A Spectrophotometric Investigation of the
Respiratory Cytochromes of
Aerobically-Grown Escherichia coli K-12

Howard Keith Withers
B.Sc., University of Birmingham, England, U.K., 1974,

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Department of **BIOCHEMISTRY**

The University of British Columbia  
Vancouver, Canada

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The cytochrome \( o \) and cytochrome \( d \) oxidase complexes provide twin termini for the branched respiratory chain of aerobically grown \textit{Escherichia coli}. Combined use of mutant strains, modulated growth conditions and high resolution analytical techniques enabled cytochromes to be resolved, identified and partially characterized. The cytochrome complement of everted membrane vesicles and detergent extracts fractionated by liquid chromatography is more complex than previously recognised.

Multiple type-\( b \) cytochromes were resolved by potentiometry and by high resolution spectrophotometry in membrane vesicles from mutant strains lacking the cytochrome \( d \) oxidase complex and grown under conditions minimising respiratory chain diversity. Cytochrome \( o \) was identified with \( E_m = +235 \text{ mV} \) (vs. NHE) as were low potential cytochromes associated with dehydrogenases. Spectrally distinct components of the cytochrome \( d \) complex yielded \( E_m \) values of \(+125 \text{ mV} \) (cytochrome \( b_{595} \)) and \(+187 \text{ mV} \) (cytochrome \( d \)). The latter displayed atypical redox behaviour with extreme hysteresis during potentiometric titrations.

Several cytochromes \( b_{556} \) displaying single, symmetrical redox \( \alpha \)-bands at 77 K were resolved from detergent extracts of vesicles. Mutant strains identified one with \( M_r = 52 \text{ 500} \) (gel filtration) and \( E_m = +20 \text{ mV} \) as the \textit{sdhC} gene product, a component of succinate dehydrogenase. DL-lactate induced another while a hydroperoxidase, \( M_r = 386 \text{ 000} \) (gel filtration) with twin \( E_m \) values of \(-2 \text{ mV} \) and \(-121 \text{ mV} \) and a split Soret absorption band at 77 K (\( \lambda_{\text{max}} = 426.0 \text{ nm} + 434.0 \text{ nm} \)) was produced under limited oxygen tension.

The Triton-solubilized and purified cytochrome \( o \) complex exhibited \( M_r = 516 \text{ 000} \) (gel filtration) with five component peptides of \( M_r = 55 \text{ 000}, 32 \text{ 000}, 31 \text{ 000}, 21 \text{ 000} \) and \( 16 \text{ 000} \) (SDS-PAGE). It displayed mid-point potentials of \(-58 \text{ mV}, +127 \text{ mV} \) and \(+260 \text{ mV} \) and three \( \alpha \)-absorption maxima at 77 K: 554.5 nm, 557.0 nm and 563.5 nm. These components were reduced equivalently during poised-potential low temperature spectrophotometric analyses. Carbon monoxide binding changed the complex’s redox \( \alpha \)-absorption spectrum minimally but shifted the high potential \( E_m \) to approximately \(+420 \text{ mV} \). Quinone analogues inhibited both reduction and reoxidation of the complex. Cytochrome \( o \) complex prepared from cloned sources contained a significantly greater proportion of the component with mid range electrochemical potential absorbing at 554.0 nm. These results are discussed in relation to possible structures of the complex, its respiratory interactions and the identity of cytochrome \( o \) itself.
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ABBREVIATIONS

