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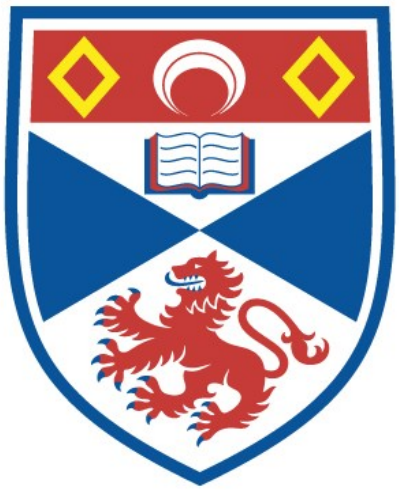

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CYTOCHROME *bd* FROM *Escherichia coli* GROWN ANAEROBICALLY BY FUMARATE RESPIRATION

by

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A thesis submitted to the University of St Andrews in application for the degree of Master of Science

April 1985

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DECLARATION

I hereby declare that the following thesis is based on work carried out by myself, that the thesis is of my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry and Microbiology of the University of St. Andrews under the direction of Dr W.J. Ingledew.

CERTIFICATE

I hereby declare that Stewart Donald Finlayson has spent four terms in research work under my direction and that he has fulfilled the condition of Ordinance No. 16 (St. Andrews), and that he is qualified to submit this thesis for the degree of Master of Science.

ACADEMIC RECORD

I matriculated at Dundee College of Technology in September 1976 and graduated with the degree of Bachelor of Science (CNAA), in Science (with commendation) in November 1979.

In May 1980, I matriculated as a research student (part-time) at the University of St. Andrews.

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SUMMARY

A cytochrome *d*-containing complex was purified from a wild-type strain of *Escherichia coli* (EMG2) grown anaerobically by fumarate respiration. A purification protocol was devised using a preliminary extraction of membrane particles with buffer containing a low concentration (0.75% v/v) of Triton X-100 before the cytochrome *bd* was solubilised with a higher concentration (6% v/v) of Triton X-100. The cytochrome was subsequently purified (to apparent homogeneity) by ion exchange chromatography on DEAE-Sephacel followed by gel filtration on Sephacryl S-300 and, finally, chromatofocusing using a pH gradient of 4-6.

The resulting cytochrome ^{d} complex, when analysed by non-denaturing PAGE, migrated as a single band which stained for iron, haem and TMPD oxidase activity. SDS-PAGE revealed two protein-staining bands which corresponded to molecular weights of 70 000 and 43 000 daltons. The values were not significantly altered by electrophoresis in gels of 7, 10 and 12.5% (w/v) acrylamide.

Analysis of the complex by isoelectric focusing gave a pI of 4.8. Low-temperature difference spectra of the purified complex showed one *b* cytochrome absorbing at 560nm, cytochrome *d* absorbing at 629nm and a broad peak at 595nm which is generally attributed to "cytochrome a_1".

The reactions of cytochrome *d* in both the membrane-bound and partially purified states, with NO, CO, and oxygen were investigated spectroscopically. Reduced cytochrome *d* was found to react with NO and CO, and reduced cytochrome *d*, when oxidised with ferricyanide in the absence of oxygen, showed a significantly different spectrum from that of aerobically-oxidsed cytochrome *d*.

In addition, some evidence for the reaction of NO and CO with the "cytochrome a_1" present in the complex was discovered.

ABBREVIATIONS

BSA	Bovine serum albumin
DOC	Deoxycholic acid
EDTA	Ethylenediamine tetraacetic acid (disodium salt)
ETP	Electron transport particle
IEF	Isoelectric Focusing
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
SDS	Sodium dodecyl (lauryl) sulphate
TEMED	N,N,N',N',-tetramethylethylenediamine
TES	2-((2-Hydroxy-1,1-bis(hydroxymethyl) ethyl) aminoethanesulphonic acid
TMBZ	3,3',5,5'-tetramethylbenzidine
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine

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CHAPTER ONE

GENERAL INTRODUCTION

Cytochrome Oxidases

Cytochrome oxidases are a diverse group of enzymes which are vitally involved in the aerobic production of cellular energy, transferring electrons to an acceptor of relatively high potential, usually oxygen. In comparison with the cytochrome *c* oxidase of eukaryotic mitochondria, prokaryotic oxidases are varied in both prosthetic groups and subunit composition. They are often simpler in structure and mode of action (Poole, 1983).

The two oxidases of *Escherichia coli* belong to different groups of cytochromes. Cytochrome *o* (for oxidase) is a *b*-type cytochrome, classed as such by having protohaem as its prosthetic group (Fig. 1a). Cytochrome *d* has a dihydroporphyrin (chlorin) iron as its prosthetic group (Fig. 1b). The shift in the absorption maxima of the pyridine ferrohaemochromogen formed from these prosthetic groups, from 556-558nm for protohaems to 600-620nm for the iron chlorin, is due to reduction in the porphyrin ring converting the protohaem into the chlorin of cytochrome *d* (Lemberg and Barret, 1970).

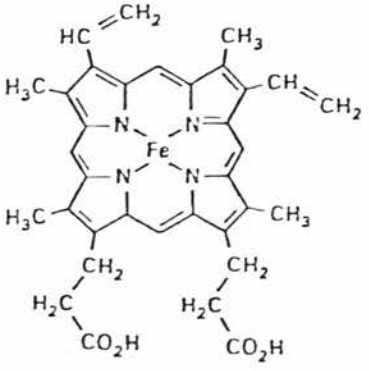
Cytochrome *d* is particularly interesting as it demonstrates a high affinity for oxygen while remaining much more resistant to cyanide inhibition than other oxidases. In addition, cytochrome *d* forms an "oxygenated compound" in its oxidised (ferric) state which is stable at room temperature (Poole *et al.*, 1983a).

FIGURE 1

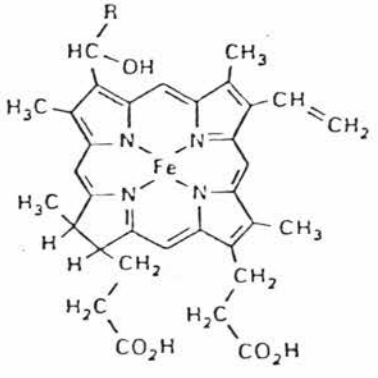
The Prosthetic Groups of Cytochromes *b* and *d*

a: Protohaem (cytochrome *b*)

b: Dihydroporphyrin (cytochrome *d*)


HC
CH2
CH3
H3C
CH
CH2
N
N
Fe
N
N
H3C
CH3
CH2
CH2
H2C
H2C
CO2H
CO2H

(a)


R
HC
OH
CH3
H3C
CH
CH2
N
N
Fe
N
N
H3C
H
CH3
H
CH2
CH2
H2C
H2C
CO2H
CO2H

(b)

The Cytochromes of *Escherichia coli*

E. coli is capable of altering the composition of its respiratory chain in response to certain extrinsic factors. Members of all four of the main groups of cytochromes, namely *a*,*b*,*c* and *d* have been reported (Haddock and Jones, 1977). Attempts to characterise the cytochrome components of the respiratory chain of *E. coli* grown under a variety of conditions, have produced some differing results. These results are difficult to compare due to the use of different strains, the difficulty in strictly defining growth conditions and the different capabilities of the techniques involved in resolving *b* and *c*-type cytochromes as these have overlapping absorbance peaks.

The pathways most extensively characterised are those that are produced under aerobic conditions and those produced anaerobically using fumarate or nitrate as the terminal oxidant (Fig. 2). The essential features of these pathways are outlined below.

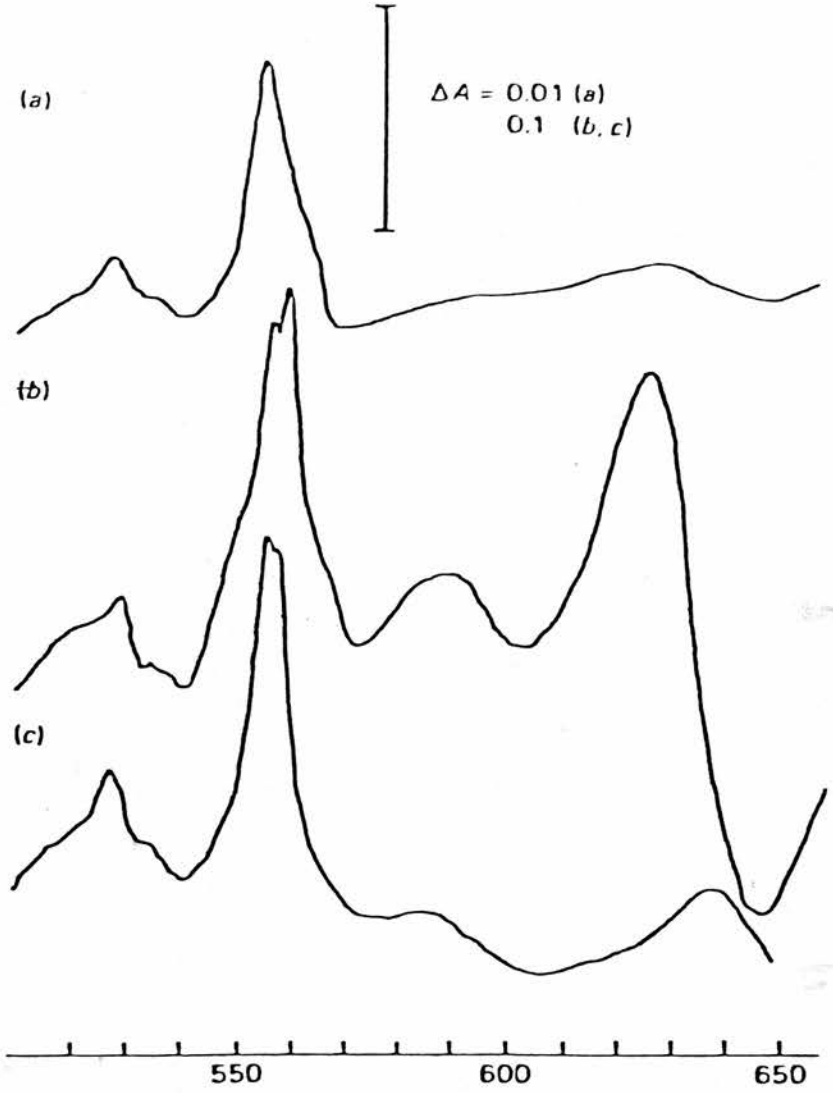
Aerobic Growth

Cells grown in highly aerobic conditions show a broad α-band around 555nm (Fig. 2a). An early investigation by Shipp (1972) of this α-band, by fourth order finite difference analysis of difference spectra, concluded that the α-band was composite and made up of absorbances due to 3 *b*-type and 2 *c*-type cytochromes. He suggested that the same components were present in cells grown under a

FIGURE 2

Optical Difference Spectra of *E. coli* Phenotypes Showing Different Cytochrome Compositions

This figure is taken from Reid and Ingledew (1979); the traces are reduced minus oxidised difference spectra and were obtained at 77K from (a) membranes from aerobically grown cells, (b) membranes derived from cells grown anaerobically with glycerol and fumarate and (c) membranes derived from cells grown anaerobically with glycerol and nitrate.


(a)
ΔA = 0.01 (a)
0.1 (b, c)
(b)
(c)
550
600
650

variety of conditions, albeit in differing relative quantities. Since then, attempts have been made to further characterise the cytochrome content by a variety of techniques including numerical analysis of difference spectra (Scott and Poole, 1982), redox potentiometry (Hendler and Schrager, 1979; Hendler *et al.*, 1975; Pudek and Bragg, 1976; Reid and Ingledew, 1979), stopped flow kinetic analysis (Haddock *et al.*, 1976), genetic mutations of cytochrome synthesis (Lorence *et al.*, 1984) and various combinations of the above (van Wielink *et al.*, 1982, 1983). All agree that the α-band is composite but there is not complete concensus over the exact composition with regard to numbers and mid-point potentials of the cytochromes.

A major source of controversy is the presence of two components, absorbing at 555nm and 562nm, with very similar mid-point potentials. These were first reported by Reid and Ingledew (1979) and subsequently by van Wielink *et al.*, (1982, 1983) and Lorence *et al.*, (1984). There is disagreement over whether they are due to one cytochrome with a split α-band or two distinct *b* cytochromes and there is also uncertainty over the CO-binding properties of these components. It is generally accepted, however, that under aerobic conditions, where the contribution from the low aeration chain (see below) would be expected to be small, the cells contain two to four spectrally and potentiometrically distinct *b* cytochromes and that cytochrome *o*, which is a *b*-type, is the major oxidase

TABLE 1

Spectral and Potentiometric Resolution of *b*-type Cytochromes in Aerobically-Grown *Escherichia coli**

Shipp (1972)	Reid & Ingledew (1979)		Scott & Poole (1982)		van Wielink *et al.* (1982,1983)		Hackett & Bragg+ (1983a)		Lorence *et al.* (1984)		Kita *et al.*{ (1979,1984a)	
			Small Intervals	Shipps' Intervals								
λ_{max}(nm)	λ_{max}(nm)	Em(mV)	λ_{max}(nm)	λ_{max}(nm)	λ_{max}(nm)	Em(mV)	λ_{max}(nm)	Em(mV)	λ_{max}(nm)	Em(mV)	λ_{max}(nm)	Em(mV)
			555	555	555.5 to 555.7	+46 to 48	555	+129	555	+165	555	+125
556	556	+80			556.7 to 556.8	+174 to 162	556	- 43	556	+ 35	556	- 45
		+260										
	558	- 50			558.4 to 558.6	- 75						
560			560		(560.2	+169)++						
	563	+260	563	563	563.5	+187 to 176	562	+196	562	+165	562	+125

*Adapted from Ingledew and Poole (1984)
+Solubilised "555-562 complex"
++Minor component described in Van Wielink (1982)
{Purified components

(Table 1). In addition, two cytochrome *c*s are formed, c_{552} and c_{548} (Shipp, 1972; Scott and Poole, 1982). Their function with respect to the electron transport chain is not known but does not seem to be important as they can be readily removed from the membrane without affecting oxidation rates.

Recent work, utilising a mutant strain thought to be incapable of synthesising the low aeration pathway, has enabled the cytochrome *o* terminated pathway to be examined in isolation (Lorence *et al*, 1984). It was described as containing two *b*-type cytochromes, one with a split α-band, high mid-point potential and the ability to bind CO (Table 1).

In conditions where the oxygen supply is limiting, a second, distinct set of *b* cytochromes is formed absorbing at around 556nm and 558nm (Haddock *et al*., 1976; Scott and Poole, 1982) and large amounts of cytochromes a_1 (so called, see later) and *d* are also synthesised. On the basis of CO-binding spectra (Castor and Chance, 1959) and oxidation kinetics (Haddock *et al*., 1976) it was concluded that cytochromes *o* and *d* could act as oxidases, but cytochrome a_1 neither bound CO nor was it kinetically competent to act as an oxidase in this instance. There is still some conflict over the function of cytochrome a_1. Cytochrome *o* synthesis appears to be discontinued under these conditions (Kranz and Gennis, 1983) but an *o*-type cytochrome exhibiting different spectral properties and a much greater affinity for O_2 has been reported

(Poole and Chance, 1981). According to Pudek and Bragg, (1975), in cells grown under low aeration, where both pathways are present, the flow of electrons is split between both branches, with both oxidases functioning simultaneously.

Just as there is dispute over the number and mid-point potentials of the *b*-cytochromes in the aerobic chain so there is controversy over the sequence of the cytochromes. Recent studies (Downie and Cox, 1978; Kita and Anraku, 1981; Kita *et al.*, 1984b), using similar techniques involving ubiquinone deficient mutants of *E. coli*, have led to contrasting results. Downie and Cox,(1978) proposed the scheme outlined in Fig. 3a, Anraku and colleagues that in Fig. 3b. Although the order shown in Fig. 3a agrees with kinetic evidence obtained by Haddock *et al.*,(1976), it disagrees with the majority of the mid-point potentials obtained for cytochromes b_{556} and b_{558}. (For a fuller discussion see Ingledew and Poole, 1984).

Nitrate Respiration

When grown on a non-fermentable carbon source with NO_3^- as the terminal electron acceptor, cells produce large quantities of *b* cytochromes absorbing at 555nm. One of these is associated with nitrate reductase ($^{nr}b_{555}$) and the other with formate dehydrogenase ($^{fdh}b_{555}$). Also synthesised are the cytochromes of the "low aeration" pathway i.e. b_{558}, a_1, and d (Fig. 2c) (Reid and Ingledew,

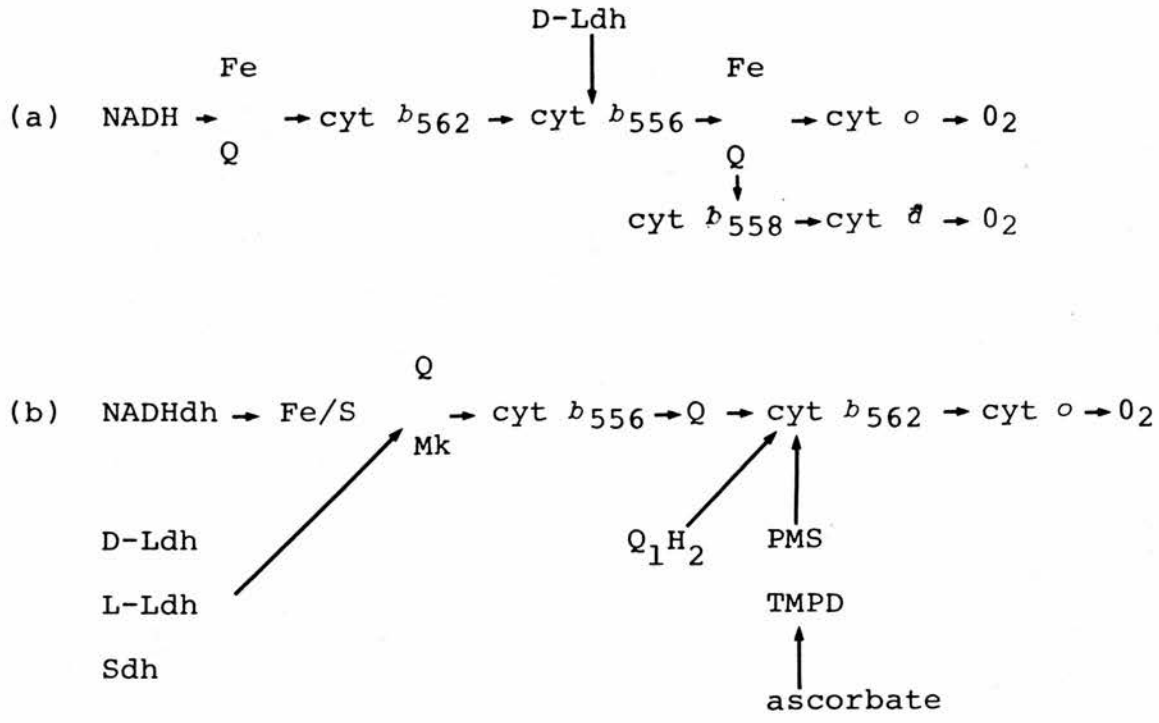
FIGURE 3

Sequence of Cytochromes in the Aerobic Respiratory Chain of *E. coli*

(a) Sequence proposed by Downie and Cox (1978).

(b) Sequence proposed by Kita and Anraku (1981).

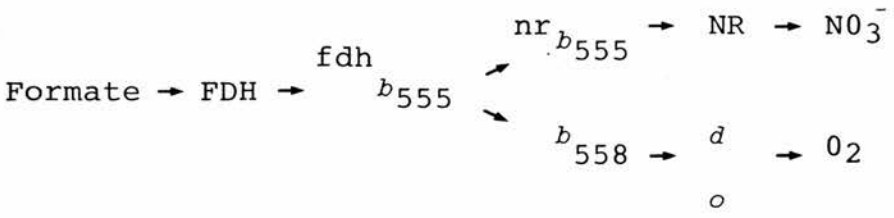
Abbreviations: cyt, cytochrome; Q, ubiquinone; MK, menaquinone; Ldh, Lactate dehydrogenase; Sdh, succinate dehydrogenase; PMS, phenazine methosulphate; TMPD, tetramethyl-p-phenylenediamine.


(a) NADH → Fe → Q → cyt b_{562} → cyt b_{556} → Fe → Q → cyt o → O_2
D-Ldh
cyt b_{558} → cyt d → O_2
(b) NADHdh → Fe/S → Q → Mk → cyt b_{556} → Q → cyt b_{562} → cyt o → O_2
D-Ldh
L-Ldh
Sdh
Q_1H_2
PMS
TMPD
ascorbate

1979; Haddock *et al.*, 1976; Crispin *et al.*, 1979). However, Hackett and Bragg (1983a,b) found only the cytochrome *o* terminated pathway in most of their strains, and Crispin *et al.*, (1979) reported a cytochrome *o* with a high affinity for 0_2 induced during nitrate respiration. A cytochrome *c* was identified by van Wielink *et al.*, (1983).

Cytochrome *d*, produced by cells grown anaerobically with nitrate, has an absorption maximum which is shifted 10nm to the red end of the spectrum (Fig. 2b). A study of the reactions of cytochrome *d* with nitrogen compounds (Hubbard *et al.*, 1983) proposes that this is due to the formation of a nitrosyl haem complex.

A suggested organisation of the above cytochromes of the "nitrate pathway" by Crispin *et al.* (1979) is shown below.



NR: Nitrate Reductase

Fumarate Respiration

When cells are grown anaerobically with a non-fermentable carbon source such as glycerol, fumarate can act as the terminal electron acceptor. There is considerable agreement as to the cytochrome composition of the electron transport chain among the various investigations (Haddock *et al.*, 1976;

Reid and Ingledew, 1979; Scott and Poole, 1982). They are in unanimous agreement that the major oxidase is cytochrome *d*, with cytochromes b_{555}, b_{558} and a_1 also being synthesised (Fig. 2c). This is similar to the "low aeration" branch of the aerobic respiratory chain. Haddock *et al.*, (1976) reported a small amount of cytochrome *o* present while neither Reid and Ingedew (1979) nor Scott and Poole (1982) found any. Scott and Poole (1982) reported the presence of a CO-binding *c*-type cytochrome absorbing at 550.5nm.

There is some dispute over the number of *c*-type cytochromes present in these cells. Haddock *et al.* (1976) found one, absorbing at 550nm while Scott and Poole (1982), by use of fourth order finite difference analysis, identified two cytochrome *c*s with absorption maxima at 548 and 550.5nm. Reid and Ingledew (1979) did not report the presence of any cytochrome *c*s. However, according to Fujita (1966) these *c*-type cytochromes are soluble and and not required for the membrane-bound electron transport process. Therefore, it is likely that the cytochrome *c*s were lost during the preparation of the membrane fragments used by Reid and Ingledew (1979).

Cytochrome *d*

The earliest report of cytochrome *d* in *E. coli* was by Yaoi and Tamiya (1928), who found that aerobically grown *E. coli* possessed an absorption band which was different

from previously described cytochromes in having an absorption maximum in the red region of the spectrum, at 630nm. Keilin (1933) designated this component cytochrome a_2, a name which remained until Barrett (1956) showed that the prosthetic group was an iron chlorin and the cytochrome was reclassified as a *d*-type. Several groups, working independently between 1933-34, revealed that the then cytochrome a_2 was autoxidisable and combined with both CO and cyanide (for references see Lemberg and Barrett, 1973).

More recently, work by Pudek and Bragg (1974, 1975), on the cyanide inhibition of oxidative phosphorylation in *E. coli*, disclosed that cytochrome *d*s' function is exceptionally cyanide resistant. Cyanide reacts with the reduced form of cytochrome *d* causing loss of the α-band absorbance. Oxygen had to be present before this reaction with cyanide could take place and the rate of disappearance of the α-band was directly proportional to the rate of electron flux through the system. These findings led Pudek and Bragg to propose that cyanide reacted with an intermediate between the oxidised (648nm) species and the reduced (628nm) species, and "trapped" it. This intermediate, "*d**", had little absorbance in the α-band region and was postulated to be analagous to the "d_x" form of cytochrome *d* from *Azotobacter vinelandii* (Kauffman and van Gelder, 1973). Later work by Poole and colleagues (Poole *et al.*, 1983a,b), using low temperature trapping and ligand exchange procedures, has led to the hypothesis that the "invisible" intermediate,

*d**, proposed by Pudek and Bragg is, in fact, the fully oxidised form of cytochrome *d* and that the form which absorbs at 648nm is an early intermediate in the reaction with oxygen. A more detailed discussion of ligand binding by cytochrome *d* is presented in Chapter 3.

The synthesis of cytochrome *d* is subject to the influence of several factors including: low oxygen tension; attainment of the stationary phase of growth on non-fermentable carbon sources; aerobic growth with glucose; anaerobic growth; growth in the presence of 0.15mM cyanide; growth under sulphate-limited conditions and growth in opposing gradients of glucose and of oxygen plus nitrate. Certain mutations in respiratory metabolism also affect cytochrome *d* production (for references see Poole, 1983).

The aims of the work described in this thesis were:-

1. To purify cytochrome *bd* from *E. coli* to a degree that would enable its properties to be investigated by spectrophotometric and electron spin resonance techniques without interference from other cytochrome components.

2. To purify cytochrome *bd* to homogeneity, to examine its structure by protein sequencing and its location and orientation in the membrane by labelling techniques.

3. To investigate and compare the electrochemical properties of cytochrome *bd* in the membrane and in solution.

It was hoped that a study of the structure and mode of action of this cytochrome would advance our understanding of oxidases in general.

A partial purification of the cytochrome *d*-containing complex of *E. coli*, grown anaerobically by fumarate respiration, had been performed previously by Reid and Ingledew (1980). During the course of this work successful purifications to homogeneity of cytochrome *d*-containing complexes from aerobically grown *E. coli* were reported by Miller and Gennis (1983) and Kita *et al.* (1984b).

CHAPTER TWO

PURIFICATION AND CHARACTERISATION OF CYTOCHROME *bd*

INTRODUCTION

Given the complex, branched nature of the electron transport chain of *E. coli* and that many of the cytochromes present have overlapping α-bands, it is desirable to fractionate the chain into individual components if a thorough study of their properties is to be made.

The ability of *E. coli* to modify the composition of its respiratory chain according to growth conditions (Fig. 2) can be used to stimulate production of the component of interest and suppress others. In this study cells were grown anaerobically with glycerol as a non-fermentable carbon source and fumarate as the terminal electron acceptor. This results in synthesis of the cytochrome *d* terminated branch and repression of the cytochrome *o* terminated branch of the respiratory chain (Reid and Ingledew, 1979; Scott and Poole, 1982).

