg, E.A., Mandel, M. & Chen, C.C. (1965). Optimal conditions for mutagenesis by N-methyl-N'-nitro-nitrosoguanidine in Escherichia coli K-12. Biochem. Biophys. Res. Commun. 18, 788 - 795.

Alexander, K. & Young, I.G. (1978b). Alternative hydroxylases for the aerobic biosynthesis of ubiquinone in Escherichia coli. Biochemistry 17, 4750 - 4755.

Bachmann, B.J. & Low, K.B. (1980). Linkage map of Escherichia coli K-12. Edition 6. Microbiol. Rev. 44, 1 - 56.

Beinert, H. (1978). E.p.r. spectroscopy of components of the mitochondrial electron transport system. Methods Enzymol. 54, 133 - 150.

Beinert, H., Orme-Johnson, W.H. & Palmer, G. (1978). Special techniques for the preparation of samples for low temperature spectroscopy. Methods Enzymol. 54, 111 - 132.

Birdsell, D.C. & Costa-Robles, E.H. (1970). Electron transport particles released upon lysis of spheroplasts of Escherichia coli by Brij 58. Biochim. Biophys. Acta 216, 250 - 261.

Bishop, D.H., Pandya, K.P. & King, H.K. (1962). Ubiquinone and Vitamin K in bacteria. Biochem. J. 83, 606 - 614

Blum, H., Poole, R.K. & Ohnishi, T. (1980). The orientation of iron-sulphur clusters in the membrane multilayers prepared from aerobically-grown Escherichia coli K-12 and a cytochrome-deficient mutant. Biochem. J. 190, 385 - 393.

Boyer, P.D. (1977). Conformational coupling in oxidative phosphorylation and photophosphorylation. Trends Biochem. Sci. 2, 38 - 41.

Boyer, P.D., Chance, B., Ernster, L., Mitchell, P., Racker, E. & Slater, E.C. (1977). Oxidative phosphorylation and photophosphorylation. Ann. Rev. Biochem. 46, 955 - 1026.

Bragg, P.D. (1965). Purification and properties of menadione reductase of Escherichia coli. Biochim. Biophys. Acta 96, 263 - 271.

Bragg, P.D. & Hou, C. (1967a). Reduced nicotinamide adenine dinucleotide oxidation in Escherichia coli particles. 1. Properties and cleavage of the electron transport chain. Arch. Biochem. Biophys. 119, 194 - 201.

Bragg, P.D. & Hou, C. (1967b). Reduced nicotinamide adenine dinucleotide oxidation in Escherichia coli particles. II. NADH dehydrogenases. Arch. Biochem. Biophys. 119, 202 - 208.

Bragg, P.D. (1979). Electron transport and energy-transducing systems of Escherichia coli. In: Membrane proteins in energy transduction; (Capaldi, R.A. ed.) Volume 2, pp.341 - 449, Academic Press, New York.

Brand, M.D., Reynafarge, B. & Lehninger, A.L. (1976). Re-evaluation of the H^+/site ratio of mitochondrial electron transport with oxygen pulse technique. J. Biol. Chem. 251, 5670 - 5679.

Brodie, A.F. (1955). DNPH cytochrome c reductase (bacterial). Methods Enzymol. 2, 693 - 699.

Brodie, A.F. & Ballantine, J. (1960). Oxidative phosphorylation in fractionated bacterial systems. The role of Vitamin K. J. Biol. Chem. 235, 226 - 231.

Castor, L.N. & Chance, B. (1959). Photochemical determination of oxidases of bacteria. J. Biol. Chem. 234, 1587 - 1592.

Chance, B., Crofts, A.R., Nishimura, M. & Price, R. (1970). Fast membrane H^+ binding in the light-activated state of chromatium chromatophores. Eur. J. Biochem. 13, 364 - 374.

Cohen, G.N. & Rickenberg, H.W. (1956). Concentrate specifique des amino-acides chez Escherichia coli. Ann. Inst. Pasteur 91, 693 - 720.