ALA  5-aminolaevulinic acid; δ-aminolaevulinic acid.
DCIP  2,6-dichlorophenolindophenol.
DMSO  dimethyl sulphoxide.
DQ  duroquinone; 2,3,5,6-tetramethyl-1,4-benzoquinone.
DQH$_2$  duroquinol; 2,3,5,6-tetramethyl-1,4-benzoquinol.
duroquinone  2,3,5,6-tetramethyl-1,4-benzoquinone.
DW2c  SLM/Aminco model DW2c dual wavelength double beam spectrophotometer.
ec  energy charge; $[\text{ATP}] + 0.5x[\text{ADP}] /[\text{ATP} + \text{ADP} + \text{AMP}]$
EDTA  ethylenediaminetetraacetic acid.
$E_h$  ambient redox potential of the system with standard hydrogen half cell as reference.
$E_m$  standard (mid-point) redox potential under experimental conditions.
$E'_m$  standard oxidation-reduction (redox) potential at neutral pH.
$E_0$  standard oxidation-reduction (redox) potential at pH 0 and unit activities.
EPR  electron paramagnetic resonance.
$\mathcal{F}$  Faraday constant (96 493 J V$^{-1}$)
GSH  glutathione, reduced form; γ-L-glutamyl-L-cysteinylglycine.
HOQNO  2-(n-heptyl)-4-hydroxyquinoline-$N$-oxide.
HPLC  high performance liquid chromatography.
mS  milliSiemens (1.0 mS = 1.0 mmho).
mV  mV vs. NHE; millivolt values are given relative to the Normal Hydrogen Electrode.
n  number of electrons transferred in an oxidation-reduction reaction.
NAD$^+$  oxidized form of nicotinamide adenine dinucleotide.
NADH  reduced form of nicotinamide adenine dinucleotide.
NPN  N-phenyl-1-naphthylamine.
ocetylglucoside  1-O-n-octyl-β-D-glucopyranoside.
PE-356  Perkin-Elmer model 356 dual wavelength double beam spectrophotometer.
PMS  phenazine methosulphate.
PMSF  phenylmethylsulphonylfluoride.

*R*  the gas constant, 8.31 J K⁻¹ mol⁻¹.

RMS  root mean square  {estimate of statistical distribution}.

[R-O]  reduced minus oxidized  {difference spectrum}.

[(R+CO) - R]  reduced plus carbon monoxide minus reduced  {difference spectrum}.

Sarkosyl  N-lauroylsarkosine ; N-dodecanoyl-N-methylglycine.

SDS  sodium dodecyl sulphate.

SDS-PAGE  sodium dodecyl sulphate polyacrylamide gel electrophoresis.

spectra:

CO-binding  reduced plus carbon monoxide minus reduced difference spectrum,

redox  reduced minus oxidized difference spectrum

*T*  temperature above absolute zero in Kelvin.

TMAO  trimethylamine N-oxide.

TMBZ  3,3',5,5'-tetramethylbenzidine.

TMPD  N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride.

Tris  tris (hydroxymethyl) aminomethane.

Triton :  polyoxyethylene p-t-octyl phenol ;  p-t-octylphenoxy polyethoxy ethanol derivative

   (trade mark, Rohm & Haas Co.).

   X-100  n = 10,  Δ = 0.
   X-114  n = 11,  Δ = 4.

*ω*⁺  wild-type  {bacterial strain}.

\(\Delta G_p\)  phosphorylation potential : eqn. (1), Introduction.

\(\Delta \mu_{H^+}\)  proton electrochemical potential ; protonmotive force.

\(\lambda\)  wavelength.

\(\Gamma\)  mass-action constant of a reaction.

\(\mu\)  conductivity  (mS cm⁻¹)

\(\mu_{\text{glc}}\)  bacterial growth rate, generations per hour: glucose as sole carbon/energy source.

\(\mu_{\text{lac}}\)  bacterial growth rate, generations per hour: lactate as sole carbon/energy source.

\(\mu_{\text{suc}}\)  bacterial growth rate, generations per hour: succinate as sole carbon/energy source.
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INTRODUCTION

“The Spectroscope seems likely to be of almost as great use in Medicine as it has already proved in terrestrial, solar and stellar Chemistry” [121]

These words of Charles A. MacMunn (1852-1911), discoverer of the ubiquitous biological pigments that he named histohaematinns, have been borne out by the multiplicity of spectroscopic and spectrophotometric techniques now applied to deciphering the complexities of respiratory chain structure, function and regulation [122]. This is especially true of those respiratory components known collectively as cytochromes, the term coined by David Keilin upon his rediscovery of the ‘histohaematinns’ in 1925 [93].