Recently, great progress has been made in isolating the various constituents of the *E. coli* respiratory chain by combining the control of growth conditions with strain selection to give maximum synthesis of the required component, e.g. cells containing the F152 episome, first described by Shipp (Shipp *el al.*, 1972), have an increased capacity for cytochrome synthesis and have been used in several purifications (see below).

b-Cytochromes (excluding cytochrome *o*)

The first published purification of a *b*-cytochrome from *E. coli* was that of Deeb and Hager (1964). They succeeded in producing crystals of cytochrome b_1 (absorbing at 558nm at 20°C) from *E. coli* strain B which had been grown aerobically on a glucose/glutamate medium. Sonication was used to extract the loosely bound cytochrome, this was followed by ammonium sulphate fractionation and chromatography on calcium phosphate columns. The crystallised cytochrome had a molecular weight of 62 000.

Using aerobically grown *E. coli* strain B and extraction with acetone/phosphate buffer followed by calcium phosphate and DEAE-cellulose chromatography, crystals of an additional cytochrome *b* (cytochrome b_{562}) have been obtained (Itagaki and Hager, 1966). This small molecule comprised 110 amino acids and had a molecular weight of 12 000.

A membrane-bound cytochrome b_{556} has been purified from *E. coli* K12 strain MR43L/F_2-*gal*. These cells contain the F152 episome and were grown aerobically on lactate medium (Kita *et al.*, 1978). A cytoplasmic membrane preparation was washed in 2% cholate and the pellet solubilised in Sarkosyl prior to chromatography on Sephadex G-200. The cytochrome b_{556} was found to be a small polypeptide with a molecular weight of approximately 17 500 which tended to form an aggregate of molecular weight 70 000.

Cytochrome *o*

A cytochrome b_{562}-*o* complex-enriched fraction was obtained from a wild type *E. coli* K12 strain MR43L by a purification protocol essentially similar to the one described above for cytochrome b_{556} (Kita *et al*, 1981). When incorporated into liposomes a membrane potential of -145mV was generated on addition of ubiquinol or phenazinemethosulphate ascorbate. This group have further purified the cytochrome *o* complex from *E. coli* K12 strain KL251/ORF4 (a strain which contains twice the cytochrome *o* of wild-type MR43) (Kita *et al*., 1984a). Cytoplasmic membranes were solubilised with Triton X-100 and the extracted cytochrome subjected to DEAE-Sepharose 6B ion-exchange chromatography. The peak fractions were concentrated by ammonium sulphate precipitation and the detergent exchanged for Sarkosyl before chromatography on Sephacryl S-200. The subsequent peak fractions were finally chromatographed on a Bio-Gel HT hydroxylapatite column. This cytochrome *o* complex showed 2 α-absorption peaks, at 555nm and 562nm, with the 555nm component combining with CO. Both components had an Em (7.4) of + 125mV which was pH dependent (-60mV/pH unit) between pH6 to 7.4. The complex was made up of equimolar amounts of two polypeptides of molecular weights 55 000 and 33 000 and contained a significant amount of copper (16.8nmol/mg of protein compared with 19.5nmol of haem per mg of protein).

Immunological characterisation of the cytochrome *o* complex from *E. coli* K12 strain GR19N, which appears

deficient in the low aeration branch of the electron transport chain (Kranz and Gennis, 1983), revealed that the subunit composition of the cytochrome complex comprised four polypeptides of approximately equal stoichiometry and apparent molecular weights of 51 000, 28 000, 18 000 and 12 700. Two absorption peaks at 555nm and 562nm were evident. A subsequent potentiometric analysis of the complex *in situ* (Lorence *et al.*, 1984) found an Em of + 165mV for both the 555nm and 562nm components and that both bound CO.

Further work on this complex by Matsushita *et al.* (1984) achieved a partial purification by first washing the membrane particles with 5M urea, followed by 6% cholate, then solubilisation with 1.25% octyl glucoside and subsequent chromatography on DEAE-Sepharose CL-6B. Subunits of molecular weights 66 000 (varying with acrylamide concentration), 35 000, 22 000, and 17 000 were identified and it was revealed that the complex contained two *b*-cytochromes with absorption peaks at 558nm and 563nm (at 20°C). When the complex was incorporated into liposomes, a membrane potential of 80mV could be generated by oxidation of ubiquinol. On incorporation into proteoliposomes containing the *lac* carrier protein, membrane potential-dependent active transport of lactate was demonstrated with ubiquinol as the electron donor.

Cytochrome a_1

A soluble haemoprotein, with spectral characteristics similar to cytochrome a_1, has recently been purified from *E. coli* K12 strain A1002, grown anaerobically on a glycerol/fumarate medium (Baines *et al.*, 1984). By a combination of differential centrifugation, ammonium sulphate fractionation and DEAE-Sephadex A-50 chromatography, a cytochrome a_1 enriched fraction, showing high catalase and peroxidase activity, was obtained. The only haem detectable was protohaem. This finding, combined with the high catalase and cytochrome *c* peroxidase activities, led the authors to suggest that the cytochrome was a high spin *b*-type cytochrome, possibily a hydroperoxidase. Further support for this theory comes from Koland *et al.* (1984). Despite strenuous attempts they were unable to extract haem *a* from a purified cytochrome *d* complex, obtaining only haems *b* (in an unexpectedly large amount) and *d*. In addition, spectral deconvolution of the reduced minus oxidised spectrum of the purified complex showed the spectrum of cytochrome a_1 to be very similar to that of cytochrome *c* peroxidase.

Cytochrome *d*

There have been three reported purifications of cytochrome *d* complexes from *E. coli*.

The earliest (Reid and Ingledew, 1980) was a partial purification from *E. coli* K12 strain EMG2 (prototroph)

grown anaerobically on a glycerol/fumarate medium. A purification protocol of DEAE-cellulose and gel filtration chromatography gave a cytochrome *d*-containing complex with a proposed cytochrome composition of cytochromes b_{555}, b_{558} and *d*.

Miller and Gennis (1983) have subsequently purified to homogeneity a cytochrome *d* complex from cells containing the F152 episome (*E. coli* K12 strain MR43L/F152) which were grown under low aeration with lactate. A procedure involving solubilisation with a zwitterionic detergent followed by two chromatographic steps on DEAE-Sepharose CL-6B and hydroxylapatite respectively resulted in a complex containing cytochromes b_{558}, a_1 and *d*. This complex was made up of two subunits of molecular weights 57 000 and 43 000. The apparent molecular weight of the smaller subunit was dependent upon acrylamide concentration.

More recently, Kita *et al.* (1984b) have isolated a complex containing cytochromes b_{558}, a_1 and *d* from a wild-type *E. coli* K12 strain MR43L using growth conditions similar to those employed by Miller and Gennis. The purification procedure involved solubilisation of inner membranes with Triton X-100 and chromatography of the extract on DEAE-Sephacel, followed by rechromatography under the same conditions. The eluted cytochrome *d* was applied to a small DEAE-Sephacel column and the detergent exchanged by flushing with Sarkosyl-containing buffer. The cytochrome was displaced by a pulse of buffer containing a high salt concentration, concentrated

by ultrafiltration, and applied to a column of Sephacryl S-300 equilibrated with Sarkosyl for chromatography and rechromatography. The purified complex was made up of two subunits having molecular weights of 51 000 and 26 000.

The results presented in this chapter describe the purification of a cytochrome *d*-containing complex from a wild type *E. coli* strain EMG2 (prototroph) grown anaerobically by fumarate respiration on glycerol.

MATERIALS

The following were obtained from the Sigma Chemical Company: BSA (Fraction V); Coomassie Brilliant Blue G250; Cooomassie Brilliant Blue R250; Deoxyribonuclease II (Type V); L-Histidine; Myoglobin (Type II); Nonidet P-40; Ovalbumin (Chicken egg, Grade V); Phosphorylase b (Rabbit muscle); D-Sorbitol; Soybean Trypsin Inhibitor (Type 1-S); TES; Triton X-100; Trypsinogen (PMSF Treated).

Acrylamide, β-mercaptoethanol, TEMED and N,N' methylene-bis-acrylamide (all electrophoresis grade) were obtained from Bio Rad Laboratories Ltd.

Deoxycholic acid (Gold Label) and 3,3',5,5'-tetramethyl-benzidine (Gold Label) were obtained from the Aldrich Chemical Co.

Casamino acids were obtained from Difco.

Gelbond was obtained from Miles Scientific Ltd.

CM 52 ion exchange cellulose was obtained from Whatman Ltd.

Agarose IEF, DEAE-Sephacel, Polybuffer 74, Polybuffer Exchanger (PBE) 94, Sephacryl S-300, Sephadex G-25, Sephadex G-75, Low pI and High pI calibration kits were obtained from Pharmacia (U.K.) Ltd.

All other chemicals were of the highest grade available from the usual commercial sources.

METHODS

SECTION 1: Assay Procedures

Protein Concentration

Protein concentration was routinely determined by the method of Lowry *et al.* (1953) with the addition of SDS to make the final solution 0.5% (w/v) with respect to SDS. The tubes were incubated at 37°C during both the hydrolysis and colour development stages. A typical standard curve obtained using Bovine Serum Albumin as a standard is shown in Fig. 4.

During and after chromatofocusing, a Coomassie Blue based assay (Bradford, 1976) was used, with Bovine Serum Albumin as a standard.

In order to minimise the interference with the assay due to Triton X-100 the following procedure was adopted. 0, 10, 20, 30, 40 and 50µl volumes of a 2mg/ml BSA solution were added, in duplicate, to tubes containing 3ml of Bradford reagent. Appropriate quantities of water were added to bring the total volume of additions to each tube to 50µl. The tubes were mixed on a vortex mixer and the same volume of Polybuffer 74 solution as contained in the sample, added to each tube and the whole vortexed once more. The absorbance at 595nm, of the standard protein solutions and the unknown samples, were read against the reagent blank to which no BSA had been added.

FIGURE 4

A Standard Curve Obtained Using the Lowry Protein Assay

This figure shows a typical standard curve obtained by the Lowry/SDS procedure described in Methods Section 1. The protein standard used was BSA.

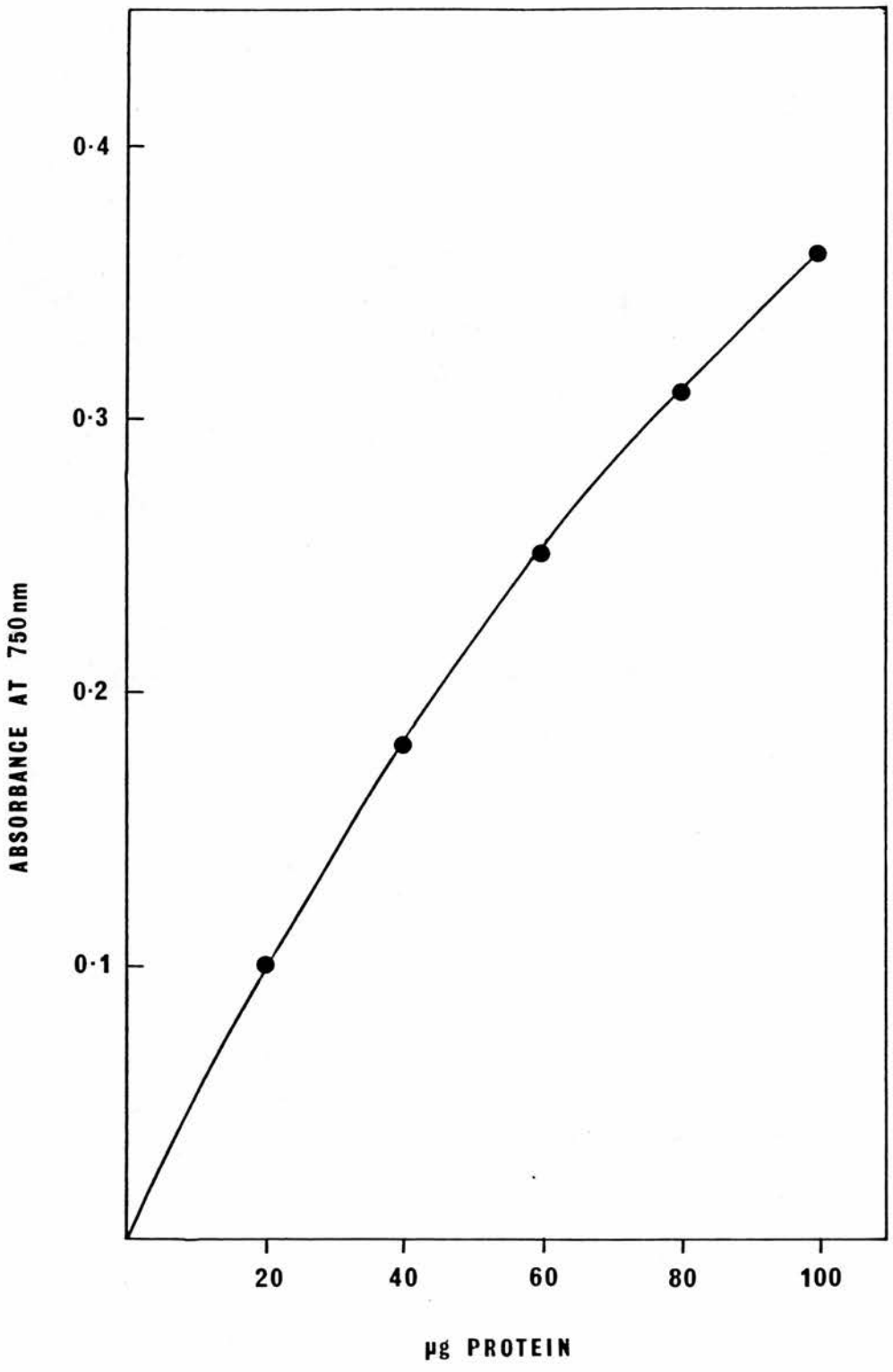
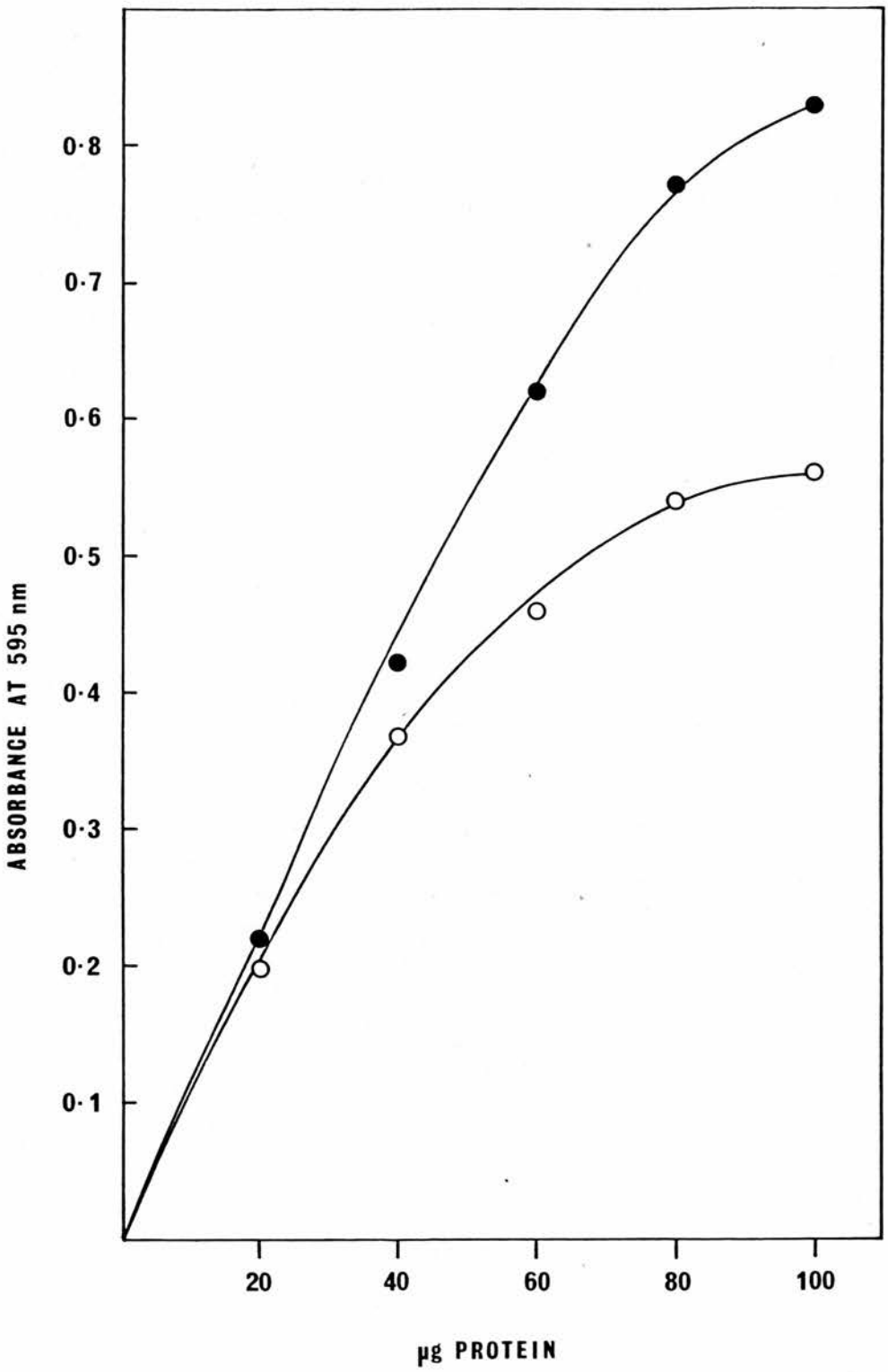

0·4
0·3
0·2
0·1
ABSORBANCE AT 750nm
20
40
60
80
100
µg PROTEIN

FIGURE 5

Standard Curves Obtained Using the Bradford Protein Assay

These curves were obtained with 50µl (●——●) and 100µl (o——o) of Polybuffer 74 solution, containing 0.5% (v/v) Triton X-100, added to each tube in addition to the BSA solution.


0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
ABSORBANCE AT 595 nm
20
40
60
80
100
µg PROTEIN

For protein estimations involving samples of two different volumes, two different standard curves, each containing the appropriate amount of Polybuffer 74, were constructed. Typical standard curves obtained using tubes containing 50µl and 100µl of Polybuffer 74 are shown in Fig. 5.

Cytochromes

The concentrations of cytochromes *b* and *d* were calculated from difference spectra obtained using a dual wavelength spectrophotometer (described in greater detail in the Methods section of Chapter 3). A millimolar extinction coefficient of 8500 for the wavelength pair of 615-630nm (Jones, 1977) was used for cytochrome *d* and a millimolar extinction coefficient of 10 800 for the wavelength pair of 562-580nm (Miller and Gennis, 1983) was used for cytochrome *d*.

Ionic Strength

Ionic Strength was monitored by measuring the conductivity of the solution and comparing the value obtained with a standard plot of known KCl concentrations in the same buffer.

SECTION 2: Purification of Cytochrome *bd*

Organism, Growth Conditions and Preparation of Cells.

Escherichia coli strain EMG2 (prototroph) was grown anaerobically at 37°C in 20l batches on a mineral salts medium (Cohen and Rickenberg, 1956) supplemented with 0.1%

(w/v) vitamin free Casmino acids. The main carbon source was glycerol (1% v/v) and potassium fumarate (100mM) was the terminal electron acceptor.

Cells were harvested in late exponential phase using an MSE continuous flow rotor running in an MSE 18 centrifuge at 18 000rpm and a flow rate of approximately 200ml/min. The cells were washed by resuspension in 50mM KPO_4 buffer, pH7.2 and collected by centrifugation at 10 000g for 15 min at 4°C. The cell paste was frozen in liquid nitrogen and stored at -30°C until required.

Cell Breakage and the Preparation of Electron Transport Particles (ETPs)

Frozen cell paste was rapidly resuspended in 100mM KPO_4/5mM EDTA, pH7.2 (2ml/g wet weight) by using a Kenwood electric blender. A small quantity of deoxyribonuclease II and 0.5ml of 1M $MgCl_2$ were added to decrease the viscosity of the suspension. The cell suspension was passed through a French pressure cell (Aminco 4-3398A, 40ml capacity) at approximately 120MPa. Unbroken cells and debris were removed by centrifugation at 10 000g for 15 min and the decanted supernatant centrifuged at 100 000g for 1h to pellet the ETP fraction. The pellet was resuspended in the KPO_4/EDTA buffer used above and recentrifuged at 100 000g for 1h. The supernatant was discarded and the pellet frozen as small particles in liquid nitrogen. These were stored at 77K until required.

Solubilisation of the Cytochrome

Thirty grammes of ETPs (1g wet weight/6ml) were suspended in 110mM KPO_4/50mM EDTA/0.75% (v/v) Triton X-100, pH7.0 buffer using a Potter homogeniser, decanted into a beaker and stirred, on ice, with a magnetic stirring bar, for 1h. The particulate membrane fraction was separated by centrifugation at 100 000g for 1h, resuspended and stirred on ice for 1h as above, except that the concentration of Triton X-100 was increased to 6% (v/v). The suspension was centrifuged at 100 000g for 1h and the supernatant used immediately, as experience showed that freezing the preparation decreased the resolution obtained from the next step.

Ion Exchange Chromatography

The solubilised cytochrome fraction was desalted by passage down a Sephadex G-25 column (25 x 4.6cm) equilibrated with 20mM KPO_4/5mM EDTA/0.2% (v/v) Triton X-100, pH6.0 (DEAE buffer) before applying to a DEAE-Sephacel column (9 x 4.6cm) equilibrated with the same buffer. The column was washed with approximately two bed volumes of DEAE buffer and the cytochrome eluted using a 0-300mM KCl gradient in 600ml of DEAE buffer. The flow rate was 50ml/h and fractions of 6ml volume were collected. Every second fraction was assayed for protein, ionic strength and A_{410}.

The peak fractions were pooled and desalted by passage down a Sephadex G-25 column (25 x 4.6cm) equilibrated

with DEAE buffer, before applying to a smaller DEAE-Sephacel column (9.5 x 2.6cm) with the aim of concentrating the sample. The latter column was washed with 50ml of DEAE buffer and the cytochrome was batch eluted with 50mM KPO_4/5mM EDTA/0.5M NaCl/0.2% (v/v) Triton X-100, pH7.0 (Sephacryl buffer).

The eluted cytochrome was further concentrated to approximately 9ml by ultrafiltration, at 0°C, in an Amicon concentration cell fitted with an XM50 membrane.

Gel Filtration

The concentrated sample, obtained in the previous section, was immediately applied to a Sephacryl S-300 column (120.5 x 2.6cm) pre-equilibrated with the previously described Sephacryl buffer and eluted with the same buffer at a flow rate of 15ml/h. Fractions of 4ml size were collected and every second fraction was assayed for protein and A_{410}.

The peak fractions were pooled and passed down a Sephadex G-25 column (25 x 4.6cm) pre-equilibrated with 25mM L-Histidine/0.5% (v/v) Triton X-100 (adjusted to pH6.0 with HCl) before immediately subjecting to chromatofocusing.

Chromatofocusing

The sample was applied to a column of Pharmacia Polybuffer Exchanger 94 (25 x 2cm) equilibrated with the 25mM L-Histidine/0.5% (v/v) Triton X-100 pH6.0 buffer. Bound

cytochrome was eluted with 75ml of Polybuffer 74 to which water, 0.1M HCl and Triton X100 had been added to produce 575ml of 0.5% (v/v) Triton X-100 Polybuffer 74 solution pH4.0. The flow rate was 50ml/h and fractions of 4ml size were collected. Every second fraction was assayed for protein and A_{410} and pH were measured.

The peak fractions were pooled and concentrated tenfold by ultrafiltration at 0°C in an Amicon concentration cell fitted with an XM50 membrane. The concentrate was subsequently rediluted to its original volume with 50mM TES/5mM EDTA, pH7.0 and reconcentrated tenfold. This process was carried out twice more prior to freezing the concentrated cytochrome and storing at 77K until required.

SECTION 3: Analytical Procedures

Non-Denaturing Polyacrylamide Gel Electrophoresis

This was performed using the Tris/Sodium acetate buffer system of Fairbanks *et al.* (1971) except that the gels and buffer contained 0.1% (v/v) Triton X-100 in place of SDS and the acrylamide concentration was increased to 7% (w/v).

Gels were cast in glass tubes (10 x 0.5cm) sealed at the bottom with parafilm and overlayed with water to achieve a flat surface. No stacking gel was used.

A small quantity (approximately 10% (v/v)) of glycerol was added to each sample and enough bromophenol blue to give a clearly visible dye front was also added. Sample (up to 50µl total volume) was applied under the running buffer.

Electrophoresis Conditions

Electrophoresis was carried out at 4°C in the dark using a Shandon electrophoresis tank and a Vokam SAE 2716 power supply.

A constant current of 1mA per gel was passed until the sample had fully entered the gel. Once this was achieved the current was increased to 4mA per gel. When the tracking dye had reached approximately 1cm from the bottom of the gel the current was switched off and the gel rimmed out of the tube by squirting water, from a hypodermic needle, between the tube wall and the gel. The position of the dye front was marked by piercing the gel with a needle dipped in Indian ink.

Haem Stain

Haem was stained using the TMBZ/H_2O_2 method of Thomas *et al.* (1976). This produced a transient light blue stain against a clear background.

Iron Stain

Iron was stained by the method published in Disc Electrophoresis by W. Maurer (1971). This is essentially the same as the haem stain except EDTA is incorporated

into the staining solution. A transient pale blue colour developed against a clear background.

TMPD Oxidase Activity

Oxidase activity was visualised by immersing the gel, immediately after electrophoresis, in a 0.1% (w/v) solution of TMPD in 50mM TES pH7.0. A pale purple colour developed, initially at the site of the oxidase activity, but eventually over the entire gel.

Protein Stain

Protein was stained by immersing the gel in a 0.1% (w/v) solution of Coomassie Brilliant Blue R in ethanol: water: acetic acid (5:5:1) for 1h and the gel destained in a solution of water: methanol: acetic acid (27:10:3) until a clear background was obtained.

SDS Polyacrylamide Gel Electrophoresis

Electrophoresis in the presence of SDS was performed using the discontinuous buffer system of Laemmli (1970) in rod gels of various acrylamide concentrations in 0.375M Tris/HCl buffer pH8.8. The acrylamide solution for the separating gel was degassed before use. The gels were cast as described previously in Methods section 3, except that after the gels had set the water overlay was removed with a tissue paper wick and 0.1ml of stacking gel (0.125M Tris/HCl, 3% (w/v) acrylamide, pH6.8) cast on top of the separating phase. This was overlayed

with water as before and the overlay removed before use.