Cole, S.T. & Guest, J.R. (1979). Production of a soluble form of fumarate reductase by multiple gene duplication in Escherichia coli K-12. Eur. J. Biochem. 102, 65 - 71.

Cox, G.B., Newton, N.A., Gibson, F., Snoswell, A.M. & Hamilton, J.A. (1970). The function of ubiquinone in Escherichia coli. Biochem. J. 117, 551 - 562.

Cox, G.B. & Gibson, F. (1974). Studies on electron transport and energy-linked reactions using mutants of Escherichia coli. Biochim. Biophys. Acta 346, 1 - 25.

Crane, R.T., Sun, I.L., & Crane, F.L. (1975). Lipophilic chelator inhibition of electron transport in Escherichia coli. J. Bacteriol. 122, 686 - 690.

Dancey, G.F., Lewine, A.E. & Shapiro, B.M. (1976). The NADH dehydrogenase of the respiratory chain of Escherichia coli. I. Properties of the membrane-bound enzyme. Its solubilisation and purification to near homogeneity. J. Biol. Chem. 251, 5911 - 5920.

Dancey, G.F. & Shapiro, B. M. (1976). The NADH dehydrogenase of the respiratory chain of Escherichia coli. II. Kinetics of the purified enzyme and the effect of antibodies elicited against it on membrane-bound and free enzyme. J. Biol. Chem. 251, 5921 - 5928.

Deeb, S.S. & Hager, L.P. (1964). Crystalline cytochrome b_1 from Escherichia coli. J. Biol. Chem. 239, 1024 - 1031.

Dutton, P.L. (1978). Redox potentiometry: Determination of midpoint potentials of oxidation-reduction components of biological electron transfer systems. Methods Enzymol. 54, 411 - 435.

Futai, M. (1973). Membrane-bound D-Lactate dehydrogenase from Escherichia coli. Purification and properties. Biochemistry 12, 2468 - 2474.

Futai, M. (1974). Orientation of membrane vesicles from Escherichia coli prepared by different procedures. J. Memb. Biol. 15, 12 - 28.

Futai, M. (1974). Reconstitution of transport dependent on D-lactate or glycerol-3-phosphate in membrane vesicles of Escherichia coli deficient in corresponding dehydrogenases. Biochemistry 13, 2327 - 2333.

Futai, M. & Tanaka, Y. (1975). Localization of D-Lactate dehydrogenase in membrane vesicles prepared by using a French press or ethylenediamine tetraacetate-lysozyme from Escherichia coli. J. Bacteriol. 124, 470 - 475.

Garvie, E. I. (1980). Bacterial lactate dehydrogenases. Microbiol. Rev. 44, 106 - 139.

Guest, J.R. & Stephen, P.E. (1980). Molecular cloning of the pyruvate dehydrogenase complex of Escherichia coli. J. Microbiol. 121, 277 - 292.

Guest, J.R. (1981a). Partial replacement of succinate dehydrogenate function by phage and plasmid-specified fumarate reductase in Escherichia coli. J. Gen. Microbiol. 122, 171 - 179.

Gutman, M., Scheffer, A. & Avi-dor, Y. (1968). The preparation and properties of the membranal DPNH dehydrogenase from Escherichia coli. Biochim. Biophys. Acta 162, 506 - 517.

Gutman, M. & Singer, T.P. (1970). Spectrophotometric observations on the oxidation-reduction circle of the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. Biochemistry 9, 4750 - 4758.

Haddock, B.A. & Schairer, H.U. (1973). Electron transport chain of Escherichia coli: Reconstitution of respiration in a 5-amino laevulinic acid requiring mutant. Eur. J. Biochem. 35, 34 - 45.

Haddock, B.A. & Jones, C.W. (1977). Bacterial respiration. Bacteriol. Rev. 41, 47 - 99.

Hall, D.O., Cammack, R., Rao, K.K., Evans, M.C.W. & Mullinger, R. (1975). Ferredoxins, blue-green bacteria and evolution. Biochem. Soc. Trans. 3, 361 - 368.