A. OVERVIEW OF BIOENERGETIC METABOLISM

(i) Cellular distribution of chemical energy

The energy required for cellular growth, for anabolic metabolism, nutrient accumulation and for electrical, mechanical and luminescent biological activities is most often distributed throughout the cell in the form of adenosine-5'-triphosphate, ATP. Cellular concentrations of this molecule are generally held away from equilibrium with those of the corresponding diphosphate, ADP, plus inorganic phosphate, P\textsubscript{i}. This status enables energetically or entropically unfavourable half-reactions to be ‘driven’ to metastable states displaced from equilibrium by enzymatic coupling to the dephosphorylation of ATP. Thus a net decrease is generated in the Gibbs (free) energy content of the coupled reactions, many of which proceed directly via the formation of a phosphorylated intermediate within the active site of the enzyme responsible for this linked catalysis. Consequently the energy state of a cell, its capability to carry out biochemical work, is frequently described in terms representing the displacement of the components of the ATP dephosphorylation reaction from equilibrium: ‘energy charge’ is equivalent to $0.5 \frac{([ADP]+2[ATP])}{[AMP]+[ADP]+[ATP]}$, and phosphorylation potential, which is often approximated as the ratio of $\frac{[ATP]}{([ADP]+P\textsubscript{i})}$
and designated $\Delta G_p$. This ratio, when expanded to include the participation of magnesium ions and protons is described by the mass-action constant of the ATP dephosphorylation reaction, $\Gamma$. Thus $\Delta G_p$ is defined as in equation (1), where $\Delta G^\circ$ is the standard Gibbs energy change for this reaction and the 'prime' associated with each variable signifies the apparent, rather than standard system conditions:

$$\Delta G_p = \Delta G^\circ = \Delta G^\circ + 2.303 RT \log_{10} \Gamma'$$

The value of $\Delta G^\circ$ may also be expressed in units of millivolts by dividing throughout equation (1) by the Faraday constant, $F$ (section I.i.e). Concise reviews of these topics may be found in references 5, 64 & 145.

The energy required for bacteria to generate ATP may be derived from many diverse reactions. Those pathways which a particular organism is capable of using are employed selectively in adaptation to environmental conditions. These reactions may be classified into two general processes for the conservation of energy: substrate level phosphorylation and electron transport phosphorylation. Many cellular activities, especially those of vectorial nature, are dependent upon membrane energization in the form of a proton electrochemical potential or proton motive force (the combination of a proton gradient and electrical potential) across a sealed membrane \[19, 87\]. ATP formed by substrate level phosphorylation may be used to generate such gradients, and conversely the proton motive force generated by electron transport processes is closely coupled to ADP phosphorylation (v.i.). In eucaryotes the mitochondrial and chloroplast inner membranes are employed for this latter purpose; procaryotes, lacking intracellular organelles, use the cytoplasmic membrane. The proton electrochemical potential is the sum of electrical and chemical forces to which the protons are subjected, as described in equation (2):

$$\frac{RT}{F} \log \frac{[H^+]^B}{[H^+]^A} = \Delta \mu_{H^+} = \Delta \psi + 2.303 \log_{10} \frac{[H^+]^B}{[H^+]^A} = \Delta \psi - 2.303 \log_{10} \frac{[H^+]}{[H^+]}$$

where $\Delta \mu_{H^+}$ is the proton electrochemical potential, $\Delta \psi$ is the electrical potential difference across the membrane, $\Delta \psi$ is the pH difference between the two compartments created by the intact membrane and where the compartments' proton concentrations are $[H^+]^A$ and $[H^+]^B$. Nicholls has provided a particularly lucid synopsis of these phenomena, both authenticated and proposed, aspects of which are described in detail below \{145\}. 

(ii) Glycolysis and fermentative growth

Relatively few substrate level phosphorylation reactions have been described, those associated with the central amphibolic pathways occurring in both of the major procaryotic glycolytic routes. Catabolism of organic substrates through either the Embden-Meyerhof-Parnas or the Entner-Doudoroff pathways generates certain common intermediates which are degraded with a sufficiently large Gibbs energy change that enzymatic catalysis of these reactions couples them to the phosphorylation of ADP. All glycolytic enzymes are soluble and that they reside freely in the cytoplasm has recently been affirmed \(124\).