Preparation of Molecular Weight Markers

Phosphorylase *b* (molecular weight 94 000), BSA (molecular weight 67 000), ovalbumin (molecular weight 45 000), trypsinogen (molecular weight 24 000) and myoglobin (molecular weight 17 000) were prepared for use as molecular weight marker proteins by dissolving 5mg of each protein in 2.5ml of electrode buffer (0.384M glycine/0.05M Tris/0.1% SDS, pH8.3) to which had been added ß-mercaptoethanol and additional SDS to give final concentrations of 1% (v/v) and 2% (w/v) respectively. The protein solution was then heated to 100°C, in a test-tube immersed in a boiling water bath, for 5 min, cooled and divided into 0.1ml aliquots for storage at -20°C. When required, an aliquot was thawed and bromophenol blue added just before use. 10µg of each standard protein was applied to the gel under the running buffer.

Preparation of Samples of Cytochrome *bd*

Samples of cytochrome were prepared for electrophoresis by making the solution 5% (w/v) with respect to SDS, 1% (v/v) with respect to ß-mercaptoethanol and incubating at 37°C for 30 min. Boiling of the sample was avoided. Samples were applied to the gel under the running buffer.

Electrophoresis Conditions

Electrophoresis was carried out in a Shandon electrophoresis tank with a Vokam 2716 power supply. A constant current of 0.5mA/gel was passed until the sample had completely entered the stacking gel when this was increased to 1mA/gel. Once the sample had fully entered the gel the current was increased to 3mA/gel and maintained at this until electrophoresis was terminated when the dye front was approximately 1cm from the bottom of the gel. Gel removal and marking of the dye front were performed as described previously in Methods section 3.

Protein Staining was Carried out as previously described in Methods section 3.

Calculation of Molecular Weights from SDS PAGE

After protein staining and destaining, the gels were scanned using a Fison's Vitatron scanning densitometer. The position on the scanner trace of the start of the separating gel and of the dye front were noted and the distance between them measured. This measurement was used to calculate the relative mobility of the protein bands by the formula:

$$R_f = \frac{\text{distance travelled by band}}{\text{distance from start of gel to dye front}}$$

A standard curve of $\log_{10}$ molecular weight versus R_f value, for the proteins of known molecular weight, was plotted and employed to estimate the molecular weight of unknown protein bands from their experimentally obtained R_f values (Fig. 18).

Isoelectric Focusing

Preparation of the Gel

Agarose gels (112 x 140mm) were cast as follows: 0.2g Agarose IEF, 2.4g sorbitol and 0.4ml Nonidet P40 were suspended in 18ml of H_2O in a boiling tube and the whole suspended in a boiling water bath until the agarose had dissolved.

While the agarose was dissolving a strip of Gelbond (125 x 155mm) was applied, hydrophilic side uppermost, to a leveling table upon which a drop of water had been placed to hold the Gelbond flat. A gel casting frame was placed upon the Gelbond and held in place with spring clips. The whole apparatus was warmed thoroughly with a hairdryer.

Once the dissolved agarose had cooled to approximately 65°C, 1.2ml of the appropriate ampholyte solution was added (LKB Ampholine pH3.5-10 or pH4-6). The tube was gently mixed and the gel poured into the gel casting frame. Care was taken to avoid the formation of bubbles. After the gel had set, a scalpel was run around the inside of the gel casting frame to loosen the gel and the frame removed. The gel was them maintained at 4°C for at least 1h in a plastic box containing damp paper towels and equipped with a tight-fitting lid.

Focusing Conditions

An LKB Multiphor 2117 electrophoresis apparatus and an LKB 2103 power source were used to perform the focusing. A few drops of 1% (v/v) Triton X-100 solution were placed on the cooling plate before applying the gel. Any excess liquid around the edge of the gel was carefully removed with a tissue paper wick. Electrode strips for use with agarose gels (Pharmacia Code No 19-3664-01) were soaked in the appropriate solutions (Cathode: 1M NaOH, Anode: 0.05M H_2SO_4) and blotted for 1 min on filter paper before placing on the gel.

Samples (20-40µg) were applied on pieces of Whatman No. 1 filter paper placed directly onto the gel, usually equidistant between the two electrodes. 25µl of the appropriate set of Pharmacia pI calibration proteins were applied at intervals along the gel unless a surface pH electrode (Russell pH, Auchtermuchty) was to be used to measure the gradient in which case they were omitted. In all cases 20µg of Soybean Trypsin Inhibitor (pI 4.55) was applied on at least two lanes to act as an internal standard.

A constant current of 10mA was used and after 700Vh the applicator strips were removed. A further 1000Vh were applied before focusing was complete.

Calculation of pH

The distance from the cathode of each of the pI standard proteins was measured and the known pI value of the protein plotted against the distance from the cathode. This formed the pH calibration curve (Fig. 6). The distance from the cathode to the component of interest was measured and the pI read from the pH calibration curve.

A similar pH calibration curve was employed in pI estimations when a surface pH electrode was used to measure the gradient. In this case the pH of the gel, at set distances from the cathode, was measured directly, using the electrode, and a pH versus distance curve drawn.

Gel Fixation and Drying

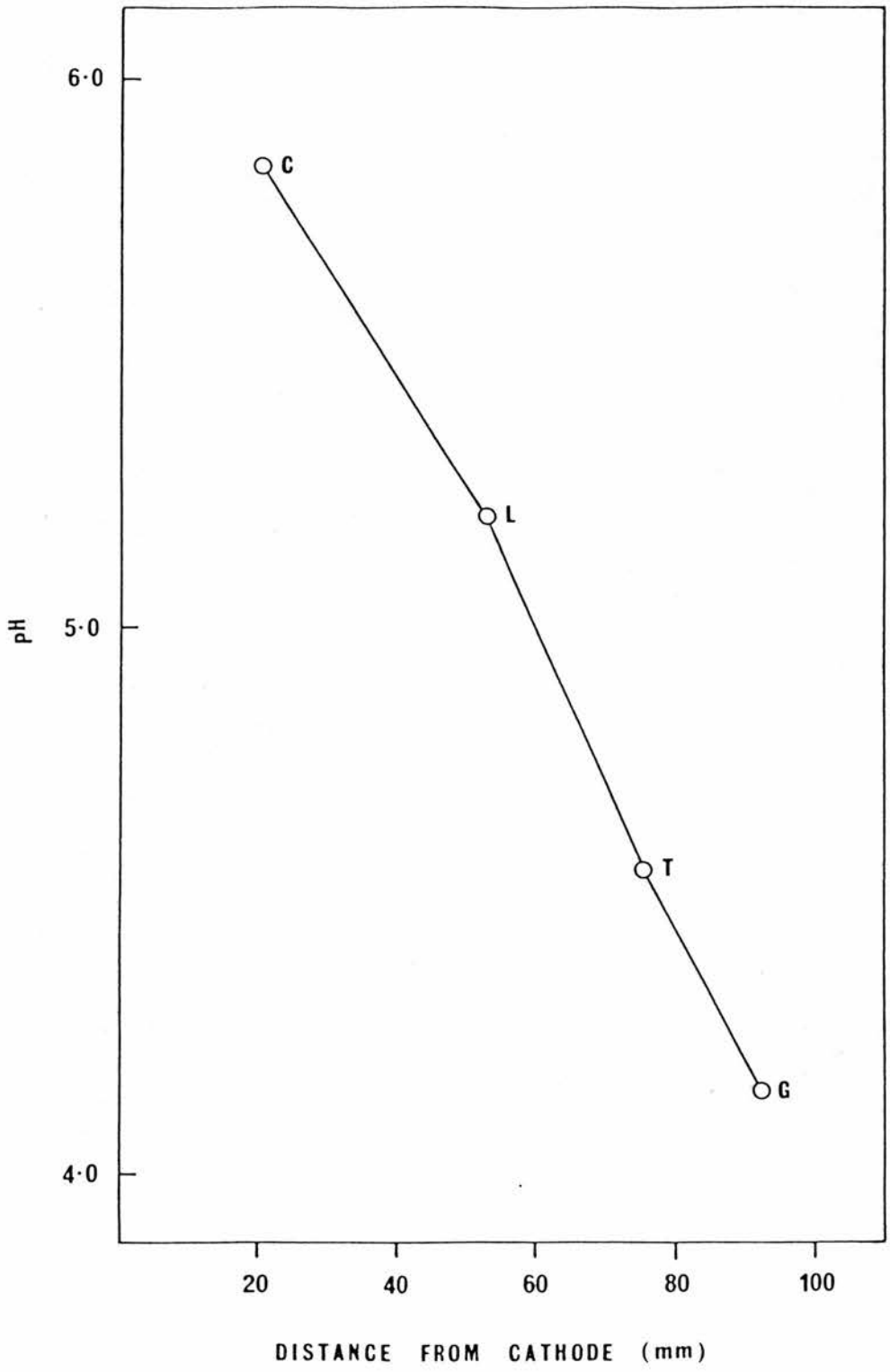
The gel was fixed by immersion in 5% (w/v) 5-Sulphosalicylic acid, 10% (w/v) Trichloroacetate for 15 min. The white opaque gel was cleared by immersion in water: acetic acid: methanol (87.5:7.5:5). After 15 min the solution was discarded and fresh solution added.

After clearing (approximately 30 min in total) the gel was dried by placing three layers of filter paper, followed by paper towels, a glass plate and a heavy object respectively on top of the gel and leaving for 15 min. Residual moisture was removed using a hair dryer.

FIGURE 6

A pH Gradient Profile Curve for an Agarose IEF Gel, pH4-6

Of the seven proteins applied, six are visible on gels of pH4-6. Pepsinogen and amyloglucosidase (Aspergillus), with pIs' of 2.8 and 3.5 respectively, migrate to the anodic end of the gradient and appear as one band. Similarly, human carbonic anhydrase B (pI 6.55) migrates to the cathodic end of the gradient leaving four proteins of calibrative use.


6·0
5·0
4·0
pH
C
L
T
G
20
40
60
80
100
DISTANCE FROM CATHODE (mm)

Protein Stain

Protein was stained by immersing the fixed and dried gel in a solution comprising 0.2% Coomassie Blue R/45% (v/v) methanol/10% (v/v) acetic acid for 10 min. The gel was quickly rinsed in distilled water and destained in the same solution as above, but minus Coomassie Blue R, until the background was clear. The gel was finally dried with a hair dryer.

Haem Stain

The focused gel was fixed as above but not dried. After fixing and clearing it was transferred to approximately 0.2M sodium acetate which had been adjusted to pH5.0 with acetic acid, for 15 min before staining for haem as described by Thomas *et al.* (1976).

If required, protein could be subsequently visualised by drying the gel and staining with Coomassie Blue R as above.

TMPD Oxidase Activity

This was visualised by immersing the focused gel immediately in 50mM TES/5mM EDTA/0.1% (w/v) TMPD, pH7.0. Oxidase activity was detected as a purple band, (the oxidation product of TMPD) against a clear background. If left, colour development continued until the entire gel was purple, therefore, the progress of the stain had to be carefully monitored.

Protein could be subsequently located by fixing, drying and staining the gel with Coomassie Blue R as described earlier.

Two Dimensional Electrophoresis

This used isoelectric focusing in the first dimension followed by SDS gel electrophoresis (SDS PAGE) in the second dimension.

First Dimension Isoelectric Focusing

The isoelectric focusing slab gel was prepared and focused as described previously in Methods section 3. After focusing, a section of the gel containing a set of standards and a sample lane was removed with scissors and stained for haem. The remainder of the gel was focused at 100V until required.

Preparation of the Sample for Second Dimension SDS PAGE

Once the location of the haem-positive band was revealed the remaining part of the gel was removed from the focusing chamber and laid alongside the stained section. The parts of the unstained gel corresponding to the haem positive band were excised from the Gelbond with a scalpel and transferred to a 1.5ml micro-centrifuge tube. An approximately equal volume of 50mM KPO_4 buffer containing 5mM EDTA/10% (w/v) SDS/2% (v/v) β-mercaptoethanol and a small quantity of bromophenol blue was added to the tube and the tube suspended in a 95°C water bath until the agarose had remelted. The solution was mixed vigorously and the tube transferred to an oven at 60°C for 30 min.

While the sample was incubating the remaining parts of the gel were stained for protein to ascertain whether the focusing and excision of bands had been successful.

A 10% (w/v) acrylamide slab gel (140 x 150 x 2mm) was cast with sample wells 1cm wide using the Laemmli buffer system described earlier (Methods section 3). A 3% (w/v) acrylamide stacking gel was used and BSA, ovalbumin and myoglobin (20µg each) employed as molecular weight marker proteins as previously described (Methods section 3).

Application of 100µl of the agarose/SDS sample mixture was carried out using an Oxford adjustable pipette, the tips for which had been pre-warmed to 60°C before use.

Electrophoresis Conditions

Electrophoresis was carried out at constant voltage, 50V until the sample had entered the separating gel, then the voltage was increased to 150V.

Once the dye had reached 1cm from the bottom of the gel the current was switched off and the front marked with Indian ink.

Silver Stain for Protein

The method used was essentially that of Wray *et al.* (1981), using ammoniacal silver nitrate to complex with protein amino groups, except that background stain was removed by destaining with Kodak Rapid Fix. The destaining solution was then rapidly removed by rinsing the gel in two changes of distilled water followed by a 20 min wash in 400ml of Kodak HE-1 hypo-eliminator to terminate destaining. The hypo-eliminator comprised water: 3% (w/v) ammonia: 3% (w/v) H_2O_2 (775:100:125).

The gel was subsequently rinsed in distilled water and transferred to 200ml of 50% (v/v) methanol for 20 min before wrapping in cling film and storing in an airtight box at 4°C until scanned.

Calculation of Molecular Weights

The protocol previously described (Methods section 3) was employed.

RESULTS

Purification of Cytochrome *bd*

Optimisation of Growth Conditions

Maximum production of cytochrome *d* was encouraged by growing *E. coli* anaerobically on a glycerol/fumarate medium supplemented with Casamino acids. This results in the synthesis of only the cytochrome *d* terminated branch of the electron transport chain (Reid and Ingledew, 1979; Scott and Poole, 1982). Harvesting the cells in the late exponential phase of growth gave a large yield of cells (approx 1.75g/l) containing a high specific content of cytochrome *d*. The cells were frozen in liquid nitrogen, immediately after washing, to minimise endoproteinase activity.

Preparation of Electron Transport Particles (ETPs)

The use of an electric blender to resuspend the frozen cell paste gave a smooth slurry thus promoting easy passage through the French pressure cell. The decrease in viscosity caused by the deoxyribonuclease II enabled a rapid and efficient centrifugal separation of unbroken cells and debris from the ETPs to be performed.

Resuspension of the pelleted ETPs in 100mM KPO_4/5mM EDTA, before recentrifugation and storage in liquid nitrogen,

TABLE 2

PREPARATION OF ETPs*

A typical ETP preparation is outlined below.

Stage	Total Content of cyt *b* (nmol)	Protein Content (mg)	Specific Content of cyt *b* (nmol haem/mg of protein)	Wet Weight (g)
Cells	8 780	16 460	0.53	100
ETP Supernatant	770	6 860	0.11	
Resuspended ETPs	7 580	14 040	0.54	
ETP Wash Supernatant	210	980	0.21	
2nd ETP Pellet	7 370	13 060	0.56	25.7

*Preparation and assays were carried out as described in Methods sections 1 and 2.

removed a quantity of contaminating protein (Table 2).

The loss of cytochromes b_{558} and *d*, was minimal (5%) during the preparation of the ETPs, therefore it was decided not to include any further proteinase inhibitors than the EDTA already present as a buffer constituent.

Solubilisation of the Cytochrome

The efficiency of various concentrations of the non-ionic detergent Triton X-100 in extracting cytochrome *bd* from the membrane was investigated (Fig. 7). The large discrepancy between the content of cytochrome *b* and cytochrome *d* in the 0.5 and 0.75% (v/v) Triton X-100 extracts was probably due to a high concentration of the membrane-associated cytochrome b_{556} solubilising before cytochrome *d* and its corresponding cytochrome b_{558}.

An extraction protocol involving a preliminary extraction of the ETPs with 110mM KPO_4/50mM EDTA, pH7.0 buffer (6ml/g wet weight) containing 0.75% (v/v) Triton X-100 was chosen as this enabled 75% of the Triton X-100-extractable protein to be removed, while only 6% of the cytochrome *d* was lost. This step also removed the majority of cytochrome b_{556} and *c*-type cytochromes from the ETPs (Figs. 8 and 9). This can be seen from the significant changes in relative signal height of the 558 and 561nm peaks (at 20°C) in the second derivative difference spectra (Fig. 9) and in the change of lineshape of the α-band around 560nm in the difference spectra (Fig. 8).

FIGURE 7

The Effect of Different Concentrations of Triton X-100 on the Extraction of Cytochromes and Protein from ETPs

ETPs (20g wet weight) were prepared as described in Methods section 2 and suspended in 110mM KPO_4/50mM EDTA, pH7.0 to a final volume of 60ml. This was divided into 5ml aliquots and the aliquots assayed for cytochrome content and protein concentration. Additions of buffer and buffer containing 25% (v/v) Triton X-100 were made to bring the final Triton X-100 concentration in the aliquot to be the equivalent of 6ml of buffer of 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2, 4, 6, 8 or 10% (v/v) Triton X-100 per g (wet weight) of ETPs. The final volume of each aliquot was 10ml.

The suspensions were homogenised, stirred on ice for 1h and centrifuged for 30 min at 40 000g and 4°C. The supernatants were decanted and assayed for cytochrome content and protein concentration.

*All yields are expressed as percentages of the total contained in the aliquot prior to detergent addition.

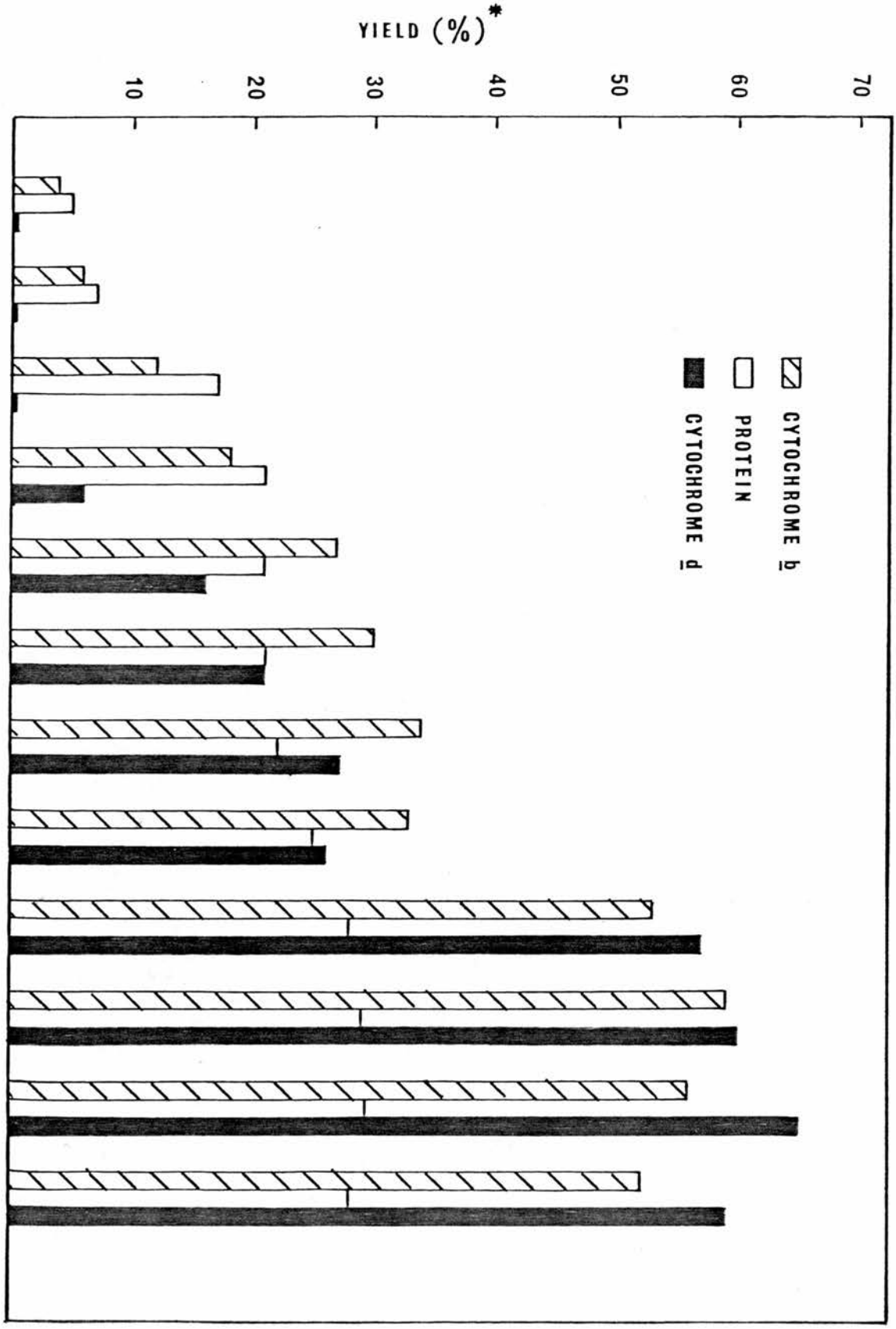

YIELD (%)*
10
20
30
40
50
60
70
CYTOCHROME b
PROTEIN
CYTOCHROME d
0
0·25
0·5
0·75
1
1·25
1·5
2
4
6
8
10
CONCENTRATION OF TRITON X-100 IN EXTRACTION BUFFER (% V/V)

FIGURE 8

Cytochrome Extraction from ETPs with a Low Concentration of Triton X-100

This figure shows the effect of extraction with buffer containing 0.75% (v/v) Triton X-100 on the cytochrome content of the residual membranes. An ETP suspension was extracted with 0.75% (v/v) Triton X-100 (6 ml/g wet weight) as described in the legend to Fig. 7. The figure shows reduced minus oxidised spectra of the following:

a: ETP suspension prior to adding detergent. Protein = 16mg/ml.

b: Supernatant from the 0.75% Triton X-100 extracted ETPs. Protein = 4.8mg/ml.

c: The resuspended, depleted membranes after Triton X-100 extraction. Protein = 12.5mg/ml.

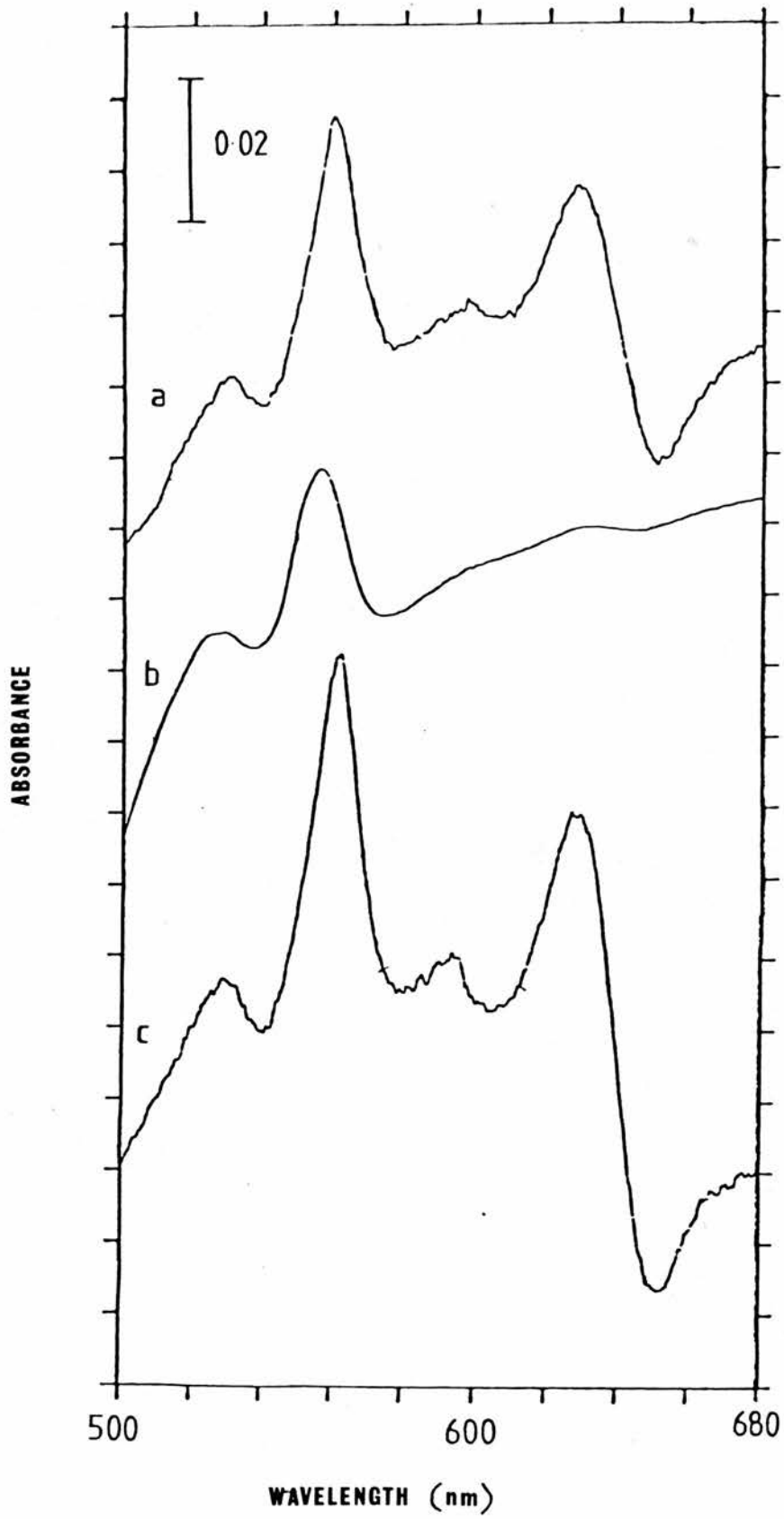

0·02
a
b
c
ABSORBANCE
500
600
680
WAVELENGTH (nm)

FIGURE 9

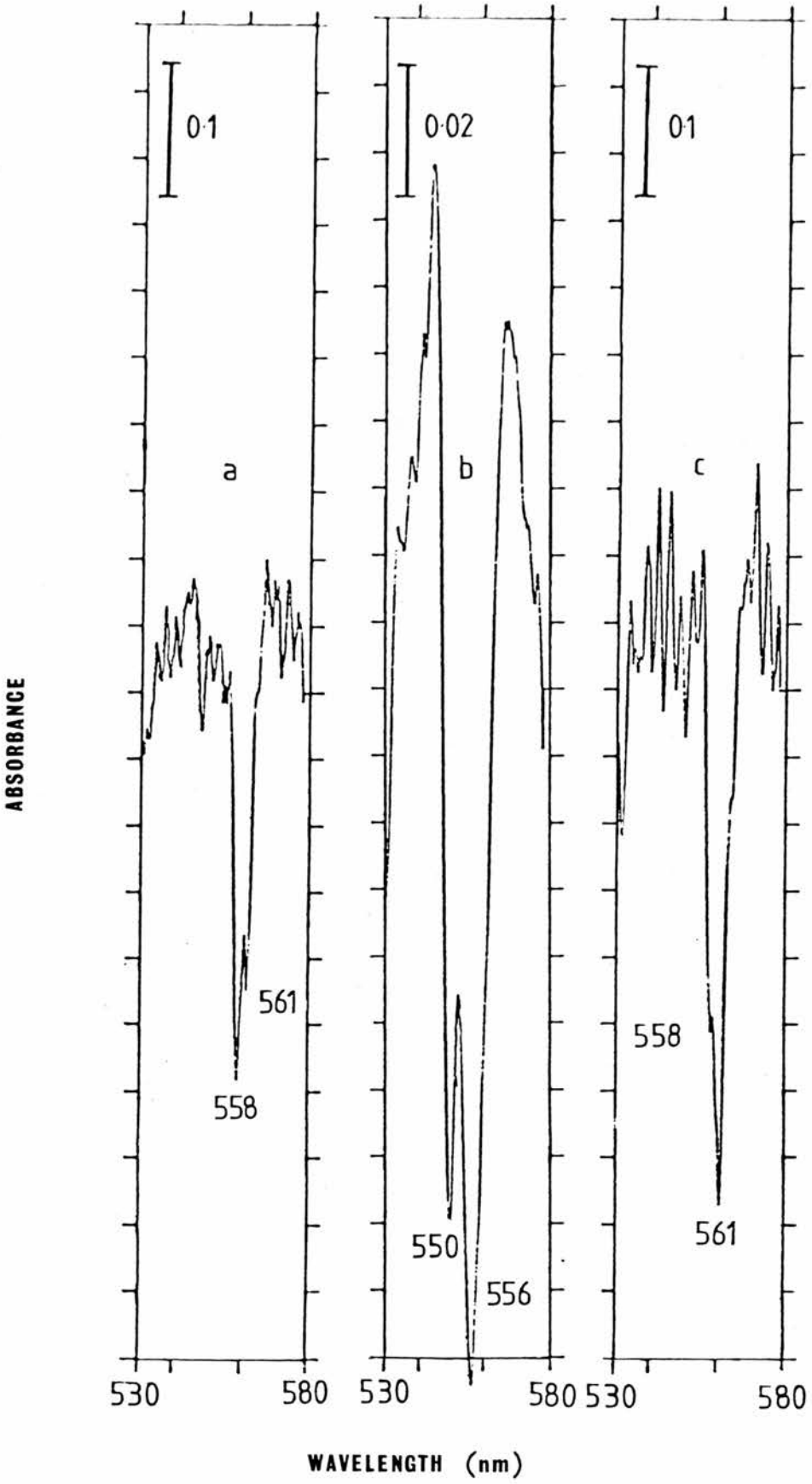
Second Derivative Difference Spectra of ETPs Before and After Extraction with 0.75% (v/v) Triton X-100

This figure shows the second derivative difference spectra of the cytochromes *b* and *c* α-band region for the extraction stages shown in Fig. 8. The spectra were performed on a Perkin-Elmer λ5 split-beam spectrophotometer, at 20°C.

a: ETP suspension prior to adding detergent. Protein = 16mg/ml.

b: Supernatant from the 0.75% Triton X-100 extracted ETPs. Protein = 4.8mg/ml.

c: The resuspended, depleted membranes after Triton X-100 extraction. Protein = 12.5mg/ml.