Horio, T., Higashi, T., Yamanaka, T., Matsubara, H. & Ohnishi, T. (1961). Purification and properties of cytochrome from Pseudomonas aeroginosa. J. Biol. Chem. 236, 944 - 951.

Ingledew, W.J., Reid, G.A., Poole, R.K., Blum, H. & Ohnishi, T. (1980). The iron-sulphur centres of aerobically-grown Escherichia coli K-12: An electron paramagnetic study. FEBS LETT. 111, 223 - 227.

Ingledew, W.J., (1983). The electron transport chain of Escherichia coli grown anaerobically with fumarate as terminal electron acceptor: An electron paramagnetic study. J. Gen. Microbiol. 129, 1651 - 1659.

Jaworowski, A., Campbell, H.D., Poulis, M.I. & Young, I.G. (1981a). Genetic identification and purification of the respiratory NADH dehydrogenase of Escherichia coli. Biochemistry 20, 2041 - 2047.

Jaworowski, A., Mayo, G., Shaw, D.C., Campbell, H.D. & Young, I.G. (1981b). Characterization of the respiratory NADH dehydrogenase of Escherichia coli and reconstitution of NADH oxidase in ndh mutant membrane vesicles. Biochemistry 20, 3621 - 3628.

Jones, C.W. & Redfearn, E.R. (1966). Electron transport in Azotobacter vinelandii. Biochim. Biophys. Acta 113, 467 - 481.

Kaback, H.R. (1972). Transport accross isolated bacterial cytoplasmic membrane. Biochim. Biophys. Acta 265, 367 - 416.

Kaback, H.R. (1974). Transport studies in bacterial membrane vesicles devoid of soluble constituents catalyse the transport of many metabolites. Science 186, 882 - 892.

Kaczorowski, G., Kohn, L.D. & Kaback, H.R. (1978). Purification of properties of D-Lactate dehydrogenase from Escherichia coli ML 308 - 225. Methods Enzymol. 53, 519 - 527.

Kashket, E.R. & Brodie, A.F. (1963). Oxidative phosphorylation in fractionated bacterial systems. X. Different roles for the natural quinones of Escherichia coli W in oxidative metabolism. J. Biol. Chem. 238, 2564 - 2570.

King, T.E. & Morris, R.O. (1967). Determination of acid labile sulphide and sulphydryl groups. Methods Enzymol. 10, 635 - 637.

Kistler, W.S. & Lin, E.C.C. (1971). Anaerobic L-α-glycero-phosphate dehydrogenase of Escherichia coli. Its genetic locus and its physiological role. J. Bacteriol. 108, 1224 - 1234.

Kline, E.S. & Mahler, H.R. (1965). Lactic dehydrogenases of Escherichia coli. Ann. N.Y. Acad. Sci. 119, 905 - 919.

Kohn, L.D. & Kaback, H.R. (1973). Mechanism of active transport in isolated bacterial membrane vesicles. XV. Purification and properties of the membrane-bound D-lactate dehydrogenase from Escherichia coli. J. Biol. Chem. 248, 7012 - 7017.

Kovatechev, S., Vaz, W.L.C. & Eibl, H. (1981). Lipid dependence of the membrane-bound D-lactate dehydrogenase of Escherichia coli. J. Biol. Chem. 256, 10369 - 10374.

Lederberg, J & Lederberg, E.M. (1952). Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63, 399 - 406.

Lester, R. L. & Crane, F.L. (1959). Natural occurrence of coenzyme Q and related compounds. J. Biol. Chem. 234, 2169 - 2175.

Lohmeier, E., Hagen, D.S., Dickie, P. & Weiner, J.H. (1980). Cloning and expression of the fumarate reductase genes of Escherichia coli. Can. J. Biochem. 59, 158 - 164.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein determination with the Folin phenol reagent. J. Biol. Chem. 193, 265 - 275.