In the absence of an external electron acceptor reoxidation of the reduced nicotinamide adenine dinucleotide formed by glycolysis is achieved by reduction of available oxidized substrates: generally by conversion of pyruvate and other glycolytic products to one or more of ethanol, lactate, acetate or other short chain organic acids. It follows that growth upon one or more of these organic acids as the sole source of carbon is not possible by fermentative processes alone — *i.e.* without an external electron acceptor being present. These fermentations permit a continued supply of ATP from substrate level phosphorylations in the presence of a suitable carbon source. Hydrolysis of this ATP is coupled to the energisation of those membranes in which the proton translocating ATP hydrolase responsible for the process is incorporated.

The principal molecular features are common to both eucaryotic and procaryotic forms of this complex enzyme. They comprise two major operational parts, the hydrophilic \(F_1\) which effects the ATP hydrolase activity at the cytoplasmic surface of the membrane, and the hydrophobic \(F_0\) which spans the membrane. The latter component provides the proton translocating mechanism, usually symbolized as a pore or pump, by which \(H^+\) may be extruded through the ion-impermeable membrane in conjunction with the ATPase activity of \(F_1\) at a rate now estimated to be \(3H^+\) per ATP hydrolysed. As alluded to above the complete vectorial reaction may approach physiological equilibrium from either direction when catalysed by the intact membrane enzyme complex. Nevertheless, the reaction kinetics and catalytic response to particular inhibitors differ between the hydrolysis and synthesis of ATP. Reviews of the structure and proposed mechanisms of the proton translocating ATPase, with emphasis upon that of *E. coli*, are provided in references \(19, 51, 55, 139, 145\). The proton electrochemical potential, \(\Delta\mu_{H^+}\), generated across the membrane by ATP hydrolysis may be utilized for active transport of ions and substrates across the cytoplasmic membrane of procaryotic cells, for transhydrogenation of nicotinamide adenine dinucleotide phosphate (NADP\(^+\)) to supply anabolic reductions, and if an electron transport pathway is present, for reverse electron transport which may generate reduced nicotinamide adenine dinucleotide (NADH) \(71, 87, 145, 196\).
Many fermentative bacteria are able to increase the efficiency with which they are able to generate a proton electrochemical potential by utilizing transport systems that export metabolic end-products down a concentration gradient into the surrounding environment. Carrier-mediated proton symport leads to an increase of transmembrane electrical potential and proton translocation to an increase in the pH gradient \(^7\).

Most microorganisms and plants are capable of performing the reactions of the glyoxylate cycle which allows them to use short-chain organic acids, including acetate, or fatty acids as sole carbon source. By-passing the two tricarboxylic acid cycle reactions in which \(\text{CO}_2\) is evolved between the condensation of acetyl-CoA with oxaloacetate to form citrate and the reformation of oxaloacetate, the glyoxylate cycle permits replenishment of the metabolic constituents through generation of succinate from isocitrate. The glyoxylate formed in this reaction undergoes condensation with a second molecule of acetyl-CoA to form malate, and thence oxaloacetate. Consequently these cells are able to use the reactions of these central pathways for anabolic purposes, especially during growth on low molecular weight carbon sources such as TCA-cycle intermediates or precursors. However, these processes generate NADH that precludes the use of oxidized short chain substrates as sole carbon source by fermentation.