0·1
0·02
0·1
a
b
c
561
558
550
556
558
561
530
580
530
580
530
580
ABSORBANCE
WAVELENGTH (nm)

A maximum yield of cytochrome *bd* of 60% was achieved with an extraction buffer containing between 6 and 8% (v/v) Triton X-100 (Fig. 7), therefore a final extraction of the ETPs with extraction buffer (6ml/g wet weight) containing 6% (v/v) Triton X-100 was decided upon as this gave a total exposure of the ETPs to 6.75% (v/v) Triton X-100. When larger amounts of ETPs were used the yield of cytochrome *bd* was reduced to 30% with a 2.4 fold purification (Table 5). The reason for the marked decrease in extraction efficiency when increasing the quantities to preparative scale is not known. A similar phenomenon was observed with deoxycholate (DOC) (data not shown). Allowing the ETP/6% (v/v) Triton X-100 suspension to stand for more than 1h did not improve the extraction.

The ability of the ionic detergent DOC to solubilise cytochrome *bd* was also investigated. However, concentrations of up to 8mg DOC/mg of protein failed to achieve more than 70% of the highest yield obtained with Triton X-100. In addition, a preliminary extraction of the membranes with a low concentration of DOC followed by extraction with a higher DOC concentration did not produce a differential extraction of the cytochromes with respect to protein.

The use of a high salt concentration in the final Triton-containing extraction buffer meant that a desalting step using a Sephadex G25 column was necessary before binding to the ion-exchange column could occur. This step could have been avoided by using a lower ionic strength extraction

buffer but an extraction with 20mM KOP_4/5mM EDTA/6% (v/v) Triton X-100, pH6.0 buffer i.e. DEAE buffer, was only 30% as efficient as the high ionic strength buffer.

Fractionation of the Electron Transport Particle Preparation

Separation of the ETP fraction into an inner membrane (cytoplasmic) fraction and an outer membrane (cell wall) fraction, by sucrose isopycnic density gradient centrifugation was attempted, with the aim of increasing the specific content of cytochromes in the membrane fraction used subsequently. The technique of separating the crude membrane fraction into two further fractions has been previously used in the purification of cytochromes b_{556}, b_{562}-o and cytochrome *bd* (Kita *et al.*, 1978, 1981, 1984a,b) and it was the method of Yamato *et al.* (1978), as modified by Kita *et al* . (1978), which was employed. Recoveries of 22% and 20% for cytochromes b_{558} and *d* respectively were achieved in the inner membrane fraction and although this fraction could be completely solubilised in the 6% (v/v) Triton-containing buffer, the final yield of 20% of the total cytochrome *d* from the ETP fraction did not compare favourably with the recovery of 30% achieve by the differential extraction of the crude ETP fraction with buffers of different Triton X-100 concentration (Table 5). However, a specific content of 1.5nmol of cytochrome *b*/mg of protein was achieved by separating the ETPs into two fractions and, as this was higher than the specific content of 1.2nmol cytochrome *b*/mg obtained by differential

extraction of ETPs, the solubilised inner membranes were subjected to further purification.

Ion-exchange and gel filtration chromatography of the solubilised inner membranes, using identical conditions to those used in the final protocol for purification, (see Methods section 2), increased the specific content of cytochrome *b* to 1.7nmol/mg. This was significantly lower than the 4.7nmol/mg achieved (see Table 5) by using ETPs, extracted as described in Methods section 2, for these steps.

Fractionation of Detergent Solubilised Cytochrome *bd* by Ammonium Sulphate and Polyethylene Glycol (PEG) 6000 and 1000

Precipitation procedures using ammonium sulphate and PEG 6000 and 1000 were investigated. Various concentrations of ammonium sulphate, up to 50% saturation, and PEGs 6000 and 1000 , up to 40% saturation, were employed. Precipitation was carried out on the cytochrome preparations immediately after extraction from the membranes (with: 6% (v/v) Triton X-100; 3mg/mg DOC and 7mg/mg DOC) and on a Triton X-100 solubilised cytochrome preparation after DEAE-Sephacel chromatography (when the Triton X-100 concentration was 0.2% (v/v)). In no case was an increase in specific content of cytochrome *b* and *d* achieved and the maximum recovery was only 70%. This lack of fractionation may be due to the precipitating agents affecting the detergent rather than the protein. As the great majority

of proteins present at this stage will be contained in a detergent micelle, any agent which precipitates the detergent will precipitate all the proteins at the same time thus failing to achieve any purification.

Ion Exchange Chromatography

The passage of the differentially solubilised cytochrome through a Sephadex G-25 column, prior to ion exchange chromatography, served to exchange this high ionic strength buffer (necessary to obtain worthwhile extraction of cytochrome) for the lower ionic strength DEAE-buffer required to facilitate binding of the cytochromes to the ion-exchange column. The use of this desalting column gave a considerable saving in time over dialysis against the DEAE buffer and would, presumably, minimise the effect of any proteolytic action.

The pH and salt gradient values were chosen on the basis of results obtained from test-tube scale experiments, (Ion Exchange Chromatography: Pharmacia, Uppsala), details of which are given in the legends to Tables 3 and 4. Essentially, these involved examining the effect of various conditions of pH and salt concentration on the binding of differentially solubilised cytochrome bd to ion-exchange matrices. Protein precipitation was evident at pH5.0 and pH5.5, therefore, as the results for these pHs and pH6.0 were very similar it was decided to perform the ion-exchange chromatography at pH6.0. It was decided

5g (wet weight) of ETPs were washed in extraction buffer containing 0.75% Triton X-100 then extracted with 6% Triton X-100 as previously described in Methods section 2. The extract was divided into 5ml aliquots. The aliquots were dialysed, for 16h at 4°C, into 400ml of a 20mM KPO_4 / 5mM EDTA / 0.2% Triton X-100 buffer of one of the following pHs: 5, 5.5, 6.0, 6.5, 7.0 or 7.5.

Quantities of DEAE-Sephacel (1.5g wet weight) were equilibrated with each of the above buffers by suspension in 50ml of the buffer followed by centrifugation at 10 000g for 10 min, repetitively, until the ionic strength of the supernatant was the same as that of the buffer.

The ionic strengths of the dialysed extracts were measured, to ensure equilibrium had been achieved with the buffer, and the pH checked. Protein and cytochrome content were assayed. Each aliquot of dialysed cytochrome preparation was added to a test-tube containing the DEAE-Sephacel of the same pH. The tube was mixed on a vortex mixer and kept on ice for 15 min to allow binding to occur. The DEAE-Sephacel was pelleted by centrifugation, at top speed, for 10 min in an MSE Minor bench-top centrifuge. The supernatant was decanted and assayed for protein and cytochrome content.

TABLE 3

Estimation of the Optimum pH for Ion Exchange Chromatography with DEAE-Sephacel

pH	Cytochrome b_{558} bound (%)*	Cytochrome *d* bound (%)*	Protein bound (%)*
5.0	100	100	82
5.5	100	100	79
6.0	100	100	79
6.5	100	100	83
7.0	100	100	84
7.5	100	100	83

*All percentages are of the total present in the aliquot before addition to the ion-exchanger.

1g (wet weight) of ETPs was sequentially extracted with 6ml of extraction buffer containing 0.75% (v/v) then 6% (v/v) Triton X-100. The 6% (v/v) Triton X-100 extract was dialysed into 500ml of 20mM KPO_4/5mM EDTA/0.2% Triton X-100, pH6.0 for 16h at 4°C.

A 1g (wet weight) portion of DEAE-Sephacel was equilibrated with the above buffer, as described in the legend to Table (3). The ionic strength, protein concentration and cytochrome content of the dialysed extract was assayed prior to the addition of a 3ml aliquot of the extract to the ion exchange gel in a test-tube. The contents were mixed on a vortex mixer and the tube kept on ice for 15 min to allow binding to take place.

The DEAE-Sephacel was pelletted by centrifugation for 10 min, at top speed, in an MSE Minor bench-top centrifuge and the supernatant retained. The gel was resuspended in 3ml of buffer, centrifuged as before, and the supernatant added to the previous one. The combined supernatants were assayed for protein and cytochrome content. This protocol of resuspension twice, followed by combination of the supernatants, was carried out for 3ml volumes of the 20mM KPO_4/5mM EDTA/0.2% Triton X-100, pH6.0 buffer which had been made 50, 100, 150 and 200mM KCl respectively. Each of the combined supernatants was assayed for cytochrome content and protein.

TABLE 4

Estimation of the KCl Concentration Required to Elute Cytochrome *bd* from DEAE-Sephacel

Salt Concentration in Supernatant (mM KCl)	Cytochrome b_{558} Content (%)*	Cytochrome *d* Content (%)*	Protein Content (%)*
Buffer	0	0	25
50	0	0	15
100	0	0	9
150	65	59	41
200	0	0	7

*All percentages are of the total present in the aliquot before addition to the ion-exchanger.

not to amend this when the pI of the cytochrome became known (pH4.8, this chapter, Results section) as a pH difference of around 1pH unit between the pI of the substance of interest and the pH of the ion-exchanger is considered optimal (Ion Exchange Chromatography: Pharmacia, Uppsala).

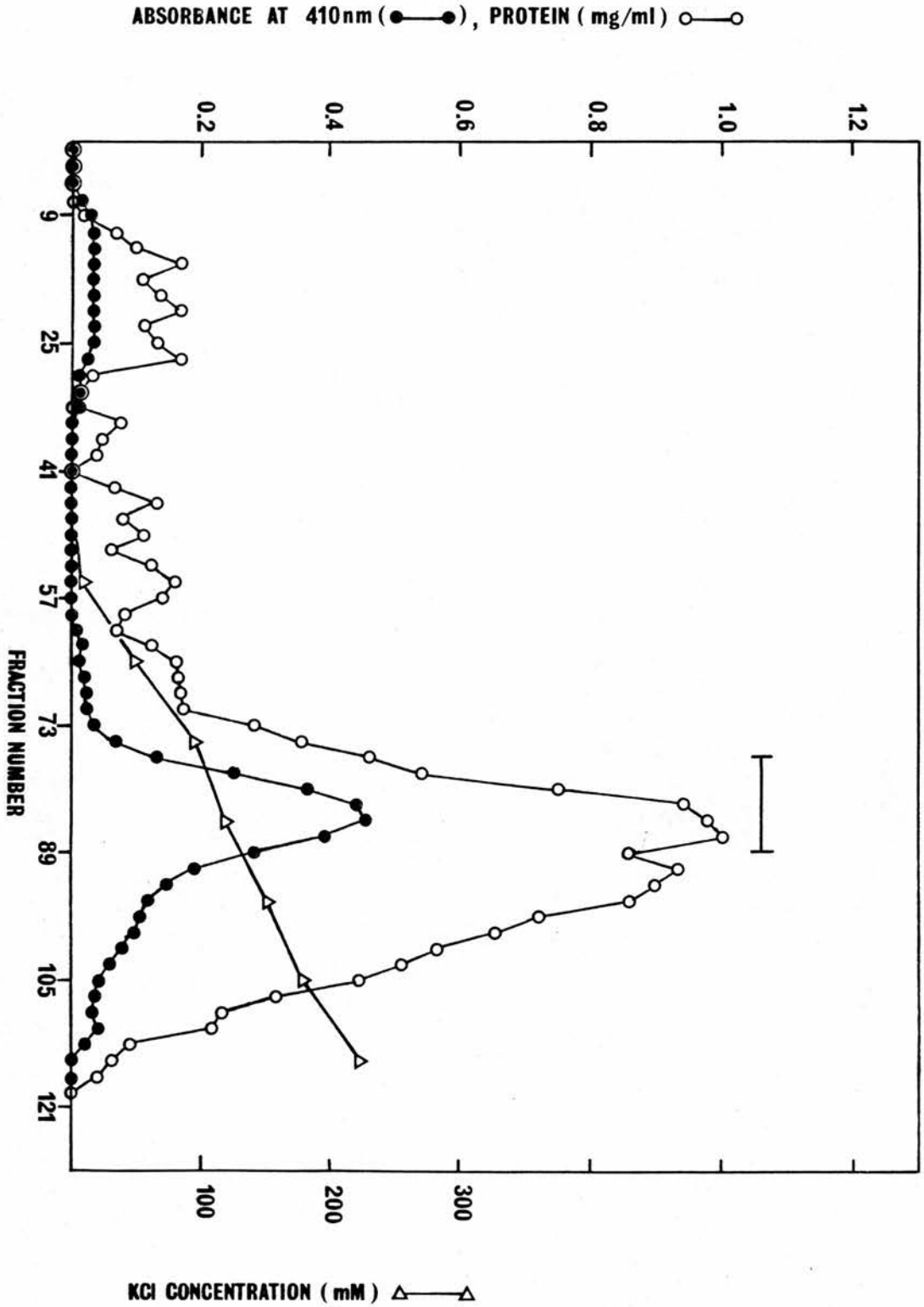
At pH6.0 the *b* and *d* cytochromes appear to be negatively charged as they bind to the DEAE-Sephacel (an anion exchanger). Any contaminating proteins which are not negatively charged at this pH should pass straight through the column. However, it is possible that contaminating proteins, which have a net positive charge at pH6.0, may still bind to the column if they contained localised areas of negative charges. Therefore, the differentially solubilised cytochromes, in DEAE-buffer, were added to CM52 ion-exchange gel (a cation exchanger) equilibrated with the same buffer to see if any contaminating protein was bound. This would provide a rapid purification step prior to DEAE-Sephacel chromatography, resulting in an increased binding capacity of the column. However, no binding of either cytochrome or protein was evident and the step was not used subsequently.

From the results of the experiment described in Table 4 it can be seen that the cytochromes were eluted from the gel by a salt concentration of 150mM KCl. As protein was eluted at concentrations lower than this, a KCl concentration gradient of 0 to 300mM in 600ml was chosen.

FIGURE 10

pH6.0 DEAE-Sephacel Chromatography of Differentially Solubilised Cytochrome *bd*

This figure shows a typical elution profile from a DEAE-Sephacel column following the application of a 0-300mM KCl concentration gradient in 600ml. The cytochrome-containing fractions which were pooled and subjected to further purification are indicated by the bar.


ABSORBANCE AT 410nm (●—●), PROTEIN (mg/ml) ○—○
0.2
0.4
0.6
0.8
1.0
1.2
9
25
41
57
73
89
105
121
FRACTION NUMBER
100
200
300
KCl CONCENTRATION (mM) △—△

The cytochrome preparation was applied to the DEAE-Sephacel column and unbound and lightly bound proteins were eluted with 2 bed volumes of buffer before applying the KCl gradient to elute the cytochromes.

Under these conditions a 2.3-fold purification and a yield of cytochrome from the column of 60% was achieved.

Neither increasing the slope of the salt gradient to 0-400mM over 600ml nor decreasing the slope to 0-300mM over 800ml gave any improvement in separation.

Gel Filtration

Sample Concentration

Further purification by gel filtration was attempted. The volume of the pooled peak fractions from the DEAE column was too large to apply directly to the gel filtration column, therefore, methods of concentrating the sample were investigated. Concentration, by ultrafiltration, of the peak fractions from the ion-exchange column was found to be slow (6h) and produced a large increase in the Triton X-100 concentration, rendering the sample viscous and resulting in a loss of resolution from the gel filtration column. A more suitable method of concentration proved to be a small scale ion-exchange step.

First, the pooled peak fractions from the DEAE-Sephacel column were desalted by passing them down a Sephadex G-25 column equilibrated with DEAE-buffer. Then the desalted

fractions were applied to a small DEAE-Sephacel column equilibrated with the same buffer. The bound cytochrome was washed with 2 bed volumes of buffer (approx. 30ml) before a batch elution was performed with the 50mM KPO_4/5mM EDTA/0.5M NaCl/0.2% (v/v) Triton X-100, pH7.0 buffer (Sephacryl buffer) with which the gel filtration column was equilibrated. This gave an approximately 5-fold concentration, an increase in the specific content of cytochrome *b* of 10% and a 90% yield from the column (Table 5). This eluate could be more quickly concentrated (30 mins) by ultrafiltration to the required volume without a significant increase in viscosity.

An attempt was made to obtain an almost detergent-free eluate from the small DEAE-Sephacel column, which may have been more amenable to purification by a precipitation step. After applying the pooled fractions to the column, the bound cytochrome was washed with 200ml of DEAE-buffer containing 0.01% (v/v) Triton X-100 and the Triton X-100 concentration of the eluting buffer was similarly reduced. The cytochrome, however, remained bound to the column despite increasing the NaCl concentration to 1M. Elution was rapidly effected by the subsequent application of Sephacryl buffer containing 1% (v/v) Triton X-100. It is thought likely that the low detergent concentration and high salt strength promoted non-specific hydrophobic interactions between the gel matrix and the bound cytochrome. A similar phenomenon occurred with the chromatofocusing column (see later).

Chromatography

A previous partial purification (Reid and Ingledew, 1980) had proposed a native molecular weight for the cytochrome-detergent complex of 400 000, as determined by gel filtration. Therefore, a filtration matrix offering selectivity in this range was required. Sephacryl S-300 was used as this has a fractionation range for proteins of 1×10^4 - 1.5×10^6 daltons.

The enclosure of the extracted membrane proteins within a detergent micelle gave a large degree of similarity to their molecular weights, necessitating high resolving power. This required the use of a long (120cm) column as shorter columns gave little resolution of the cytochrome from the main protein peak.

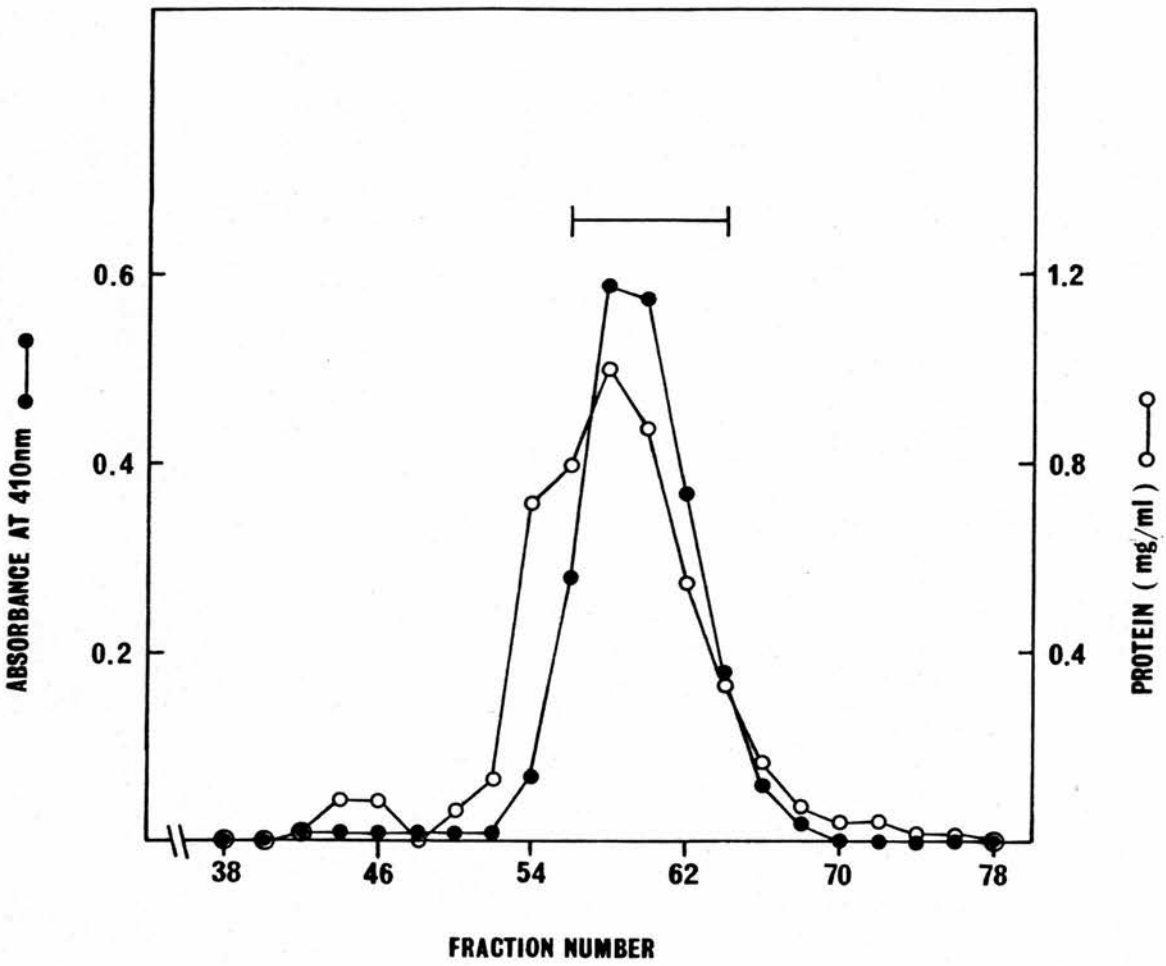
The rigid particle structure of Sephacryl S-300 enabled the use of a large hydrostatic head to achieve a flow rate of 15ml/h, allowing this step to be carried out overnight. Resolution was found to be dependent on sample volume and viscosity. A compromise sample volume of 9ml, with a protein concentration of 9mg/ml, was ultimately employed. This was equivalent to 1.4% of the total bed volume. Reducing the sample volume below 1% of the bed volume does not normally improve resolution (Gel Filtration: Pharmacia, Uppsala).

The gel filtration elution medium employed comprised, 100mM KPO_4, pH7.0, to buffer the pH and 0.5M NaCl to prevent any ionic interactions between the sample and the gel matrix.

FIGURE 11

Gel Filtration Chromatography of Pooled Peak Fractions from the DEAE-Sephacel Column on Sephacryl S-300

The concentrated batch elution from the small DEAE-Sephacel column was applied to a Sephacryl S-300 column and chromatographed as described in the Methods section 2. The cytochrome-containing fractions indicated by the bar were pooled for further purification. Only a limited number of fractions after the cytochrome-containing peak were collected and it is assumed that the remaining protein applied to the column would be eluted after these fractions.


ABSORBANCE AT 410nm
0.6
0.4
0.2
1.2
0.8
0.4
PROTEIN (mg/ml)
38
46
54
62
70
78
FRACTION NUMBER

A typical elution profile for a Sephacryl S-300 column, under these conditions, is shown in Fig 11. Use of this protocol gave an increase in the specific content of cytochrome *b* of 50% with a yield of 62% from the column and was adopted as routine.

Preparative Sucrose Density Gradient Centrifugation of Cytochrome *bd*

An attempt to further purify the pooled peak fractions from the Sephacryl S-300 column by sucrose density gradient centrifugation was made. A gradient of 5 to 21% (w/v) sucrose, was not successful in separating the cytochrome (A_{410}) and protein peaks.

Chromatofocusing

As separation according to size had been used and separation according to density had been unsuccessful, isoelectric focusing (IEF) of the preparation was carried out to investigate the possibility of further separation according to isoelectric point. Preliminary results (Fig. 12) indicated that this might be successful since at least three protein components were resolved by this technique.

In order to be able to process milligramme quantities of cytochrome, chromatofocusing was chosen over preparative isoelectric focusing as it was simpler and could be performed overnight. A pH gradient of 4-6 was selected as the haem-staining band of the focused gel migrated to a position corresponding to pH4.8.