Massari, S. & Azzone, G.F. (1970). Mechanism of ion translocation in mitochondria. 1. Coupling of K^+ and H^+ fluxes. Eur. J. Biochem. 12, 301 - 309.

Meijer, E.M., Wevar, R. & Stouthamer, A.H. (1977). The role of iron-sulphur centre 2 in electron transport and energy conservation in the NADH-ubiquinone segment of Paracoccus denitrificans. Eur. J. Biochem. 81, 267 - 275.

Mitchell, P. (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature (London) 191, 144 - 148.

Mitchell, P. (1966). Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. 41, 445 - 502.

Mitchell, P. & Moyle, J. (1967). Respiration driven proton translocation in rat liver mitochondria. Biochem. J. 105, 1147 - 1162.

Mitchell, P. & Moyle, J. (1968). Proton translocation coupled to ATP hydrolysis in rat liver mitochondria. Eur. J. Biochem. 4, 530 - 539.

Mitchell, P. (1974). A chemiosmotic mechanism for proton-translocating adenosine triphosphate. FEBS LETT. 43, 189 - 194

Mitchell, P. (1975a). Protonmotive redox mechanism of the cytochrome b-c complex in the respiratory chain: Protonmotive ubiquinone cycle. FEBS. LETT. 56, 1 - 6.

Mitchell, P. (1977b). Protonmotive Q cycle: A general formulation. FEBS. LETT. 59, 137 - 139.

Mitchell, P. (1976). Vectorial chemistry and the molecular mechanism of chemiosmotic coupling: Power transmission by protocity. Biochem. Soc. Trans. 4, 399 - 430.

Mitchell, P. (1977). A commentary on alternative hypothesis of protonic coupling in the membrane systems catalysing oxidative and photosynthetic phosphorylation. FEBS. LETT. 78, 1 - 20.

Mitchell, P. (1979). Compartmentation and communication in living systems. Ligand conduction: A general catalytic principle in chemical, osmotic and chemiosmotic reaction systems. Eur. J. Biochem. 95, 1 - 20.

Moyle, J. & Mitchell, P. (1978). Cytochrome c oxidase is not a proton pump. FEBS LETT. 88, 268 - 272.

Nishimura, Y., Tan, I.K.P., Ohgami, Y., Kohgami, K. & Kamihara, T. (1983). Induction of membrane-bound L-Lactate dehydrogenase in Escherichia coli under conditions of nitrate respiration, fumarate reduction and trimethylamine-N-oxide reductase. FEM. Microbiol. Lett. 17, 283 - 286.

Ohnishi, T., Salerno, J.C., Winter, D.B., Lim, J., Yu, C.A. & King, T.E. (1976a). Thermodynamic and e.p.r. characterization of two ferredoxin-type iron-sulphur centres in the succinate-ubiquinone segment of the respiratory chain. J. Biol. Chem. 251, 2094 - 2104.

Ohnishi, T., Lim, J., Winter, D.B. & King, T.E. (1976b). Thermodynamic and e.p.r. characterization of a Hipip-type iron-sulphur centre in the succinate dehydrogenase of the respiratory chain. J. Biol. Chem. 251, 2105 - 2109.

Orme-Johnson, W.H. & Orme-Johnson, W.R. (1978). Overview of iron sulphur proteins. Methods Enzymol. 53, 259 - 268.

Pandya, K. P. & King, H.K. (1966). Ubiquinone and menaquinone in bacterial systems. Arch. Biochem. Biophys. 114, 154 - 157.

Papa, S. (1976). Proton-translocating reactions in the respiratory chains. Biochim. Biophys. Acta 456, 39 - 84.

Pascal, M. & Pichinoty, F. (1965). Regulation de la biosynthese et de la function des D-et L.lactate -dehydrogenases chez Aerobacter aerogenes. Biochim. Biophys. Acta 105, 54 - 69.

Polgase, W.J., Pun, W.T. & Withaar, J. (1966). Short communications: Lipoquinones of Escherichia coli. Biochim. Biophys. Acta 118, 425 - 426.