FIGURE 12

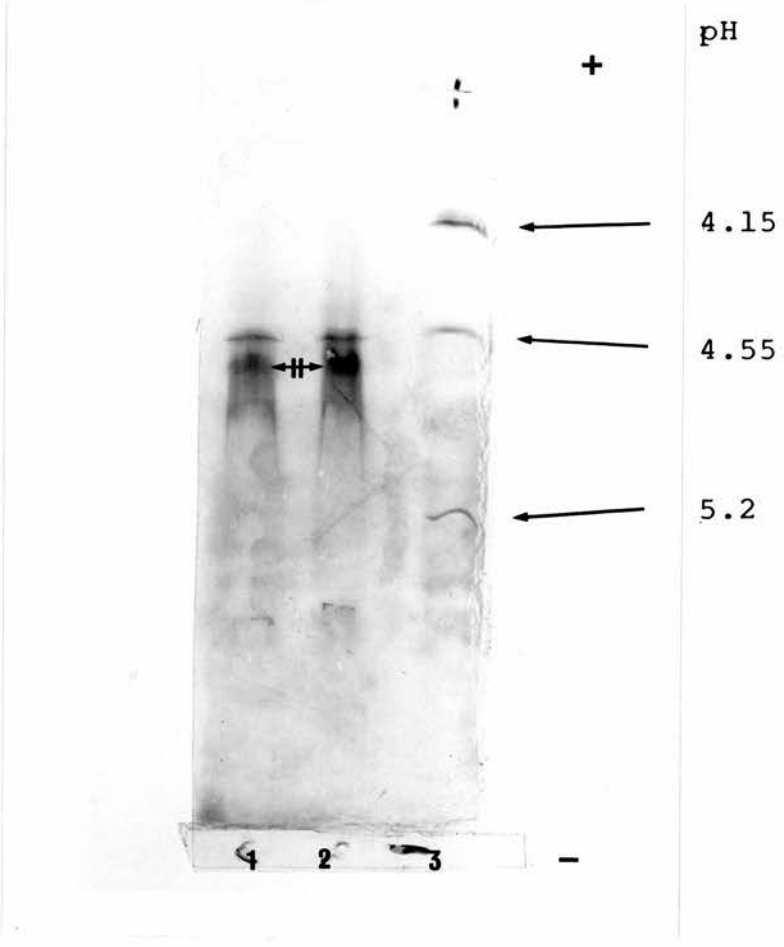
Isoelectric Focusing (pH4-6) of Pooled Peak Fractions from a Sephacryl S-300 Chromatography Column

Pooled peak fractions from a Sephacryl S-300 column were focused, as described in Methods section 3, on a pH gradient of 4-6. The gel was then fixed and stained for haem. The position of the haem-positive band was noted before the gel was stained for protein (shown). **H** indicates the position of the haem-staining band.

Lane 1: Pooled peak fractions from the Sephacryl S-300 column, 30µg of protein loaded.

Lane 2: As lane 1.

Lane 3: pI standard proteins.


pH
+
4.15
4.55
5.2
1
2
3
4
-

A typical elution profile from the chromatofocusing column is shown in Fig. 13. The cytochrome was eluted in Polybuffer 74 of pH5.0. Although elution from the column is in order of decreasing isoelectric point, the elution pH of a component is not necessarily that of the protein's isoelectric point. The elution pH is affected by a combination of factors which include displacement effects, the surface potential of the protein and the solubility of the protein at its isoelectric point (for further discussion see Chromatofocusing: Pharmacia, Uppsala). It should also be noted that chromatofocusing was carried out at 4°C while IEF was performed with a cooling water temperature of 15°C. It has been found that the pI of a protein is affected by temperature. Typically, the pI of a basic protein will vary by 0.03 pH units per degree Celsius. while an acidic protein will alter its pI by 0.005pH units per degree Celsius (Isoelectric Focusing, Principles and Methods, Pharmacia, Uppsala).

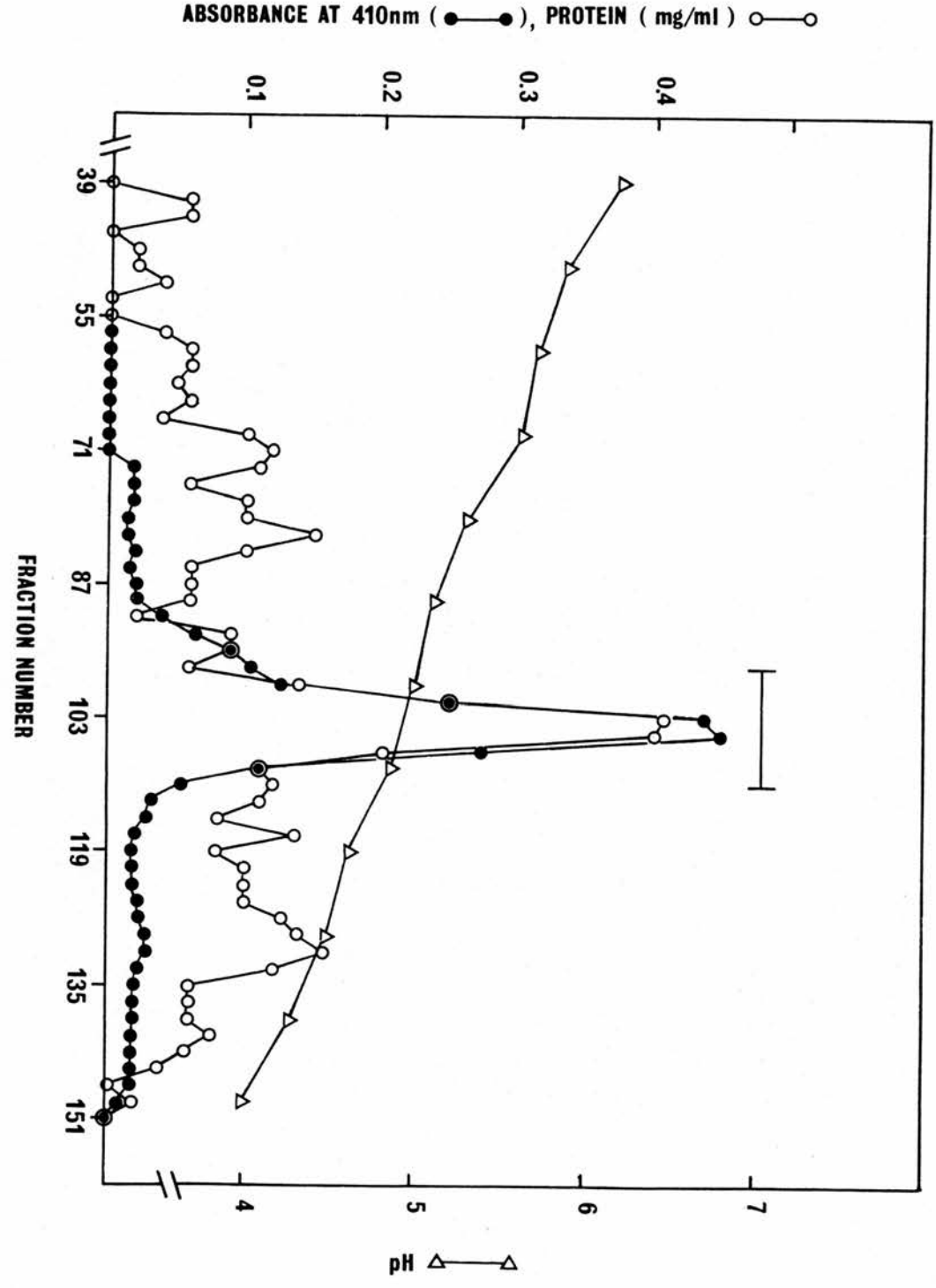
Presumably these factors explain the disparity between the elution pH of 5.0 for cytochrome *bd* and the isoelectric point of 4.8 estimated by isoelectric focusing.

A dilution factor of 1:6.66 for Polybuffer 74: water was found to give sharper resolution of the cytochrome than the recommended ratio of 1:8. The diluted Polybuffer 74 was degassed, immediately before being used to elute the cytochrome, to minimise any interference, by dissolved CO_2, with the pH gradient.

FIGURE 13

Chromatofocusing of Pooled Peak Fractions
from the Sephacryl S-300 Column

The pooled peak fractions from the Sephacryl S-300 column were applied, after buffer exchange on a Sephadex G-25 column, to the chromatofocusing column. The cytochrome was eluted by a pH gradient from pH6.0 to 4.0. The cytochrome-containing fractions which were pooled are indicated by the bar.


ABSORBANCE AT 410nm (●—●), PROTEIN (mg/ml) ○—○
0.1
0.2
0.3
0.4
39
55
71
87
103
119
135
151
FRACTION NUMBER
4
5
6
7
pH △—△

Protein Estimation of Chromatofocusing Fractions

The protein assay described by Bradford (1976) was used to monitor protein elution from the column and to estimate the protein concentration of the final pooled fractions. (Use of the Lowry and Biuret protein assays was not possible as the Polybuffer 74 interfered strongly with these assays). A blank containing the same quantity of Triton X-100 as the sample was used to compensate for the interference caused by the detergent binding to the dye used in the assay in a manner similar to proteins. The presence of larger amounts of detergent in the sample mixture had the effect of "overloading" the assay and taking the sensitivity out of the linear range (Fig. 5) thus, for samples of different volumes, different standard curves had to be prepared.

In an attempt to diminish the interference with the protein assay, various low Triton X-100 concentrations in the Polybuffer were tried. At a Triton X-100 concentration of 0.1% (v/v), the cytochrome remained bound to the column after passage of the pH gradient. A detergent concentration of 0.2% (w/v) gave a yield of 10% from the column. Increasing the concentration to 0.5% (v/v) gave a good recovery from the column (44%). Although ensuring an adequate concentration of Triton X-100 to prevent excessive binding to the column, this concentration did not alleviate the interference with the assay.

Protein estimations were performed on pooled peak chromatofocusing fractions before any subsequent concentration step since, after concentration, it was difficult to determine the exact Triton X-100 concentration required for the assay blank. Despite these precautions it is likely that the protein concentration estimated by the Bradford technique is higher than the actual concentration.

The difficulty experienced in extracting the cytochrome from the membrane, its reported high polarity percentage of 35% (Miller and Gennis, 1983) and the requirement for a Triton X-100 concentration which is well in excess of the critical micelle concentration of 0.016% (v/v) (Clarke, 1975) in order to prevent excessively strong binding to ion-exchange media, all indicate that the enzyme is highly hydrophobic and is therefore likely to bind a substantial quantity of detergent. This bound detergent will react with the Bradford assay and give an anomalously high estimate of protein concentration.

Removal of Polybuffer

Attempts were made to remove the Polybuffer 74 in order to permit the use of a less detergent-sensitive protein assay such as the Lowry/SDS assay described in the Methods, section 1. These are described below:-

One method, recommended by Pharmacia, for removing Polybuffer 74 from a sample is chromatography on Sephadex G-75. Therefore, pooled peak fractions from the chromatofocusing column were concentrated to 5ml, by ultrafiltration on ice in an Amicon concentration cell fitted with an XM50 membrane, and applied to a Sephadex G-75 column (38 x 2.6m) equilibrated with 50mM TES/5mM EDTA/0.1% (v/v) Triton X-100, pH7.0. The pooled peak cytochrome-containing fractions from the Sephadex G-75 column gave a specific content of 2.1nmol cytochrome *b* per mg of protein, using the Lowry/SDS protein assay, and a yield of 87% from the column was achieved. This specific content of cytochrome *b* differed markedly from the specific content previously obtained for the same peak fractions, before chromatography on Sephadex G-75, of 7.1nmol of cytochrome *b* per mg of protein by using the Bradford assay. The use of the Bradford assay on the fractions obtained from the G-75 column was not possible as the concentration by ultrafiltration had increased the Triton X-100 concentration to an unknown level and micelles of the detergent would have co-migrated down the column with the cytochrome. Clearly, something (presumably the Polybuffer) was interfering with the Lowry assay as the result for protein content of the pooled fractions after filtration on Sephadex G-75 was more than three times that of the pre-Sephadex G-75 filtration fractions.

To investigate whether the Polybuffer was not being removed by gel filtration because of its association with either the Triton X-100 micelles or the cytochrome itself a model system was devised to represent the cytochrome preparation at this stage. This system comprised a 0.5mg/ml solution of BSA in Polybuffer 74 of the same concentration, pH and Triton X-100 concentration as that in which the cytochrome was eluted from the chromatofocusing column. This test solution was subjected to repeated cycles of concentration by ultrafiltration and redilution with 50mM TES/5mM EDTA, pH7.0, as described in the legend to Fig. 14, to remove all the Polybuffer 74. Successful removal of the Polybuffer 74 was achieved within six cycles, strongly indicating that the Polybuffer was not binding to the Triton X-100 present.

The pooled, peak fractions from a chromatofocusing column, having a specific content of 7.0nmol cytochrome *b* (using the Bradford protein assay) was subjected to seven cycles of ten-fold concentration and redilution, as described in the legend to Fig. 14. However, estimating the protein content of the final concentrate using the SDS/Lowry protocol gave a specific content for cytochrome *b* of 1.9nmol/mg with a recovery of 98%. This represented a 3.5 fold increase in the total protein.

It seemed likely that the Polybuffer 74 component, which interfered with the SDS/Lowry protein assay, was binding

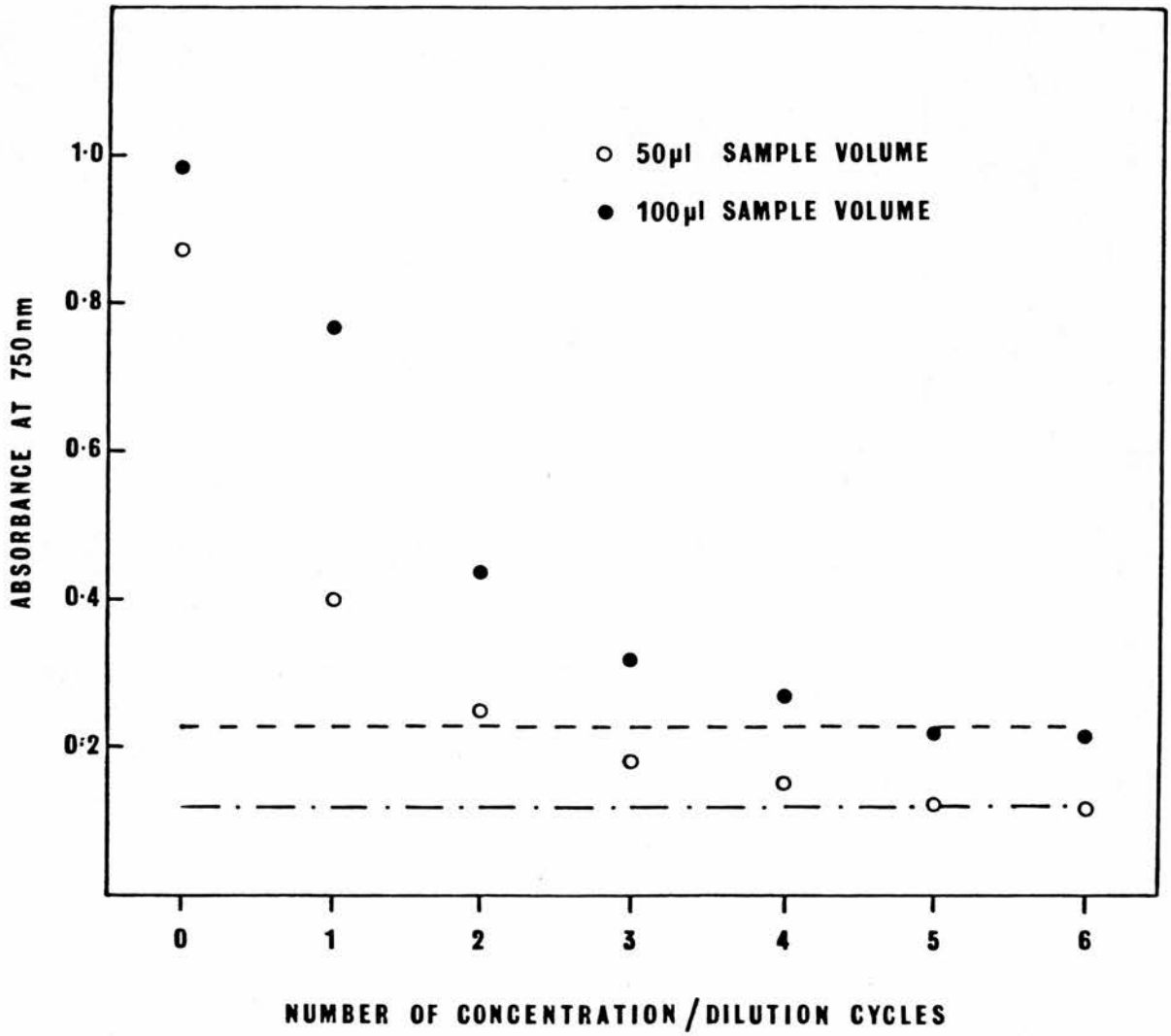
FIGURE 14

Removal of Polybuffer 74 from a Solution of BSA, by Ultrafiltration

3.0ml of Polybuffer 74 stock solution were made 0.5% (v/v) with respect to Triton X-100, adjusted to pH5.0 with 1M HCl and 10mg of BSA were added. The final volume was adjusted to 20ml with water. Duplicate samples of 50 and 100µl volumes were taken and assayed for protein using the modified procedure of Lowry described in Methods section 1.

The BSA solution was concentrated to a volume of 2ml in an Amicon concentration cell fitted with an XM 50 membrane, and rediluted with 18ml of 50mM TES/ 5mM EDTA, pH7.0. Again, 50 and 100µl samples were taken, in duplicate, and assayed for protein concentration. This process was repeated until consistent results were obtained.

A standard curve, using BSA as the protein standard and the same batch of reagent solutions, was used to estimate the correct A_{750} for 25 and 50µg of protein.


50µl SAMPLE VOLUME
100µl SAMPLE VOLUME
ABSORBANCE AT 750nm
1·0
0·8
0·6
0·4
0·2
0
1
2
3
4
5
6
NUMBER OF CONCENTRATION / DILUTION CYCLES

to the cytochrome and could not be removed by buffer - exchange steps since the only difference between the model system and the one involving the pooled chromato-focusing fractions was the substitution of cytochrome *bd* for BSA.

A routine procedure involving three cycles of ten-fold concentration by ultrafiltration followed by redilution with 50mM TES/5mM EDTA, pH7.0 and a final ten-fold concentration was adopted to remove the Polybuffer 74. This was rapid, gave a good yield of cytochrome (approx. 100%), removed much of the Polybuffer 74 and obviated the need for a concentration step prior to the various analytical procedures employed for characterising the cytochrome.

EDTA (5mM) was included in the buffers, wherever possible, to complex any metal ions which may have interfered with EPR spectroscopy and also to inhibit *E. coli* proteinases (Goldberg *et al.*, 1981).

It was found necessary to carry out the purification without interruption as freezing and thawing of the impure preparation resulted in a loss of resolution and yield from the next stage.

In summary, by using the purification protocol described in this chapter, a cytochrome *bd* preparation was obtained with a specific content of cytochrome *b* of 7.1 nmol haem/mg of protein. This represented a 14-fold

increase over the specific content of cytochrome *b* which was present in the ETP fraction used as the starting point of the purification. The final recovery of cytochrome *bd* was 4.4% of the total contained in the ETP fraction and this preparation was called "purified cytochrome *bd* ". Further details of the purification factors and yields from the various stages of the purification are presented in Table 5.

TABLE 5

A Summary of the Purification of Cytochrome *bd*

A typical purification is outlined below

Stage	Total Cyt b_{558} (nmol)	Total Protein (mg)	Specific Content of Cyt b_{558} (nmol haem/mg protein)	Yield (%)	Purification
0.75% Triton X-100 Suspension	2471	4950	0.5	100	0
6% Triton X-100 Suspension	2042	4100	0.53	83	1.06
6% Triton X-100 Extract	747	620	1.2	30	2.4
First DEAE-Sephacel Pool	454	164	2.8	18	5.6
Second DEAE-Sephacel Pool	404	130	3.1	16	6.2
Sephacryl S-300 Pool	257	53	4.7	10	9.4
Chromatofocusing Pool	109	155	7.1	4.4	14.2

Characterisation of purified cytochrome *bd*

Isoelectric focusing

Agarose was chosen as the support medium for IEF because of its ease of handling compared with acrylamide and its rapid staining and destaining properties once dried. The placing of a few drops of 1% (v/v) Triton X-100 solution onto the cooling plate of the focusing chamber before applying the gel, helped to maintain good contact between the gel and the plate, thus aiding cooling. Passage of a current greater than 10mA through the gel created localised overheating, leading to drying of the gel and distortion of the pH gradient.

The extent of focusing obtained for a given set of conditions was monitored by applying identical samples at different distances from the cathode. Once focusing had been achieved the respective bands were colinear and parallel to the cathode. The protocol for isoelectric focusing described in Methods section 3 achieved this consistently. Precipitation of protein at the site of sample application was apparent, particularly with samples from the early stages of the purification. In these cases it was likely to be due to the presence of cell wall membrane glycoproteins. For preparations of cytochromes from later stages of the purification (including that of the purified cytochrome) it was not known whether the precipitation was due to the focusing conditions or simply present in the sample prior

to application. The focused band of cytochrome stained for both haem and TMPD oxidase activity while the precipitated protein stained only for haem, suggesting that it may have been denatured cytochrome.

The pI obtained for cytochrome *bd* from these experiments was 4.8 and this value remained constant throughout the purification (Fig. 15). This indicates that the Polybuffer constituent which bound to the cytochrome did not affect its pI.

Analysis of the focused band by SDS-PAGE gave two protein staining bands of apparent molecular weights 72 000 and 43 000. These are similar values to those found for the purified cytochrome *bd* (see Fig. 16). A silver stain was employed to visualise the protein bands in the second dimension SDS gel since staining with Coomassie Blue R was not sensitive enough to clearly reveal the banding pattern.

Polyacrylamide Gel Electrophoresis under Non-Denaturing Conditions

The buffer system of Fairbanks *et al.* (1971) was employed as it gave similar resolution to the discontinuous buffer system of Laemmli in gels of 5, 6 and 7% (w/v) acrylamide concentration, while providing more optimal conditions for haem staining. A single band staining for protein was evident when approximately 30µg of the purified enzyme was electrophoresed under non-denaturing conditions

FIGURE 15

An Isoelectric Focusing Gel (pH4-6) of Cytochrome *bd* Preparations

The loadings were as follows:

Lane 7: 25µl of Low pI standard proteins

Lane 8: 20µg of Soybean Trypsin Inhibitor

Lane 9: 40µg of pooled peak fractions from the DEAE column

Lane 10: Approx. 40µg of purified cytochrome

Lanes 7 and 8 were stained for protein while Lanes 9 and 10 were stained for TMPD oxidase activity.

Note: the broadening of the TMPD oxidase staining band in Lane 9 is due to freezing and thawing the sample prior to use.

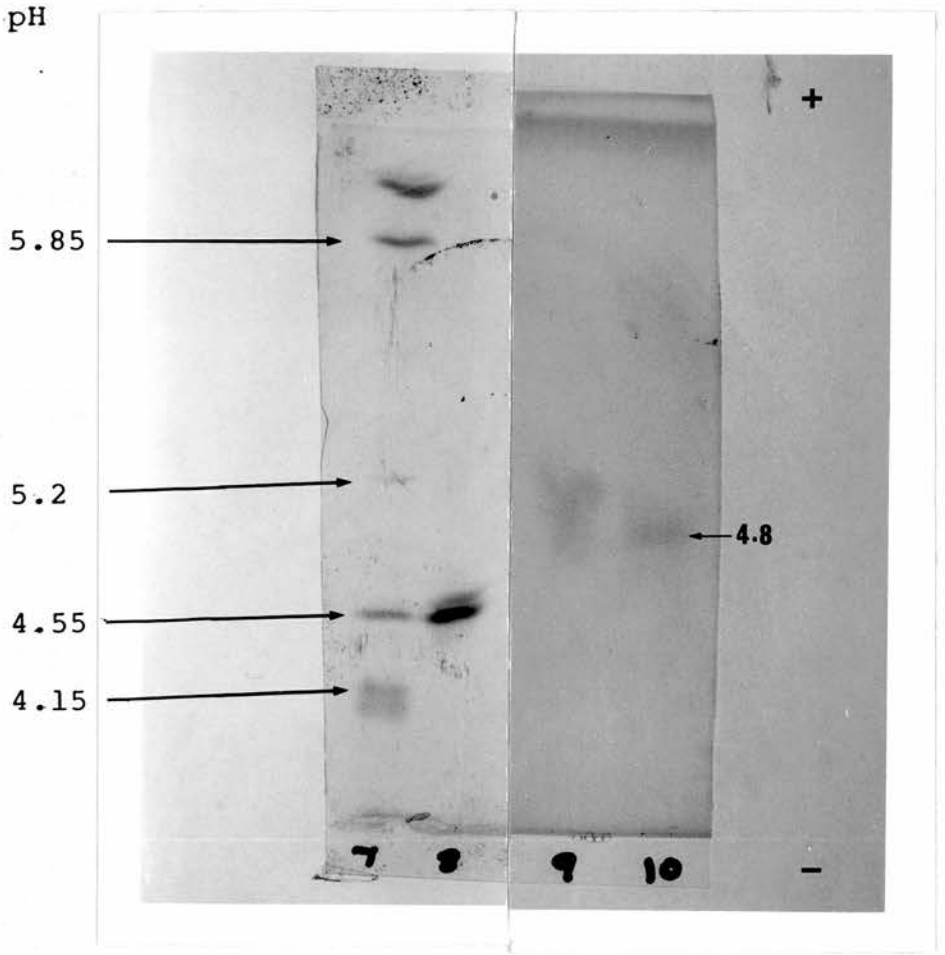

pH
5.85
5.2
4.55
4.15
4.8
+
−
7
8
9
10

FIGURE 16

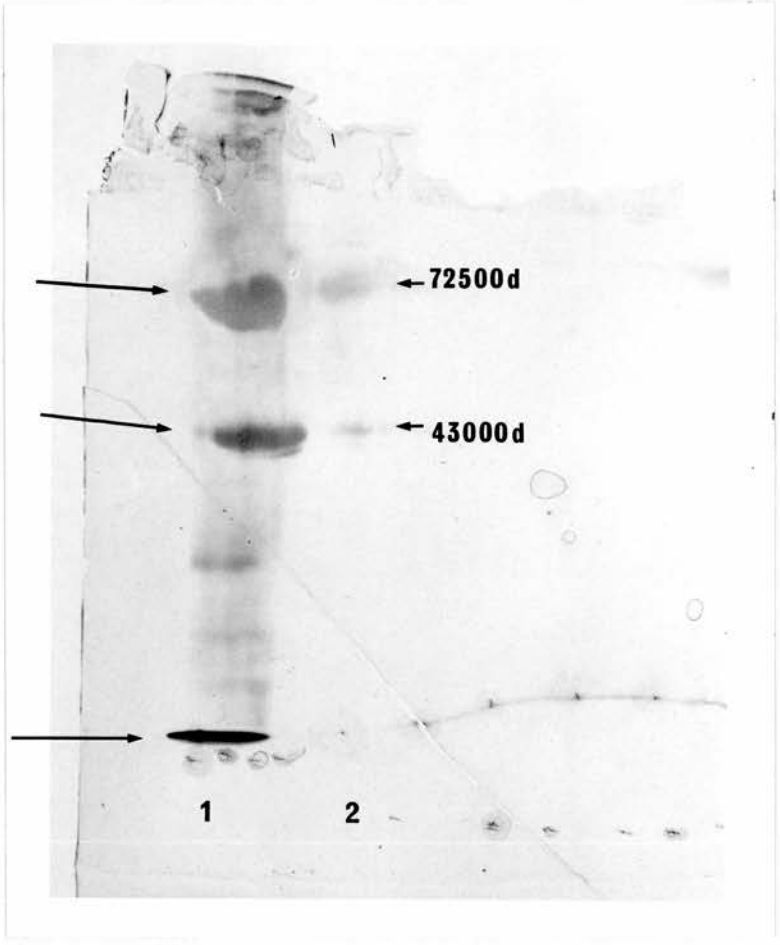
Second Dimension SDS-PAGE of the Haem-Positive Band from IEF of Peak Fractions from Gel Filtration

The haem staining band from an IEF gel, pH4-6, of pooled peak fractions from a Sephacryl S-300 column was excised and applied to a 10% (w/v) polyacrylamide gel containing 0.1% SDS, as described in Methods section 3.

Lane 1 contains molecular weight marker proteins; BSA, ovalbumin and myoglobin

Lane 2 contains 100µl of excised gel/SDS mixture

The gel was stained using the silver stain described in Methods section 3.


67 000d
45 000d
17 000d
72500d
43000d
1
2
Dye Front

(Fig. 17). This band also stained for haem, iron and TMPD oxidase activity. Some aggregated enzyme was usually observed at the top of the gel. Although this aggregated material stained heavily for haem it did not stain for TMPD oxidase activity suggesting that, as for isoelectric focusing, the enzyme was denatured.

Electrophoresis Under Dissociating Conditions

An SDS concentration of 5% (w/v) in the sample preparation buffer was found to be the minimum required for effective dissociation of the cytochrome. Boiling of the sample caused irreversible aggregation of the enzyme, while incubation at 37°C for 30 min gave good dissociation. A similar phenomenon of aggregation following boiling in the presence of SDS has been reported for cytochrome *bd* (Miller and Gennis, 1983) and also for the *b* cytochrome from *E. coli* nitrate reductase (Chaudry and MacGregor, 1983).

Under the conditions described in the Methods, section 3 two protein staining bands, of apparent molecular weight 70 000 and 43 000 were revealed (Fig. 19). The molecular weights of the subunits remained within 5% of these values on gels of 7, 10 and 12.5% (w/v) acrylamide concentration and throughout the purification.

The specific content of cytochrome b_{558}, using the protocol described in the Methods, section 1, was 7.1nmol haem/mg of protein.

FIGURE 17

Non-Denaturing Gel Electrophoresis of the Purified Cytochrome *d* Complex

Approx. 30µg of pooled peak fractions from the chromatofocusing step were electrophoresed on a 7% (w/v) acrylamide gel as described in Methods section 3. The gel was stained with Coomassie Blue R and the dye front marked with indian ink.

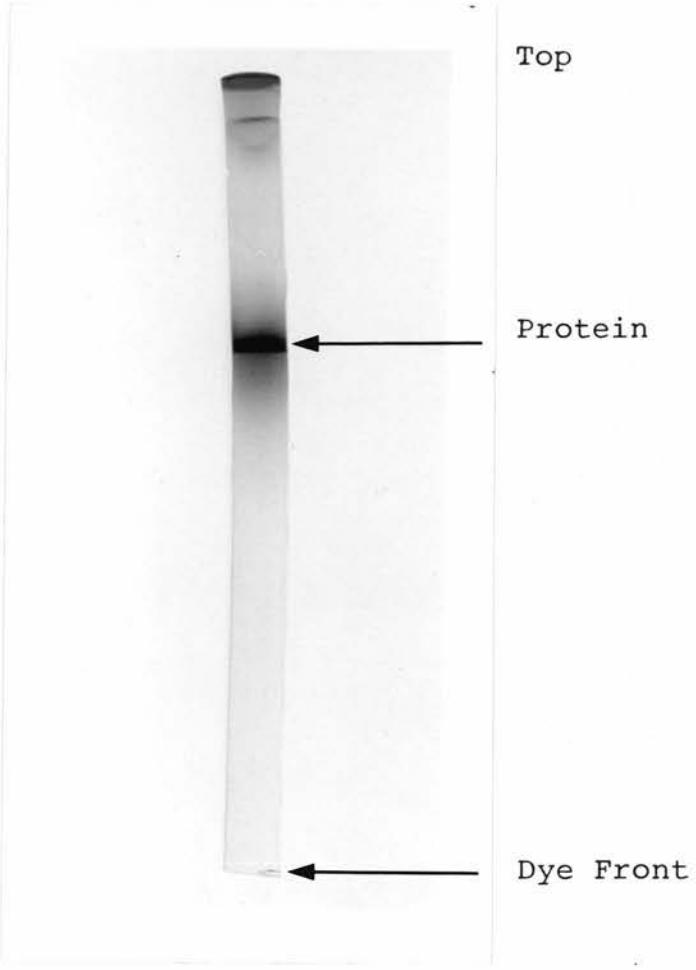

Top
Protein
Dye Front

FIGURE 18

Molecular Weight Calibration Plot for Electrophoresis in a 10% (w/v) Acrylamide, 0.1% (w/v) SDS Gel

This figure shows the relationship between the relative mobility (R_f) of protein standards in a 10% (w/v) acrylamide SDS gel and their molecular weight. Letters refer to the protein standards as follows: P, phosphorylase b (92 500 MW); B, BSA (67 000 MW); O, ovalbumin (45 000 MW); T, trypsinogen (24 000 MW); M, myoglobin (17 000 MW). The R_f values obtained for the two subunits of cytochrome *bd* are marked on the standard curve.

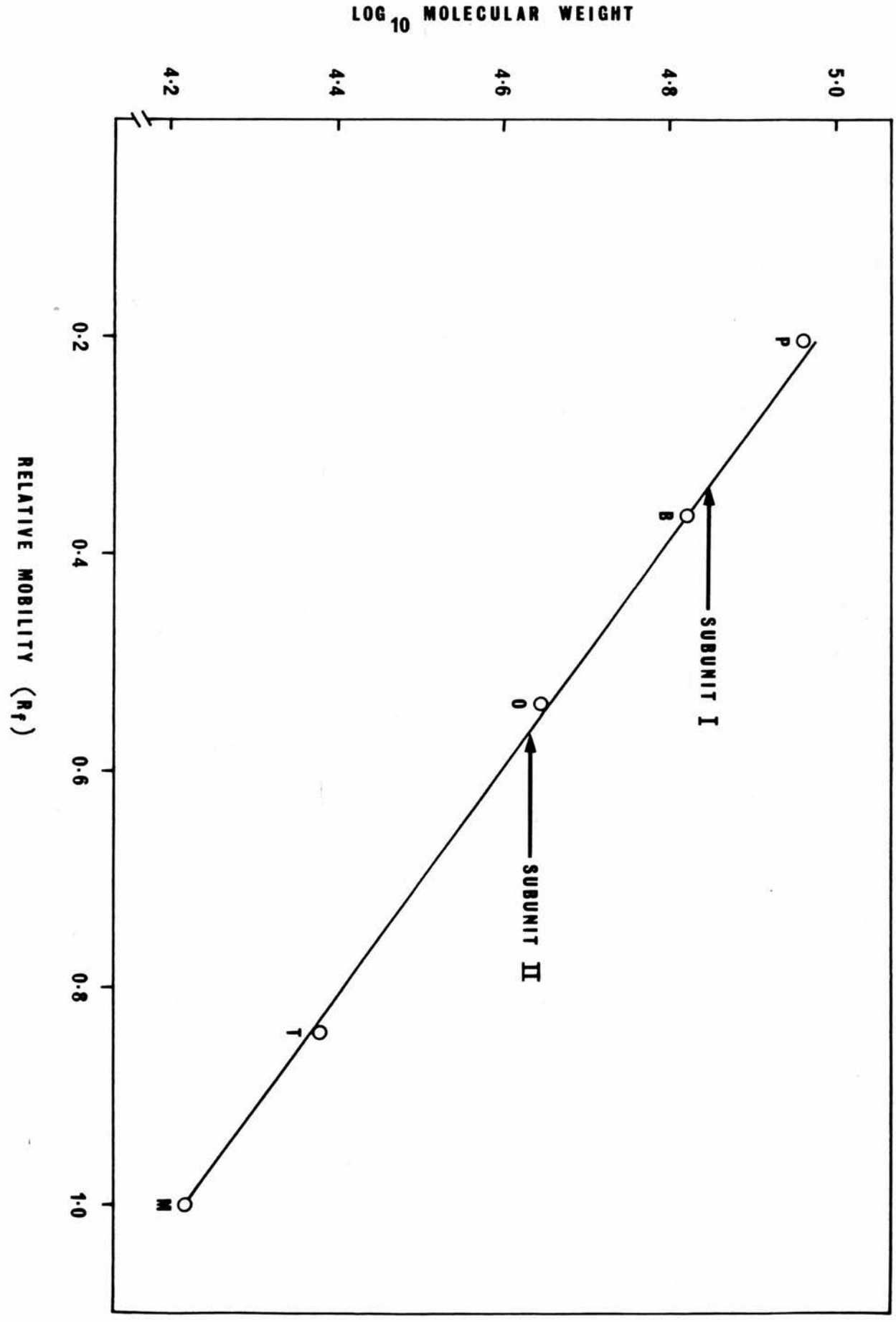
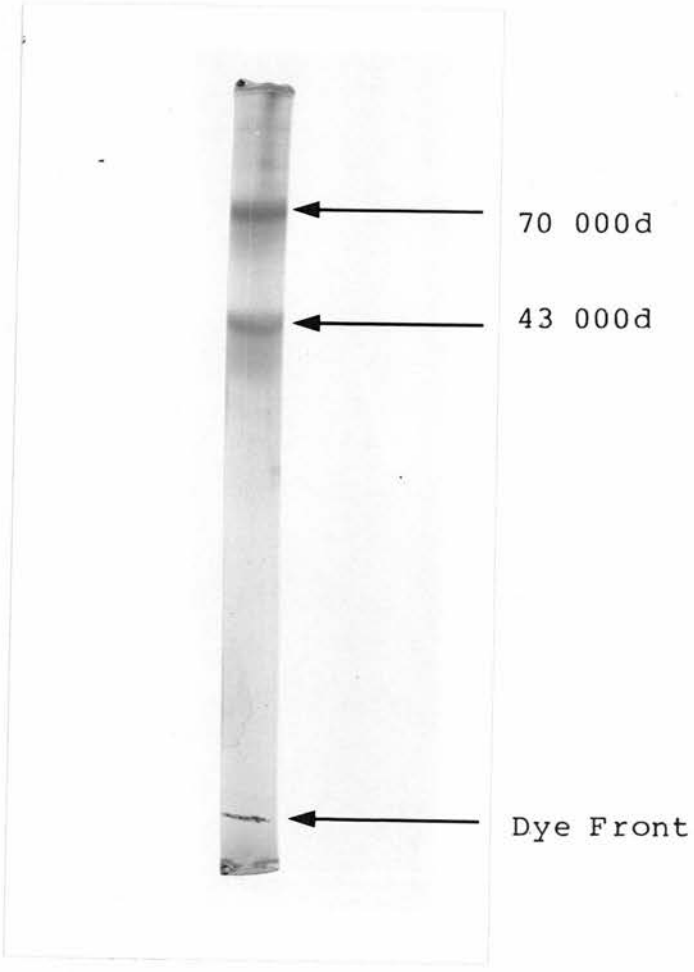

LOG 10 MOLECULAR WEIGHT
5·0
4·8
4·6
4·4
4·2
RELATIVE MOBILITY (Rf)
0·2
0·4
0·6
0·8
1·0
P
B
O
T
M
SUBUNIT I
SUBUNIT II

FIGURE 19

SDS Polyacrylamide Gel Electrophoresis of the Purified Cytochrome d Complex

Approx. 30µg of pooled peak chromatofocusing fractions, treated with 5% (w/v) SDS, 1% (v/v) β-mercaptoethanol; were electrophoresed on a 10% (w/v) acrylamide gel as described in Methods section 3. The dye front was marked and the gel stained with Coomassie Blue.


70 000d
43 000d
Dye Front

The reduced minus oxidised difference spectrum of the purified complex at 77K (Fig. 21) revealed a single peak at 560nm indicating the presence of only one *b*-cytochrome while the broad absorbance centred at 595nm suggests cytochrome a_1. However, the identity of this component is currently the subject of debate (Koland *et al.*, 1984, Baines *et al.*, 1984). The peak at 629nm and the trough at 652nm are due to the reduced and oxygenated forms of cytochrome *d*, respectively.

DISCUSSION

The two previously published complete purifications of cytochrome *d* complexes (Miller and Gennis, 1983; Kita *et al*. 1984b) and the work described here report isolated complexes of similar cytochrome composition, namely, a *b*-cytochrome absorbing between 558 and 560nm, a cytochrome a_1 and cytochrome *d*. The optical properties of these are discussed in greater detail in Chapter 3.

While there is agreement over the number of different subunits comprising the complex, estimations of their respective molecular weights by SDS PAGE have produced different results. The complex described here has a large subunit with a molecular weight of 70 000 and a smaller subunit of 43 000 daltons. The values obtained were similar in gels of 7, 10 and 12.5% (w/v) acrylamide concentration. The apparent molecular weights of the enzymes subunits obtained by Kita *et al* . (1984b) of 51 000 and 26 000 daltons were also unaffected by varying the acrylamide concentration in the gel, but these differed markedly in size from those described here. Miller and Gennis (1983) reported subunit molecular weights of 57 000 and 43 000 for their cytochrome *d* complex. Only the smaller of the subunits is comparable with the cytochrome *bd* complex described herein, and neither are similar in size to the subunits described by Kita *et al*. In addition, Miller and Gennis report that the 43 000 dalton subunit behaved as a 28 000 dalton species on gels of 12.5% (w/v) acrylamide concentration, while no variance in

TABLE 6

Reported Molecular Weight for the Subunits of Cytochrome *bd* from *E. coli*

	Large Subunit	Small Subunit
This work	70 000	43 000
Miller and Gennis (1983)	57 000	43 000*
Kita *et al* . (1984b)	51 000	26 000

*This subunit behaved as a 28 000 dalton species in gels of 12.5% (v/v) acrylamide concentration.

estimated molecular weight with acrylamide concentration was found during the work described in this thesis.

There are three possible reasons for the disparity in the reported molecular weights. One is the use of different strains of *E. coli*, another is the use of different growth conditions affecting the cytochrome and the third is different proteinase activities, as a consequence of the other differences, affecting the purification procedure to varying extents.

Miller and Gennis (1983) and Kita *et al.* (1984b) employed aerobic growth to late log phase, with low aeration, on lactate medium while the cells used in this work were grown anaerobically on a glycerol/fumarate medium. The transition from aerobiosis to anaerobiosis effects major changes in the cellular protein composition of *E. coli* (Smith and Neidhardt, 1983a,b) including stimulating production of cytochrome *d* (Kranz and Gennis, 1983) and may result in the repression of proteinases which may have been responsible for the significant degree of proteinase activity reported by Miller and Gennis (1983). This proteinase activity was considered by these authors to be high enough to necessitate the inclusion of phenylmethylsulphonyl fluoride in their buffers and possibly contributed to the loss of 33% of the total cytochrome b_{558} during cell breakage and pelleting of the membrane fragments. Later work by this group (Kranz and Gennis, 1984) on cytochrome *bd* has shown that the large subunit is susceptible to proteinase degradation from 57 000 to 30 000 daltons while the smaller subunit is much more

resistant. No figures are given for the recovery of cytochrome *bd* during the membrane particle preparation stages by Kita *et al.* (1984b), nor was any proteinase activity reported.

Factors other than strain differences and growth conditions may be involved in this controversy. A comparison of two recent purifications of cytochrome ^{o} from *E. coli* (Kranz and Gennis, 1983, Matsushita *et al.* 1984) is interesting. Both groups used the same strain of *E. coli* grown under similar conditions but harvested the cells at different growth phases and employed different purification protocols. Kranz and Gennis (1983) reported subunits of 51 000, 28 000, 18 000 and 12 700 daltons while Matsushita *et al.* (1984) reported subunits of 66 000 (varying with acrylamide concentration), 35 000, 22 000 and 17 000 daltons.

It is likely that the difference between the pI of the cytochrome *bd* reported here, of 4.8 and the value of 5.3 reported by Miller and Gennis (1983) is due to the different composition of the subunits. It may also be due, in part, to different quantities of phospholipid being associated with the complex. Kranz and Gennis (1984) report that glycolipid is still associated with the cytochrome *d* complex after purification by isoelectric focusing.

The specific content of cytochrome b_{558} of 7.1nmol/mg reported here is considerably lower than the 18.9nmol/mg

and 12.3nmol/mg reported by Miller and Gennis (1983) and Kita *et al.* (1984b) respectively.

These specific contents were calculated from the pyridine ferrohaemochromogen spectra of their purified preparations and if, as is currently suggested (Koland *et al.*, 1984, Baines *et al.*, 1984), the cytochrome a_1 apparent in the complex is in fact a *b*-type cytochrome then the cytochrome "a_1" will contribute greatly to the absorbance of the pyridine ferrohaemochromogen but contribute little to the cytochrome *b* α-band at approx. 560nm. The result will be an anomalously high protohaem concentration accorded to cytochrome b_{558} alone with the consequent calculation of a high specific content. However, this does not explain the difference between the specific content reported here and those published elsewhere as the millimolar extinction coefficient used to calculate the cytochrome b_{558} content of the purified cytochrome *bd* preparation reported here was taken from Miller and Gennis (1983) and thus contains the possible error discussed above.

A likely reason for the large differences in specific content is the influence of Triton X-100 on the protein estimation procedure used for the purified preparation. As discussed in the Results section of this chapter, this will produce a lower-than-actual specific content by overestimating the protein content. In addition, assuming similar subunit and haem stoichiometres, the larger size of the enzyme described here would give a lower specific content.

CHAPTER THREE

THE EFFECT OF LIGAND BINDING ON THE SPECTRAL PROPERTIES OF CYTOCHROME *bd*

INTRODUCTION

The Spectral Properties of Cytochrome *d* of the Cytochrome *bd* Complex

The spectral properties of cytochrome *d* are distinctive due to the presence of an iron chlorin as the prosthetic group. In the absolute spectra a peak at around 630nm is seen in the reduced form while a further peak, at around 650nm, is now attributed to an oxygenated form of the enzyme. This latter feature was thought to be due to the fully oxidised form of cytochrome *d* . The Soret band of cytochrome *d* is now generally accepted to be weak and diffuse (Chance, 1953; Reid and Ingledew, 1979, 1980; Poole and Chance, 1981; Poole *et al.*, 1981, 1982a). However, as cytochrome *d* has yet to be isolated from any organism free from other cytochromes, this must be based on indirect evidence. Recent support for the view that the cytochrome *d* Soret band is weak has come from several investigations: (a) the selective photolysis of carbonmonoxy-cytochrome *d*, with a HeNe laser at low temperatures, in the presence of oxygen, produces significant spectral changes at 646-650 nm and 633-635 nm. These are due to the formation of the oxygenated form and disappearance of the reduced form respectively. There are, however, no significant changes in the Soret region (Poole and Chance, 1981); (b) a suspension of *E. coli* in which all the cytochromes except cytochrome *d* were reduced, still showed a prominent Soret shoulder at around 448 nm (Poole and

Chance, 1981); (c) the photodissociation reflectance spectrum of *E. coli*, at 4K, reveals a prominent α-band attributed to cytochrome *d*, but only a small peak in the Soret region (Poole *et al.*, 1982a); (d) a CO-liganded, reduced *E. coli* suspension which is photolysed, at -121°C, in the absence of oxygen does not reveal any spectral features of cytochrome *d*. However, in the photodissociation spectra, a variation in the magnitude of a peak at 436nm with the number of light flashes was observed. This peak has been attributed to carbon-monoxy-cytochrome *d* (Rice and Hempfling, 1978). The change in intensity was identical whether oxygen was present (when a cytochrome *d* α-band was produced) or not and was, therefore, likely to be independent of cytochrome *d* (Poole and Chance, 1981); (e) a band absorbing at 448nm in the reduced-plus-CO minus reduced spectrum, previously thought to be due to cytochrome *d*, has been assigned to cytochrome a_1 (Poole *et al.*, 1981).

A further absorption band at 675-680nm has been reported (Pudek and Bragg, 1974; Hendler and Schrager, 1979; Poole *et al.*, 1983a). When a CO-liganded sample of cytochrome *d* was photolysed at low temperatures this 675nm feature appeared and increased as the absorbance at 650nm, due to the oxygenated form, was lost. These two features are unlikely to be directly related because their respective rates of formation and loss were different, with no clear isobestic point between the 650nm and 675nm components being apparent (Poole *et al.*, 1983a).

The Reaction of Cytochrome *d* with Oxygen

Cytochrome *d* is a terminal oxidase in *E. coli*, as deduced from photochemical action spectra (Castor and Chance, 1959) and kinetic data obtained by stopped-flow spectrophotometry (Haddock *et al.*, 1976). Its reaction with oxygen is unusual in that a stable oxygenated compound, cytochrome d_{650}, distinct from the oxidised state of the cytochrome, is formed (Poole *et al.*, 1983a). This hypothesis is based largely on evidence obtained by Poole and colleagues using low temperature trapping and ligand exchange procedures. The main points of their argument are: (a) flash photolysis, at -130°C, of substrate-reduced and CO-bound membranes in the presence of O_2, generated a sharp symetrical band at 650-652nm (where the reference spectrum was that of the CO-liganded, reduced form) (Poole *et al.*, 1983a); (b) when this trapped state was investigated by EPR spectroscopy its spectrum was identical to that of the CO-bound form (Poole *et al.*, 1983a) and there was no evidence of the g=6 high spin resonances of oxidised cytochrome *d* (Der Vartanian *et al.*, 1973); (c) at relatively high temperatures (-80° to -90°C), *b*-type cytochromes were oxidised simultaneously with the disappearance of cytochrome d_{650}. If the cytochrome d_{650} was the fully oxidised enzyme then electron transfer from the *b*-type cytochromes would reform the reduced oxidase. This, however, was not observed (Poole *et al.*, (1983b); (d) cytochrome d_{650} can be formed at room temperature

by aeration but not by ferricyanide in the absence of O_2 (Pudek and Bragg, 1976; Poole *et al.*, 1983a) nor by electrodic potentiometry (Koland *et al.*, 1984); (e) resonance Raman spectroscopy (Poole *et al.*, 1982b) of aerated, solubilised cytochrome *d* shows absorptions (1078 to 1105cm^{-1}) attributed to the oxygen-oxygen stretching frequency of a haem-oxygen adduct, similar to oxyhaemoglobin and oxymyoglobin.

Consideration of these results, along with the unusual potentiometric and ligand binding properties of cytochrome d_{650}, led Poole *et al.* (1983) to propose that reduced cytochrome *d* reacted with oxygen to form the species absorbing at 650nm.

$$(Fe^{2+})_{d630} \xrightarrow{O_2} (Fe^{2+}.O_2)_{d650} \underset{h\gamma+O_2}{\overset{CO}{\rightleftharpoons}} d_{639}$$

This is an intermediate in the oxidoreduction of cytochrome *d* and can react with CO or go on to form the fully oxidised form of cytochrome *d* which has no specific absorbance in the red region of the spectrum.

$$(Fe^{2+}.O_2)_{d650} \longrightarrow (Fe^{3+})_{d}$$

This is the form of cytochrome *d* which should be equated with the invisible species, d^*, described by Pudek and Bragg (1974). Cyanide reacts with this species.

$$(Fe^{3+})_{d} \underset{Na_2S_2O_4}{\overset{CN^-}{\rightleftharpoons}} (Fe^{3+}.CN)_{d}$$

NADH

The cyanocytochrome *d* is "trapped" in the fully oxidised, "invisible" state and is, therefore, colourless itself.

The Reaction of Cytochrome *d* with Nitrogen Compounds

Meyer (1973) reported that if a succinate-reduced suspension of ETPs, prepared from stationary phase *E. coli* grown under low aeration, was bubbled with NO or treated with HNO_2, spectra resulted which were similar, in the region 610-720nm, to those produced by aeration. However, differences in the spectra for HNO_2 and NO-treated samples at wavelengths below this were evident, particularly in the Soret region. He suggested that these may have been due to NO reacting with cytochromes *o* and a_1 as well as cytochrome *d*, while HNO_2 reacted with cytochrome *d* to produce *d*-NO. This NO was tightly bound and, therefore, not free to react with other cytochromes.

An investigation into the effect of silver ions on the respiratory chain of *E. coli* by Bragg and Rainnie (1974) showed that the addition of $AgNO_3$ to reduced ETP suspensions gave a decrease in the 628nm peak of cytochrome *d* and a slower decrease in the trough at 650nm. However, this did not occur on addition of $NaNO_3$ and thus the effect was thought to be caused by silver ions converting the cytochrome into a form which had little absorption at 628nm.

Haddock *et al*. (1976) and Reid and Ingledew (1979) reported that cells grown anaerobically in the presence of NO_3^- displayed a shift of the absorption maximum of reduced cytochrome *d* from 630nm to 636nm and a diminished α-band.

Recently, Hubbard *et al*.(1983) have presented evidence that the spectral changes to cytochrome *d* caused by the addition of $AgNO_3$ and seen in nitrate-grown cells is due to the formation of a nitrosyl species. They found that the same spectral effect was produced on adding $NaNO_3$, KNO_3 and, at different rates, by the addition of NO_2^-, $N_2O_3^{2-}$ and NO. The addition of silver ions in the form of $AgSO_4$ had no effect.

The Reaction of Cytochrome *d* with CO

The binding of CO by cytochrome *d* has been shown to cause a red shift of the reduced cytochrome *d* peak, by approx. 6nm, for both the membrane-bound and solubilised enzyme (Poole and Chance, 1981; Miller and Gennis, 1983; Kita *et al*., 1984b). This reaction of cytochrome *d* differs from that of all other reported cytochrome oxidases in that following flash photolysis of the carbonmonoxycytochrome, between 5K and 120K, CO is rebound even in the presence of O_2. The speed of CO rebinding is such that, in order to obtain spectra of the photolysed compound, temperatures close to that of liquid helium must be used (Poole *et al*., 1982a). If the CO-liganded cytochrome is photolysed, in the presence

of O_2, at 5K, and the temperature is then raised to 205K before recooling to 5K, the CO is still bound. The rebinding of CO at 5K is a first order process while at 20K it is complex. This, plus the rapid rebinding of CO, is similar to the behaviour of myoglobin and haemoglobin (Poole, 1983).