Poole, R.K., Waring, A.J. & Chance, B. (1979b). The reaction of cytochrome o in Escherichia coli with oxygen: Low temperature kinetics and spectral properties. Biochem. J. 184, 379 - 389.

Poole, R.K. & Haddock, B.A. (1975). Effects of sulphate limited growth in continuous culture in the electron transport chain and energy conservation in Escherichia coli K-12. Biochem. J. 152, 537 - 546.

Poole, R.K., Scott, R.I. & Chance, B. (1980). Low temperature spectral and kinetic properties of cytochromes in Escherichia coli K-12 grown at lowered oxygen tension. Biochim. Biophys. Acta 591, 471 - 482.

Poulis, M.I., Shaw, D.C., Campbell, H.D. & Young, I.G.(1981). In-vitro synthesis of the respiratory NADH dehydrogenases of Escherichia coli: Role of UUG as initiation codon. Biochemistry 20, 4178 - 4185.

Pratt, E. A., Fung, L.W.N., Flowers, J.A. & Ho, C. (1979). Membrane-bound D-lactate dehydrogenase from Escherichia coli: Purification and properties. Biochemistry 18, 312 - 316.

Pratt, E. A., Jones, J.A., Cottam, P.F., Dowd, S.R. & Ho, C. (1983). A biochemical study of the reconstitution of D-lactate dehydrogenase-deficient membrane vesicles using fluoride-labelled components. Biochim. Biophys. Acta 729, 167 - 175.

Rabinowitz, J.C. (1978). Analysis of acid labile sulphide and sulphydryl groups. Methods Enzymol. 53, 275 - 278.

Ragan, C.I. (1976). NADH-ubiquinone oxido-reductase. Biochim. Biophys. Acta 456, 249 - 290.

Ramos, S., Schuldiner, S. & Kaback, H.R. (1976). The electrochemical gradient of protons and its relationship to active transport in Escherichia coli membrane vesicles. Proc. Natl. Acad. Sci. U.S.A. 73, 1892 - 1896.

Rao, N.A., Felton, S.P. & Huennekens, F.M. (1967). Quantitative determination of mitochondrial favins. Methods Enzymol. 10, 494 - 497.

Reid, G.A. & Ingledew, W.J. (1979). Characterization and genotypic control of the cytochrome content of Escherichia coli. Biochem. J. 182, 465 - 472.

Reynafarge, B., Brand, M.D. & Lehninger, A.L. (1976). Evaluation of the H^+/site ratio of mitochondrial electron transport from rate measurement. J. Biol. Chem. 251, 7442 - 7451.

Rieske, J.S. (1976). Composition, structure and function of complex III of the respiratory chain. Biochim. Biophys. Acta 456, 195 - 247.

Salerno, J.C., Blum, H. & Ohnishi, T. (1979). The orientation of iron-sulphur clusters and a spin-couple ubiquinone pair in the mitochondrial membrane. Biochim. Biophys. Acta 547, 270 - 281.

Senior, A.E. (1973). The structure of ATPase complexes of mitochondrial ATPase. Biochim. Biophys. Acta 301, 249 - 277.

Shipp, W.S. (1972). Cytochromes of Escherichia coli. Arch. Biochem. Biophys. 150, 459 - 472.

Short, S. A., Kaback, H.R. & Kohn, L.D. (1974). D-lactate dehydrogenase binding in Escherichia coli dld⁻ membrane vesicles for active transport. Proc. Natl. Acad. Sci. U.S.A. 71, 1461 - 1465.

Short, S.A., Kaback, H.R., Hawkings, T. & Kohn, L.D. (1975a). Immunological properties of the membrane-bound D-lactate dehydrogenase from Escherichia coli. J. Biol Chem. 250, 4285 - 4290.

Slater, E.C. (1953). Mechanism of phosphorylation in the respiratory chain. Nature (London) 172, 975 - 978.

Slater, E.C. (1967). An evaluation of the Mitchell hypothesis of Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Eur. J. Biochem. 1, 317 - 326.

Slater, E.C. (1967). Application of inhibitors and uncouplers for a study of oxidative phosphorylation. Methods Enzymol. 10, 48 - 56.

Stroobant, P. & Kaback, H.R. (1975). Ubiquinone-mediated coupling of NADH dehydrogenase to active transport in membrane vesicles from Escherichia coli. Proc. Natl. Sci. U.S.A. 72, 3970 - 3974.

Tager, J.M., Wanders, R.J.A., Groen, A.K., Kunz, W., Bohnensack, R., Kuster, U., Letko, G., Bohme, G., Duszunski, J. & Wojtczak, L. (1983). Control of mitrochondrial respiration. FEBS LETT. 151, 1 - 9.

Taniguchi, S. & Kamen, M.D. (1965). The oxidase system of heterotropically-grown Rhodospirillum rubrum. Biochim. Biophys. Acta 96, 395 - 428.

Tarmy, E.M. & Kaplan, N.O. (1968a). Chemical characterization of D-lactate dehydrogenase from Escherichia coli B. J. Biol. Chem. 243, 2579 - 2586.

Tarmy, E.M. & Kaplan, N.O. (1968b). Kenetics of Escherichia coli B D-lactate dehydrogenase and evidence for pyruvate-controlled change in conformation. J. Biol Chem. 243, 2587 - 2596.

Wallace, B.J. & Young, I.G. (1977a). Aerobic respiration in mutant of Escherichia coli accumulating quinone analogues of ubiquinone. Biochim. Biophys. Acta 461, 75 - 83.

Wallace, B.J. & Young, I.G. (1977b). Role of quinone in electron transport to oxygen and nitrate in Escherichia coli: Studies with a ubi A⁻ men A⁻ double quinone mutant. Biochim. Biophys. Acta 461, 84 - 100.

Wang, C. & Smith, R.L. (1975). Lowry determination of protein in the presence of Triton X-100. Anal. Biochem. 63, 414 - 417.

Watari, A., Kearney, E.B. & Singer, T.P. (1963). Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. J. Biol. Chem. 238, 4063 - 4073.

White, D.C. (1965). Synthesis of 2-demethyl vitamin K_2 and the cytochrome system in Haemophilus. J. Bacteriol. 89, 299 - 305.

Wikström, M.K.F. (1973). The different cytochrome b components in the respiratory chain of animal mitochondria and their role in electron transport and energy conservation. Biochim. Biophys. Acta 301, 155 - 193.

Wikström, M.K.F. & Pentillä, T. (1982). Critical evaluation of the proton translocating property of cytochrome oxidase in rat liver mitochondria. FEBS LETT. 144, 183 - 189.

Williams, R.J.P. (1961). Possible functions of chains of catalysts. J. Theor. Biol. 1, 1 - 17.

Williams, R.J.P. (1978). The history and the hypothesis concerning ATP-formation by energised protons. FEBS LETT. 85, 9 - 19.

Wilson, G.S. (1978). Determination of oxidation-reduction potentials. Methods Enzymol. 54, 396 - 410.

Yoch, D.C. & Carithers, R.P. (1979). Bacterial iron-sulphur proteins. Microbiol. Rev. 43, 384 - 421.

Young, I.G. & Wallace, B.J. (1976). Mutations affecting the reduced nicotinamide adenine dinucleotide dehydrogenase complex of Escherichia coli. Biochim. Biophys. Acta 449, 376 - 385.

Young, I.G., Jaworowski, A. & Poulis, M.I. (1978). Amplification of the respiratory NADH dehydrogenase of Escherichia coli by gene cloning. Gene 4, 25 - 36.

Young, I.G., Jaworowski, A. & Poulis, M.I. (1982). Cloning of the gene for the respiratory D-Lactate dehydrogenase of Escherichia coli. Biochem. 21, 2092 - 2095.