The Reaction of Cytochrome *d* with Cyanide

Pudek and Bragg (1974) were the first to report the unusual cyanide resistance of cytochrome *d* from *E. coli*. They found that stationary phase cells were less susceptible to KCN inhibition of NADH oxidation than cells harvested in log phase. This resistance to inhibition could be directly related to the amount of cytochrome *d* present. On further investigation they found that the reaction of KCN with cytochrome *d* removed its α-band. Addition of KCN to a reduced suspension of membrane vesicles resulted in the loss of the 628nm absorption peak, due to the reduced cytochrome *d*, only while O_2 was present. On attainment of anaerobic conditions the decrease in peak size stopped. Addition of KCN to an anaerobic vesicle suspension plus NADH had no effect on the spectrum of cytochrome *d*. However, subsequent aeration of the suspension caused a rapid disappearance of the 628nm band without formation of the 648nm absorption band of the oxygenated enzyme. Removal of the 648nm absorption (without formation of another band) was noted on addition of KCN to an oxygenated membrane suspension without substrate but its rate of disappearance was much slower than the rate of

substrate oxidation inhibition seen on addition of substrate to a similar suspension plus KCN. The disappearance of the 648nm absorbance in the presence of oxygen, KCN and substrate i.e. when there was passage of electrons through the system, proceeded at a rate similar to that of the inhibition of substrate oxidation. This led Pudek and Bragg to propose that cyanide was reacting with an intermediate between the oxidised and reduced states.

Manipulation of the rate of electron flux through the system, by the use of various substrates and their competitive inhibitors, gave a correlation between the rate of substrate oxidation and the rate of cyano-cytochrome formation. This led to the proposal of an intermediate form of cytochrome *d* (*d**) between the reduced (628nm) and oxidised (648nm) species. This was the cyanide binding form, had little absorbance in the α-band region and was analagous to the d^{x}-form proposed for the cytochrome *d* of *Azotobacter vinelandii* (Kauffmann and Van Gelder, 1973).

MATERIALS

Tetrachlorohydroquinone was obtained from the Sigma Chemical Co.

Carbon monoxide and nitric oxide were obtained from BOC Ltd.

Hydroquinone was obtained from British Drug Houses.

All other chemicals were of the highest quality available from the usual commercial sources.

METHODS

Spectrophotometer

All the spectra in this chapter were obtained using a split-beam, wavelength-scanning spectrophotometer which was built in the departmental workshops. In essence it was comprised of a light source (usually tungsten; sometimes Xenon arc), an Applied Photophysics monochromator, Bulova Time Products oscillator and an EMI photomultiplier with supporting power supplies and circuitry.

Partial Purification of Cytochrome *bd* for use in Ligand-Binding Spectral Studies

The method of purification was, essentially, that described in Methods section 2, Chapter 1, for cytochrome solubilisation and ion-exchange chromatography.

After the peak cytochrome-containing fractions from the large DEAE-Sephacel column were pooled, they were passed down a Sephadex G-25 column equilibrated with 50mM TES/5mM EDTA/0.1% Triton X-100, pH7.0 and then concentrated, approx. five-fold, in an Amicon concentration cell fitted with an XM50 membrane. After concentration the cytochrome preparation was stored at 77K until required. The buffer-exchange step after ion-exchange chromatography enabled both the ETPs and solubilised cytochrome to be investigated in the same buffer, thus lessening the possibility that any visible

differences between the spectra of the membrane-bound and solubilised cytochromes were due to the reaction of a buffer component with the ligand or cytochrome.

Both ETP suspensions and a partially purified cytochrome *bd* preparation were used in the ligand binding studies detailed below. A comparison of the spectra obtained from these two samples will show the effect (if any) of solubilisation on the ligand reactions of cytochrome *d*. Use of a partially purified cytochrome *bd* preparation allowed the investigation of the ligand binding of the cytochrome *bd* complex free from the spectral interference due to cytochrome b_{556} which is present in the ETPs but absent from the partially purified preparation.

Reduced Minus Oxidised Difference Spectra, at 77K, of Partially Purified and Purified Cytochrome *bd*

These spectra were obtained using an aluminium "spade" cuvette with perspex "windows". The sample was maintained at 77K by submerging the bottom of the aluminium blade of the cuvette in liquid nitrogen which was contained in an unsilvered Dewar flask through which the light-path passed.

Reduced Minus Reduced-Plus-NO Difference Spectra

Electron Transport Particle Suspension

ETPs were suspended in 50mM TES/5mM EDTA pH7.0 and placed in stoppered 3ml cuvettes. Both cuvettes were oxidised by vigorous stirring in air and a baseline spectrum scanned. The reference cuvette was reduced with a few grains of $Na_2S_2O_4$ and the difference spectrum scanned. The sample cuvette was then reduced with the minimum of $Na_2S_2O_4$ necessary to produce a featureless reduced minus reduced spectrum before NO was bubbled through the suspension for 10 sec. The cuvette was flushed with NO before being tightly stoppered to exclude oxygen. The reduced minus reduced-plus-NO spectrum was then scanned.

Partially Purified Cytochrome *bd*

An essentially similar procedure to that used for ETPs was followed. NO was introduced by the injection of 10µl of NO-saturated buffer into the tightly-stoppered, reduced, sample cuvette via a small hole in the stopper. Mixing was effected by agitating a small glass bead contained within the cuvette. The difference spectrum was scanned and a further 10µl injected, with mixing, before rescanning.

The NO-saturated buffer was prepared as follows: 20ml of boiled 10mM TES/5mM EDTA, pH7, were bubbled with NO

for 30 min. The pH of the solution was adjusted to pH7.0 with boiled 3M NaOH and the solution bubbled with NO for a further 30 min prior to use.

Reduced Minus Reduced-Plus-CO-Difference Spectra

Electron Transport Particle Suspension

A similar procedure to that used to obtain the reduced minus reduced-plus-NO spectrum of ETPs was employed except that CO gas was bubbled through the reduced sample for 1 minute before scanning.

Partially Purified Cytochrome *bd*

This spectrum was obtained employing the same procedure as the ETP-plus-CO spectrum.

Potassium Ferricyanide-Oxidised Difference Spectra

Electron Transport Particle Suspension

ETPs were suspended in 50mM TES/5mM EDTA, pH7.0 buffer and the suspension made 10μM with respect to hydroquinone and tetrachlorohydroquinone. Stoppered quartz cuvettes, of 3ml capacity, were filled with the suspension and both samples vigorously stirred in air before the oxidised minus oxidised baseline spectrum was scanned. The reference cuvette was then reduced with a few grains of sodium dithionite and tightly stoppered before the reduced minus oxidised spectrum was scanned. The sample cuvette was tightly stoppered and reduced with the minimum

quantity of a 10mg/ml sodium dithionite solution, introduced through a small hole in the stopper, required to produce a featureless reduced minus reduced spectrum. The sample was then reoxidised by injecting 40µl of 50mM TES/5mM EDTA, pH7.0 buffer, which had been saturated with potassium ferricyanide, boiled and subsequently bubbled with oxygen-free N_2 for 60 min. Mixing was achieved by agitating a small glass bead contained within the cuvette. The spectrum was rescanned.

Partially Purified Cytochrome *bd*

The protocol described above for the anaerobically oxidised ETPs suspension was employed.

RESULTS

Reduced Minus Oxidised Difference Spectra of Purified Cytochrome *bd*

Spectra were scanned at room temperature and with the sample maintained at 77K. This low temperature produces a sharpening and intensification of absorbance peaks which enables a more detailed examination of spectral fine structure to be made. The sharpening of the absorption bands (and some degree of intensification) is caused by the reduction in thermal energy of the system resulting in fewer π-electrons being in the excited state at any one time. Thus, a greater proportion of the "excitable" π-electrons of the chromophore are available to absorb light and be promoted to a higher energy level. In addition, these electrons lose their energy less quickly and, as spectral linewidth is inversely proportional to the lifetime of the excited state, the longer lifetime gives a sharper band. The majority of the spectral intensification occurs when the sample freezes into a mass of ice crystals. These provide a myriad of reflective surfaces throughout the sample, increasing the lightpath through the sample with a consequent increase in absorbance. A further effect of cooling the sample to 77K is the "blue shift". This is the displacement of absorption maxima to shorter wavelengths.

All these effects can be seen by a comparison of the difference spectrum of purified cytochrome *bd* obtained at 20°C (Fig. 20) with that obtained at 77K (Fig. 21). At 20°C the Soret band comprised a broad peak centred around 430nm while at 77K this was clearly resolved into two components, one absorbing at 431nm, the other, of almost equal intensity, absorbing at 440nm. In the 77K spectrum there is a general sharpening of the cytochrome *b* α and β-bands with the α-band being displaced from 562 to 560nm. A marked sharpening of the cytochrome a_1 α-band at 595nm is also evident. The absorbance peak at 630nm, due to the reduced form of cytochrome *d*, is sharpened and displaced to 629nm by cooling to 77K and a striking difference is evident, between 650 and 750nm, with the low temperature spectrum clearly defining the trough at 655nm of the oxygenated cytochrome *d* and revealing a further absorption band at approx. 680nm which is not evident from the 20°C spectrum.

The Partial Purification of Cytochrome *bd*

Because of the lengthy procedure involved in preparing purified cytochrome *bd* and the low yield obtained, it was decided to use a partially purified cytochrome *bd* preparation for the ligand binding spectra of the solubilised enzyme. This was considered an acceptable alternative as the major contaminating cytochrome, cytochrome b_{556}, was removed by the preliminary extraction

FIGURE 20

A Reduced Minus Oxidised Difference Spectrum of Purified Cytochrome *bd* at 20°C

Specific Content = 7.1 nmol b_{558}/mg

Protein Concentration = 0.55mg/ml

Pathlength = 0.5cm

Scan Speed = 0.5nm/sec

Bandwidth = 1nm

Chart Speed = 0.5mm/sec

The reference cuvette was oxidised by stirring vigorously in air while the sample cuvette was reduced with a few grains of sodium dithionite.

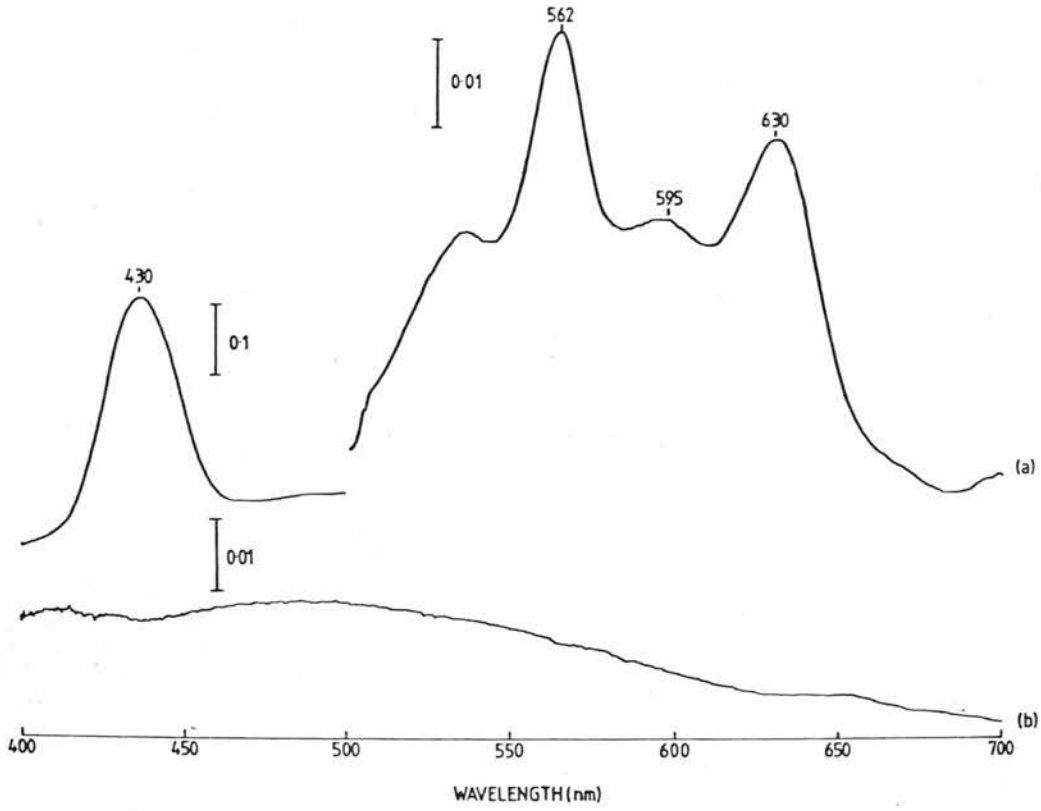

562
0·01
630
595
430
0·1
(a)
0·01
(b)
400
450
500
550
600
650
700
WAVELENGTH (nm)

FIGURE 21

A Reduced Minus Oxidised Difference Spectrum of Purified Cytochrome *bd* at 77K

Specific Content = 7.1nmol b_{558}/mg

Protein Concentration = 0.55mg/ml

Pathlength = 0.2cm

Scan Speed: 400-500nm = 0.7nm/sec

500-700nm = 0.5nm/sec

Bandwidth = 1nm

Chart Speed = 0.5mm/sec

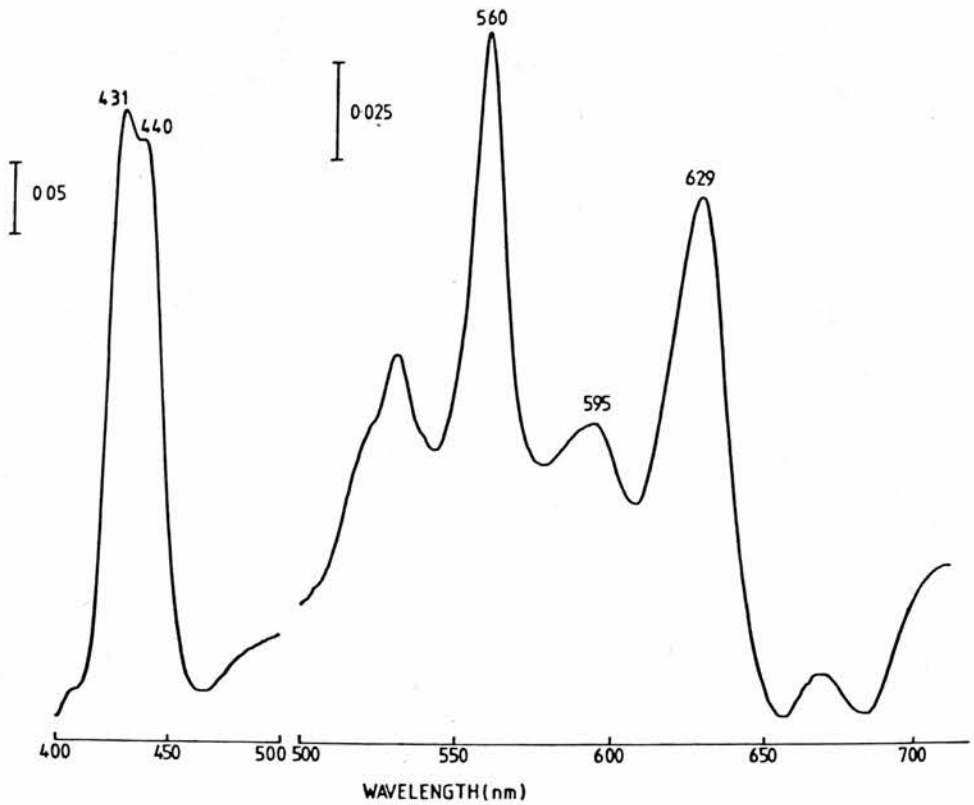

0·05
431
440
0·025
560
595
629
400
450
500
500
550
600
650
700
WAVELENGTH(nm)

FIGURE 22

A Reduced Minus Oxidised Difference Spectrum of Partially Purified Cytochrome *bd* at 77K

Specific Content = 2.8nmol cytochrome *b*/mg

Protein Concentration = 1.5mg/ml

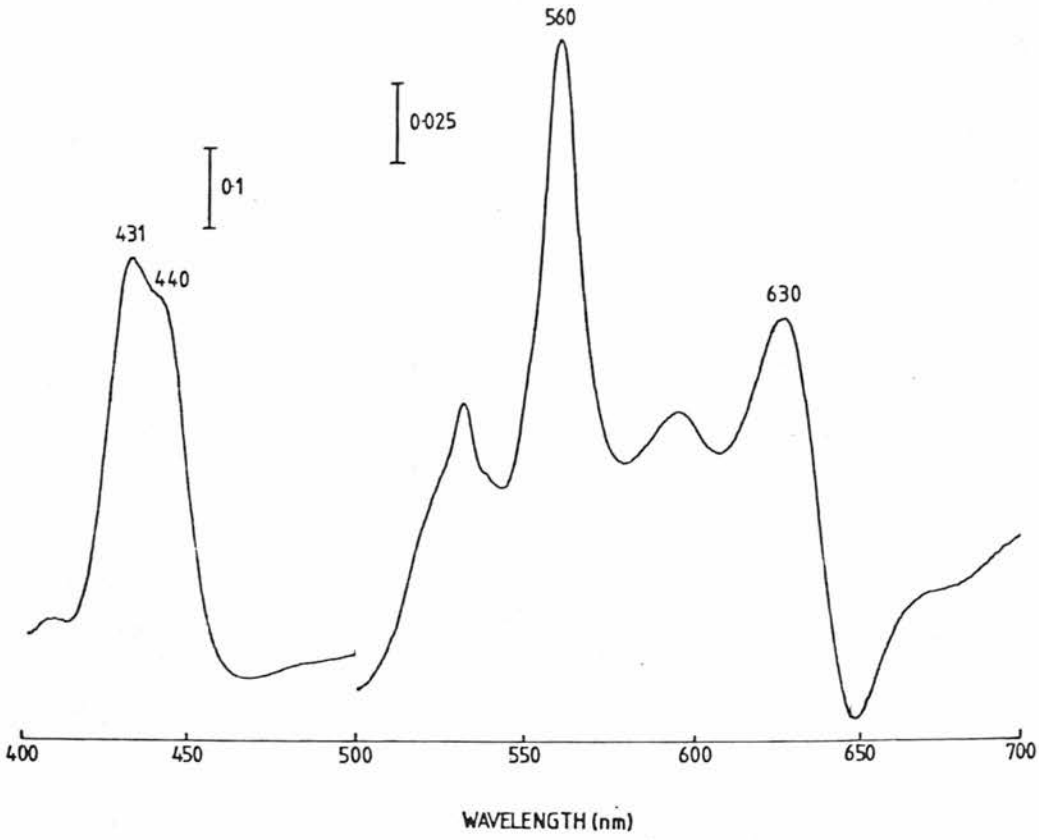
Pathlength = 0.2cm

Scan Speed = 0.5nm/sec

Bandwidth = 1nm

Chart Speed = 0.5mm/sec

The reference cuvette was oxidised by stirring vigorously in air while the sample cuvette was reduced with a few grains of sodium dithionite prior to freezing in liquid nitrogen.


560
0·025
0·1
431
440
630
400
450
500
550
600
650
700
WAVELENGTH (nm)

of the ETPs with 0.75% (v/v) Triton X-100-containing buffer. In addition, the low temperature difference spectrum of the pooled peak cytochrome-containing fractions from the DEAE-Sephacel column (Fig. 22) showed no major differences from the low temperature difference spectrum of the purified cytochrome (Fig. 21).

The partially purified cytochrome was much easier to obtain in quantities that enabled the use of a cytochrome concentration compatible with lower spectrophotometer gain. Consequently, smoother, less noisy spectra were obtained.

Reduced Minus Reduced-Plus-NO Difference Spectra

Bubbling the ETP suspension with NO for longer than 10 seconds caused excessive acidification of the suspension, resulting in denaturation of the cytochrome. Similarly, bubbling NO through the partially purified cytochrome preparation was not possible as even the briefest exposure resulted in enzyme denaturation. This problem was overcome by adding small quantities of NO-saturated buffer to the reduced cytochrome although this was less efficient at excluding oxygen.

Bubbling a reduced ETP suspension with NO resulted in a significant change in the Soret region (Fig. 23) from a peak at 433nm with a strong shoulder at approx. 443 to a single peak at 443nm. The α and β-bands of cytochrome b_{558}, at 562 and 535nm respectively, were

not evident and the broad peak at 595nm due to cytochrome a_1 was shifted to approximately 605nm. The peak at 630nm, due to reduced cytochrome *d*, appeared to be attenuated but this may have been due to the shift in the absorbance of cytochrome a_1 from 595 to 605nm, as the peak-to-trough distance from 630nm to 653nm was the same in both the reduced minus oxidised and reduced minus reduced-plus-NO spectra. No changes in the absorption maxima wavelengths of the α-band of cytochrome *d* were apparent.

The results obtained for the partially purified cytochrome *bd* preparation were essentially similar to those of the ETP suspension (Fig. 24). The addition of 10µl of NO-saturated buffer is clearly not enough to react with all the cytochrome as the peak-to-trough distance for the cytochrome *d* α-band was much less than that for the reduced minus oxidised spectrum. In this case the addition of a further 10µl of NO-saturated buffer appears to be accompanied by the admission of some oxygen, judging by the change in lineshape in the Soret region producing a large shoulder at 433nm, due to cytochrome b_{558}, accompanied by a small increase in absorbance at 562nm. This effect was very difficult to avoid when adding larger volumes of NO-saturated buffer to the cuvette.

FIGURE 23

A Reduced Minus Reduced-Plus-NO Difference Spectrum of an Electron Transport Particle Suspension at 20°C

Specific Content = 0.5nmol cytochrome *b*/mg

Protein Concentration = 5.3mg/ml

Pathlength = 1cm

Scan Speed = 0.5nm/sec

Bandwidth = 1nm

Chart Speed = 0.5mm/sec

a = reduced minus oxidised spectrum.

b = reduced minus reduced-plus-NO spectrum.

c = oxidised minus oxidised baseline.

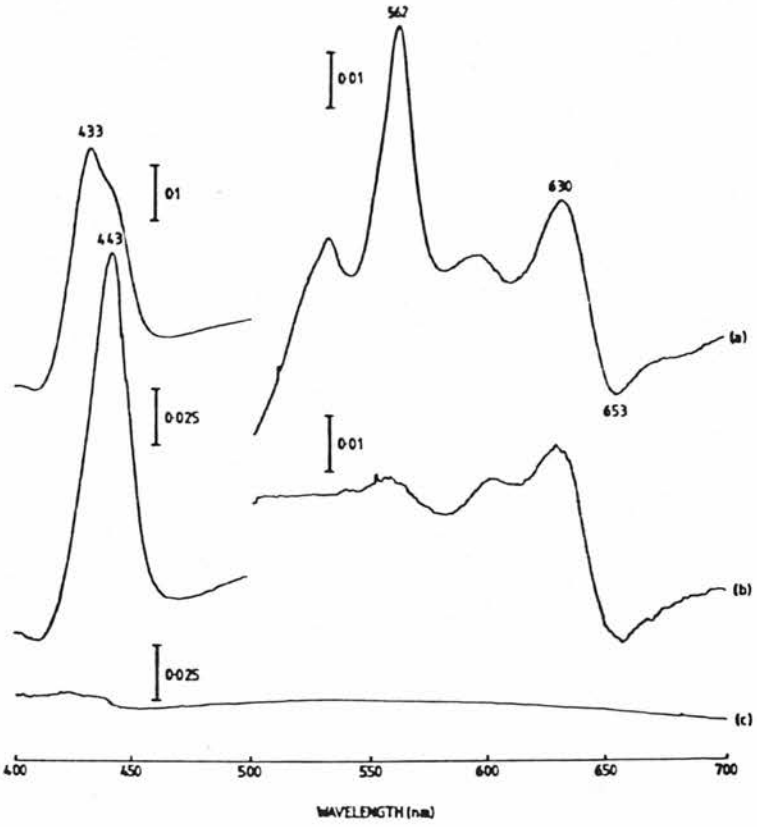

562
0·01
433
0·1
630
443
653
(a)
0·025
0·01
(b)
0·025
(c)
400
450
500
550
600
650
700
WAVELENGTH (nm)

FIGURE 24

A Reduced Minus Reduced-Plus-NO Difference Spectrum of Partially Purified Cytochrome *bd* at 20°C

Specific Content = 2.8nmol cytochrome *b*/mg

Protein Concentration = 1.5mg/ml

Pathlength = 1cm

Scan Speed = 0.5nm/sec

Bandwidth = 1nm

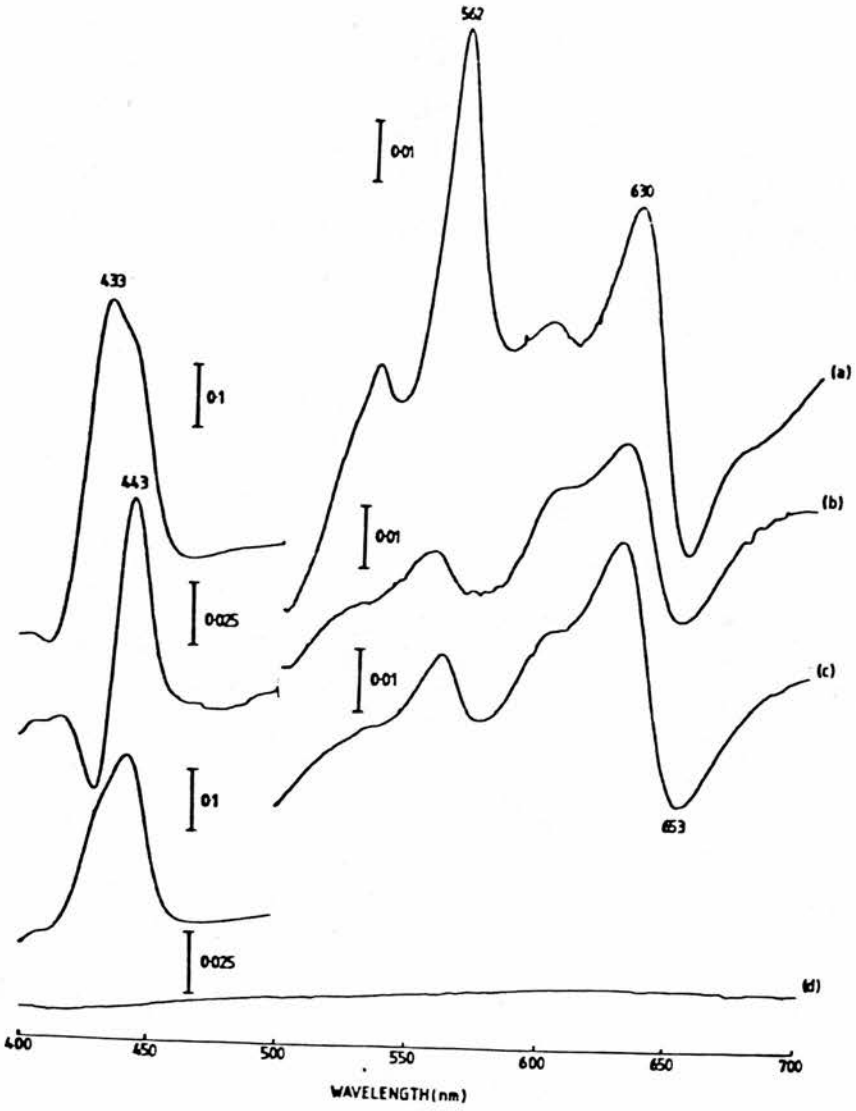
Chart Speed = 0.5mm/sec

a = reduced minus oxidised spectrum.

b = reduced minus reduced plus 10µl NO saturated buffer.

c = reduced minus reduced plus 20µl NO saturated buffer.

d = oxidised minus oxidised baseline.


562
630
433
443
653
0·01
0·1
0·025
0·01
0·01
0·1
0·025
(a)
(b)
(c)
(d)
400
450
500
550
600
650
700
WAVELENGTH(nm)

Reduced Minus Reduced-Plus-CO Difference Spectra

A split Soret band is evident in the reduced minus reduced-plus-CO difference spectrum of an ETP suspension (Fig. 25) with peaks at 433nm and 444nm. A further peak at 570nm can be seen as can a broad absorption around 600nm. The peak and trough due to cytochrome *d* occur at 625nm and 647nm respectively. This is a displacement towards the blue end of the spectrum compared with the reduced minus oxidised spectrum.

The CO-difference spectrum obtained for the partially purified cytochrome preparation shows more detail in the Soret region than that of the ETP suspension (Fig. 26). A sharp trough at 421.5nm is followed by two distinct, sharp peaks at 432nm and 447nm. Again, a small peak at 570nm can be seen and also a broad absorption centred at 600nm approx. The cytochrome *d* absorbances are blue shifted, as with the ETP suspension, to 625nm for the peak due to the reduced form, and 643nm for the trough due to the CO-liganded species. There is a decrease in the wavelength difference between the cytochrome *d* peak and trough from 22nm, for the CO-treated ETP suspension, to 18nm for the purified enzyme.

FIGURE 25

A Reduced Minus Reduced-Plus-CO Difference Spectrum of an Electron Transport Particle Suspension at 20°C

Specific Content = 0.5nmol cytochrome *b*/mg

Protein Concentration = 2.6mg/ml

Pathlength = 1cm

Scan Speed = 0.5nm/sec

Bandwidth = 1nm

Chart Speed = 0.5mm/sec

a = reduced minus reduced-plus-CO spectrum.

b = oxidised minus oxidised baseline.

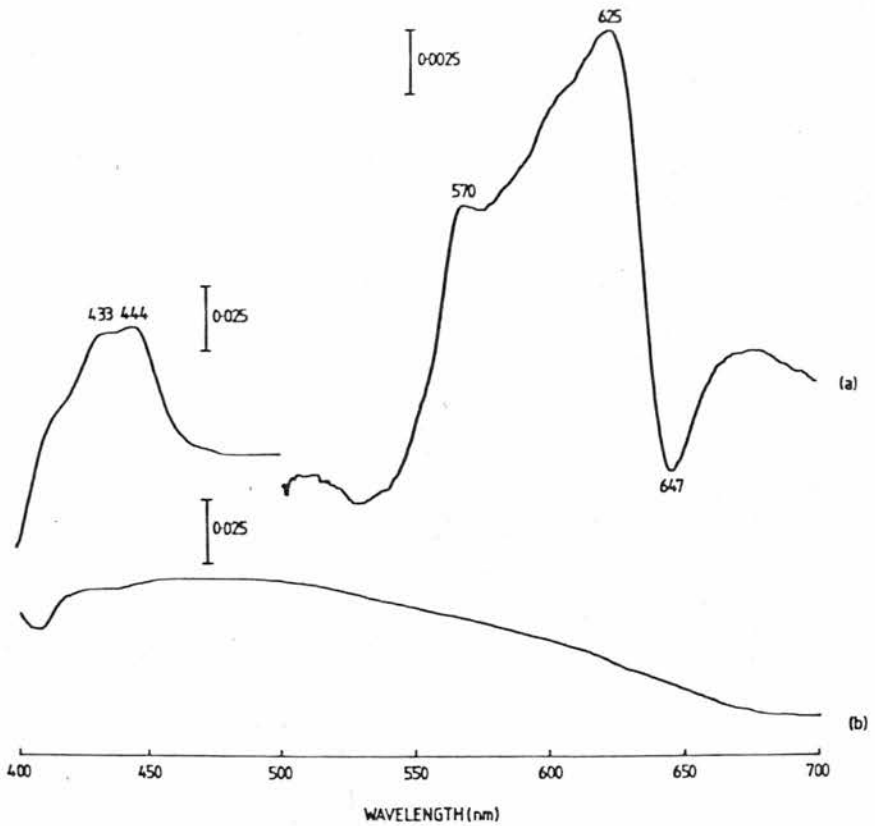

625
0·0025
570
433 444
0·025
647
(a)
0·025
(b)
400
450
500
550
600
650
700
WAVELENGTH (nm)

FIGURE 26

A Reduced Minus Reduced-Plus-CO Difference Spectrum of Partially Purified Cytochrome *bd* at 20°C

Specific Content = 2.8nmol cytochrome *b*/mg

Protein Concentration = 1.7mg/ml

Pathlength = 1cm

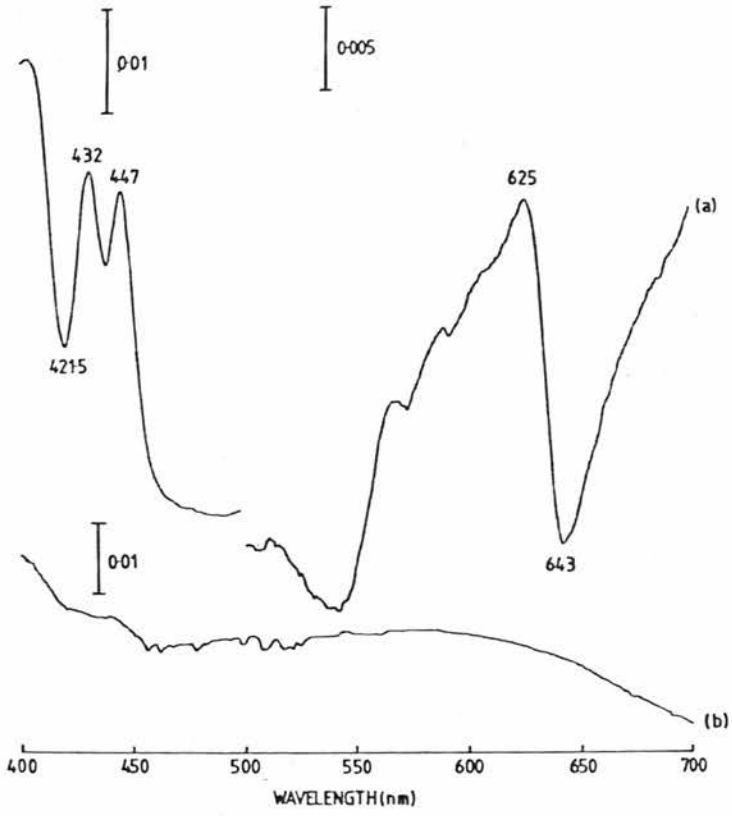
Scan Speed = 0.5nm/sec

Bandwidth = 1nm

Chart Speed = 0.5mm/sec

a = reduced minus reduced-plus-CO spectrum.

b = oxidised minus oxidised baseline.


0·01
0·005
432
447
421·5
625
(a)
643
0·01
(b)
400
450
500
550
600
650
700
WAVELENGTH(nm)

Reduced Minus Ferricyanide-Oxidised Difference Spectra

Hydroxyquinone (Em + 280mV) and tetrahydroquinone (Em + 340mV) were included in the samples to act as redox mediators and assist in the transfer of electrons from the reduced cytochrome *d* (Em + 260mV) to oxidised ferricyanide (Em + 430). They were chosen on the basis of their mid-point potentials being between +280 and +430mV and because they had little absorbance between 500 and 700nm at a concentration of 10μM (not shown). Their use gave increased efficiency of oxidation compared with ferricyanide alone. This greater efficiency permitted the use of smaller volumes of ferricyanide-saturated buffer with a consequent decrease in the likelihood of introducing unwanted oxygen into the reduced sample.

The Soret region is not shown here as ferricyanide absorbs strongly at 434nm and this large peak masked all other details.

For both the ETP suspension (Fig. 27) and the partially purified cytochrome (Fig. 28), oxidation with potassium ferricyanide resulted in a marked decrease in the trough at 655nm, compared with the aerobic spectra, while giving similar spectra in all other respects. However, if the ferricyanide-oxidised sample was subsequently stirred in air, a spectrum identical to that of the aerobic spectra was obtained (data not shown).

FIGURE 27

A Comparison of the Reduced Minus Ferricyanide-Oxidised and Reduced Minus Aerobically-Oxidised Difference Spectra of an Electron Transport Particle Suspension at 20°C

Specific Content = 0.5nmol cytochrome *b*/mg

Protein concentration = 2.6mg/ml

Pathlength = 1cm

Scan Speed = 0.5nm/sec

Bandwidth = 1nm

Chart Speed = 0.5mm/sec

a = reduced minus aerobically-oxidised spectrum.

b = reduced minus ferricyanide-oxidised spectrum.

c = oxidised minus oxidised baseline.

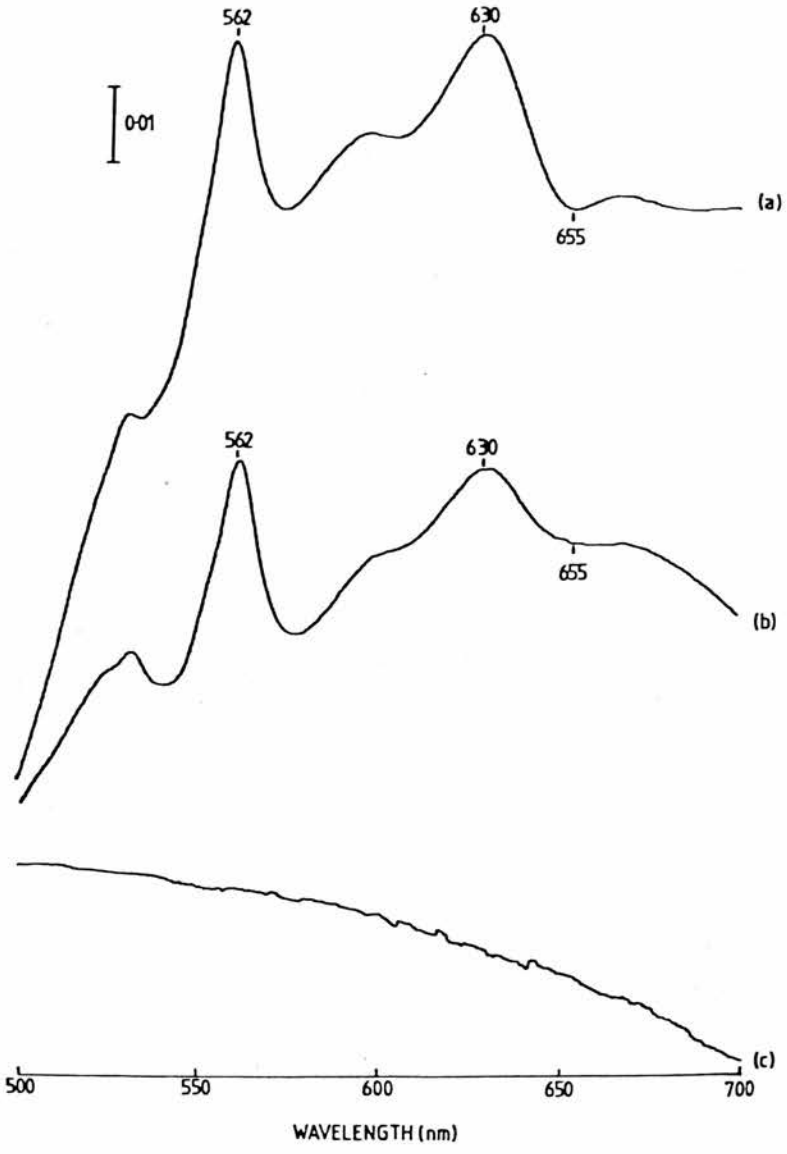

562
630
0·01
655
(a)
562
630
655
(b)
(c)
500
550
600
650
700
WAVELENGTH (nm)

FIGURE 28

A Comparison of the Reduced Minus Ferricyanide-Oxidised and Reduced Minus Aerobically-Oxidised Difference Spectra of Partially Purified Cytochrome *bd* , at 20°C

Specific Content = 2.8nmol cytochrome *b*/mg

Protein Concentration = 2mg/ml

Pathlength = 1cm

Scan Speed = 0.5nm/sec

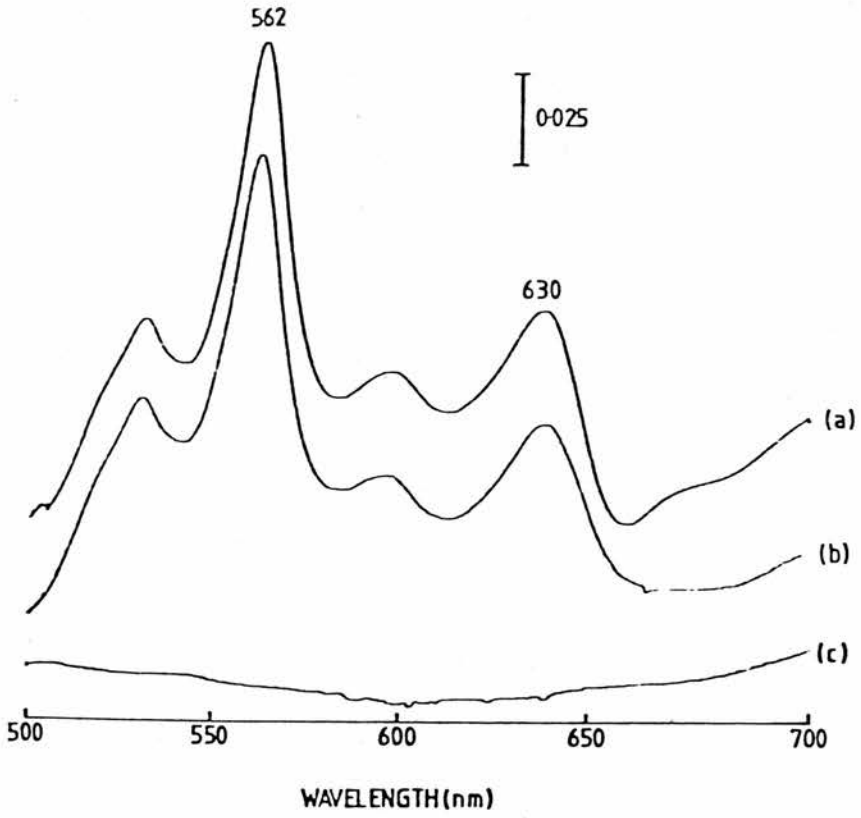
Bandwidth = 1nm

Chart Speed = 0.5mm/sec

a = Reduced minus aerobically-oxidised spectrum.

b = Reduced minus ferricyanide-oxidised spectrum.

c = Oxidised minus oxidised spectrum.


562
0·025
630
(a)
(b)
(c)
500
550
600
650
700
WAVELENGTH(nm)

These results suggest that the species absorbing at 655nm is not the oxidised form of cytochrome *d* as, if it were, oxidation with ferricyanide would produce a trough of similar size to that of the aerobically oxidised spectra. However, it appears that oxygen is required for the formation of the 655nm species. The absence of any other differences between the aerobically and ferricyanide-oxidised spectra indicates that the species of cytochrome *d* formed on oxidation with ferricyanide has little absorbance between 500 and 700nm.

DISCUSSION

Purified Cytochrome *bd* Difference Spectra

The previously published spectra of purified cytochrome *d* complexes (Reid and Ingledew, 1980; Miller and Gennis, 1983; Kita *et al.*, 1984b) are in general agreement with the results presented here. There is some dissimilarity in the exact wavelengths at which the spectral features occur (Table 7) but the general outlines of the spectra are similar.

The single Soret peak, centred around 430nm, which is seen at room temperature is further resolved, at 77K, into two separate components, one absorbing at 430nm the other around 440nm. Poole *et al.* (1983a) have allocated a peak at 430nm, formed after laser photolysis of CO-liganded cytochrome *d*, to cytochrome b_{558} and this is the likely source of the 430nm peak reported here. A peak at 445nm, previously attributed to a *b*-cytochrome, has been attributed to cytochrome a_1 (Poole *et al.*, 1981) and a partially purified cytochrome a_1 preparation from *E. coli* grown anaerobically on glycerol/fumarate medium had a Soret peak (at 20°C) of 444nm (Baines *et al.*, 1984). As the Soret absorbance of cytochrome *d* is weak and diffuse it seems probable that the peak around 440nm is due to the cytochrome a_1 present in the complex.

A shoulder on the cytochrome *b* α-band, at around 550nm, is visible in all the spectra. This is the region where

TABLE 7

A Summary of the Major Reported Spectral Features of Low Temperature (77K) Difference Spectra of Purified Cytochrome *bd*

	Reid and Ingledew 1980	Miller and Gennis 1983	Kita *et al.* 1984b	This Thesis
Wavelength (nm)				
400-500	-	429,436.5	-	431,440
501-600	555,558,595*	558.5,595	558,594	560,595
601-700	626,650*	624,645,681	624,645*	629,655,680

*These values were obtained by inspection of the published spectra and are, therefore, approximate.

c-type cytochromes absorb, however, Miller and Gennis (1983) failed to detect any covalently-bound haem by haem-staining the subunits of the complex after SDS-PAGE and no haem *c* was released by silver from the protein following extraction with acetone. Subsequent work by Koland *et al*. (1984), employing spectral deconvolution of the reduced minus oxidised spectrum of the purified cytochrome *d* complex, showed that cytochrome a_1 had a strong absorbance, centred around 560nm, which was usually masked by the cytochrome b_{558} α-band. This is the likely source of the shoulder at 550nm.

A single peak around 560nm in both room temperature and 77K spectra is attributed to cytochrome b_{558} in all the previous investigations (except Reid and Ingledew, 1980 who reported a split peak with absorbances at 555nm and 558nm for their partial purification which they attributed to two distinct *b*-cytochromes) and a single peak at 560nm is evident in the results presented here.

An absorption band around 595nm is common to all the reported spectra but the wavelengths reported here, for the absorbance peak and trough due to cytochrome *d*, are some 5nm higher than those reported in the other three previous purifications (Reid and Ingledew, 1980; Miller and Gennis, 1983; Kita *et al*., 1984b). The reason for this is not known.

A spectral component absorbing around 680nm is clearly evident in the low temperature spectrum of the purified

cytochrome *bd* (Fig. 21) and a weaker, but still distinct, feature is visible at this wavelength, in both the room temperature and 77K difference spectra shown by Miller and Gennis (1983). Neither Reid and Ingledew (1980) nor Kita *et al*. (1984b) scanned beyond approx. 670nm. This absorption band has previously been reported by various authors (Hendler and Schrager, 1979; Pudek and Bragg, 1974; Poole *et al*., 1983a) but its origins are as yet unknown.

NO-Liganded Difference Spectra

The reduced minus reduced-plus-NO spectra obtained from an ETP suspension and the partially purified cytochrome *bd* were very similar to those reported by Meyer (1973), with virtually identical changes in the Soret region, a shift in the cytochrome a_1 α-band to approx. 605nm and no decrease in intensity or change in wavelength maxima for the α-band of cytochrome *d*. This is in contrast to the decrease in the intensity of the cytochrome *d* absorption which has been reported by other workers (Bragg and Rainnie, 1973; Haddock *et al*., 1976; Reid and Ingledew, 1979; Hubbard *et al*., 1983) often accompanied by a shift in absorption from 630nm to 636nm.

The Soret trough and peak at around 415nm and 443nm respectively are unlikely to be due to cytochrome *d*, which has a weak and diffuse absorbance in this region. A trough at 415nm followed by a sharp peak at 447nm and broader peaks at 564nm and 598nm have been described for

the reduced-plus-NO difference spectrum of a soluble a_1-type cytochrome from *E. coli* (Baines *et al.*, 1984) and similar features can be found in the spectra described here. It is possible that the (so called) cytochrome a_1, present in the complex, was reacting with NO and was the source of the Soret features. Loss of the peak at 433nm, which is visible in the reduced minus oxidised spectrum, is due to cytochrome b_{558} remaining reduced on addition of NO.

Repeated scanning of the spectrum over one hour revealed no changes. This suggests that, as reported by Hubbard *et al.* (1984), the reaction with NO is rapid with the NO being strongly bound. The resulting nitrosyl compound has similar spectral properties to the oxygenated form of the enzyme, not the oxidised form which has no significant absorption in the red region of the spectrum (Poole *et al.*, 1983a). Formation of the oxidised form would result in a decrease in the absorption at 653nm due to the oxygenated form.

CO-Liganded Difference Spectra

The effects on the peak and trough due to the reduced and oxidised forms of cytochrome *d* respectively, caused by bubbling a reduced suspension of ETPs with CO, (as shown in Fig. 25) are in accordance with those published previously for cells grown anaerobically on a glycerol/ fumarate medium (Reid and Ingledew, 1980). However, there are marked differences at shorter wavelengths.

Reid and Ingledew (1980) found the reduced-plus-CO minus reduced difference spectrum of an ETP suspension to be essentially featureless from 400nm up to the trough due to reduced cytochrome *d* at approx. 630nm while the spectrum described here has a significant split Soret band and a small peak at 570nm. The peak at 444nm is likely to be due to cytochrome a_1 as this has been shown to bind CO, giving a trough in reduced-plus-CO minus reduced spectra at 445nm (Poole *et al.*, 1981). Also, a soluble a_1-type haemoprotein has been partially purified from *E. coli* grown anaerobically on a glycerol/fumarate medium by Baines *et al.* (1984) and this showed an absorption at 447nm for the CO-liganded form in difference spectra. The peak at 433nm is typical of a cytochrome *o* but no trough at around 420nm, characteristic of the *E. coli* cytochrome *o* and o_{436} (Poole *et al.*, 1980; Poole and Chance, 1981), is noticeable. The identity of the peak at 570nm is not known. The cytochrome *o*-CO complex produces a peak at 574nm in reduced-plus-CO minus reduced spectra (Poole *et al.*, 1981) but this would appear as a trough in the reduced minus reduced-plus-CO spectrum presented here and no such feature is present. Neither Reid and Ingledew (1979, 1980) nor Scott and Poole (1982) found a CO-binding *b*-cytochrome in cells grown anaerobically.

The difference spectra of CO-liganded, purified cytochrome *bd*, which have been previously published, again agree closely with that shown in Fig. 26 over the region 600-700nm but differences are evident at shorter wavelengths.

No significant absorbance differences were found by Reid and Ingledew (1980) in the Soret-region for their partially purified preparation while absorption differences at approx. 420, 430 and 445nm were visible for the CO-treated, purified cytochrome *bd* preparations of Miller and Gennis (1983) and Kita *et al.* (1984b) and are also reported here. As with the CO-liganded cytochrome *d* in ETPs the band at approx. 445nm is most likely to be due to the cytochrome a_1-CO complex. The presence of a trough at 420nm, a peak at around 430nm and a trough at 540nm suggest an *o*-type cytochrome in all three purified preparations. Miller and Gennis (1983) found a small peak at 561nm which they suggested may be due to CO binding to denatured cytochrome *b*. They also found that CO bound to the air-oxidised cytochrome. This may be due to CO displacing oxygen from the oxygenated intermediate form of cytochrome *d* as opposed to binding to the oxidised form (Poole *et al.*, 1983a). Kita *et al.* (1984b) report that the cytochrome *b* in their preparation bound CO extensively, giving a peak at 560nm comparable in size with that of the reduced minus oxidised spectrum. This did not occur in the presence of excess soybean phospholipids. The effect of this addition on the shape of the Soret band was not recorded. A peak at 570nm is evident from the CO-difference spectra shown here (Figs. 25, 26). It is possible that this may be due to denatured cytochrome *b* but the great similarity between the overall shape of the reduced minus reduced-plus-CO spectra of ETPs and the purified cytochrome suggest that

similar proportions of denatured cytochrome *b* would need to be present in both preparations.

As already mentioned, the CO-liganded spectra of purified cytochrome *d* previously published are in good agreement with that presented here (Fig. 26). Miller and Gennis (1983) and Kita *et al.* (1984b) report absorbances at 622 and 642nm, due to the reduced and CO-liganded forms of cytochrome *d* respectively. These are similar to the values of 625 and 643nm reported here. No wavelengths are given for these absorbances by Reid and Ingledew (1980) but examination of the spectra indicates that they do not occur until after 630nm.

From the results shown it appears that the reaction of CO with reduced cytochrome *bd* forms a cytochrome *d*-CO complex and, possibly, a cytochrome *b*-CO species. However, only one absorption band is visible between 555 and 565nm in the 77K reduced minus oxidised difference spectrum (Fig. 21) so the presence of an *o*-type cytochrome in addition to the cytochrome b_{558} of the complex is unlikely. Neither Reid and Ingledew (1979, 1980) nor Scott and Poole (1982) found a *b*-type cytochrome which bound CO in ETPs and whole cells respectively. However, both Miller and Gennis (1983) and Kita *et al.* (1984b) report some CO binding by a *b*-cytochrome in their purified preparations. It may be possible that the *b* cytochrome of the *bd* complex only binds CO when it has been purified.

Ferricyanide-Oxidised Difference Spectra

The spectra obtained by oxidising a reduced ETP suspension and a reduced, partially purified cytochrome *d* preparation with potassium ferricyanide are in close agreement with the previously published data. Pudek and Bragg (1976), found that, even at potentials of greater than +400mV (generated by the addition of ferricyanide), the trough at 650nm was not formed. However, its appearance followed the addition of pulses of water saturated with oxygen or H_2O_2. Similarly, Koland *et al*. (1984), using electrodic potentiometry, found that the oxidised spectrum of a purified cytochrome *d* complex showed a large decrease in the size of the trough at approx. 650nm compared with the aerated spectrum. The absolute spectrum of an *E. coli* suspension, oxidised with ferricyanide, was reported by Poole *et al*., (1983a) to be featureless from 520 to 700nm while that of a vigorously aerated suspension showed, among others, a large peak at 652nm.

All of these results indicate that oxygen is necessary for the formation of the species absorbing at around 650nm and that the anaerobically oxidised species of cytochrome *d* has little absorbance between 500 and 700nm.

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