(iii) **Electron transport phosphorylation**

Oxidative phosphorylation describes ATP synthesis coupled to respiratory electron transport; photophosphorylation is that accompanying electron transport during photosynthetic energy transduction. Both processes employ molecular constituents described above in relation to the generation of a proton electrochemical gradient with a proton translocating ATP hydrolase: an intact, ion-impermeable membrane separating two compartments with a proton translocating ATPase attached to one membrane surface. An electron transport mechanism creates the proton electrochemical gradient across the membrane which then forces proton translocation through the \(F_0\) portion of the ATPase resulting in tightly coupled ATP synthetase activity at the \(F_1\) enzyme assembly. Consequently the ATPase reactions implemented during fermentation are reversed, albeit with some kinetic and inhibition response modifications as mentioned earlier. Without respiratory or photosynthetic electron transport to maintain the proton gradient synthesis of ATP by the enzyme complex is unable to proceed. The other facet of this coupling is that if ATP synthesis is blocked or prevented, as when substrate is exhausted, the established proton electrochemical potential will prevent further electron transport (v.i.).
Photosynthetic electron transfer causes the formation of a proton electrochemical potential which is exploited by a proton translocating ATP synthetase activity. In brief, light induces vectorial ejection of an electron at low potential through the reaction centre located within the membrane and the return of an electron to this reaction centre at higher potential is accomplished by a proton translocating electron transfer chain which thereby generates the proton electrochemical gradient. These essential principles are common to both bacterial (cyclic) and chloroplast (non-cyclic) photosystems [12, 44, 45, 145]. Light is also the energy source for the simplest known proton pump, the bacteriorhodopsin inserted through the energy transducing purple membrane of *Halobacterium halobium*. Photons bleach the pigment and cause protons to be released vectorially from the outer membrane surface; regeneration of the pigment is accomplished by protons from the inner surface, thereby generating a proton gradient. This proton electrochemical potential is used to drive a proton translocating ATPase and functional ATP synthetase activity may also be obtained in vesicles reconstituted with bacteriorhodopsin, phospholipid and the proton translocating ATPase from different sources, including that of higher animals [145, 195].

As an alternative to light as an external energy source for the electron transport mechanisms generating a proton electrochemical gradient, a redox potential may be used for this purpose using reduced substrates that may be exogenous in origin or generated internally, as in the case of NADH. Oxidative phosphorylation also requires the presence of an external electron acceptor of greater potential than that of the molecule donating the reducing equivalents. Generally such a respiratory electron transport pathway requires that both donor and acceptor react at one face of the membrane. A series of enzyme complexes function as an alternating sequence of hydrogen carrier and electron carrier from one face of the membrane to the other in proton translocating loops connecting the donor indirectly to the acceptor (Fig. 1a). The number of loops, or proton translocating segments, that are possible is dependent upon the potential difference between donor and acceptor. Thus dioxygen, with a high mid-point potential for the \( \text{O}_2/2\text{H}_2\text{O} \) redox couple of +820 mV vs. NHE, is particularly suited to the rôle of acceptor and is the favoured molecule for this function in many species. Some of the alternatives available to facultative anaerobes are illustrated in Figure 2. Certain *Clostridia* species, growing anaerobically on carbon monoxide, generate a proton electrochemical potential used to drive L-alanine uptake which varies in nature from electrical potential plus proton gradient below pH 7.5 to a potential which is solely electrical in nature above this pH [86].

Refer to [15, 33, 59, 136, 137, 145 & 210] for explanations of proposed mechanisms of electron transport phosphorylation. In the past contradictory interpretations of a variety of experimental results were highly controversial, yet most evidence is now accepted as agreeing in principle with the functioning of a chemiosmotic process, originally described by Mitchell [136].
Fig. 1: Models of the arrangement of respiratory components in electron transport pathways.

Abbreviations: FMN & FP, flavoproteins; QH$_2$, ubiquinol; cyt., cytochrome; Fe/S, iron-sulphur proteins; Q, ubiquinone; e$^-$, electron.

a. Generalised scheme proposed for the arrangement of respiratory components found in many aerobic bacteria. These respiratory chains translocate protons less efficiently than those incorporating cytochromes $a, a_3$ and $c$ which are thought to resemble the mitochondrial system.

After Haddock & Jones, 1977 (71).

b. i. The ‘protonmotive Q-cycle’ illustrated in general form with electrons passing from component x to component y. The terms cyt. $b^a$ and cyt. $b^b$ imply species or forms of cytochrome $b$ accepting and donating an electron to quinol and quinone respectively.


ii. Incorporation of the ‘protonmotive Q-cycle’ into a scheme for mitochondrial electron transport, illustrating greater efficiency of proton translocation than the scheme in ‘a’.

After Haddock & Jones, 1977 (71).
Fig. 2: Respiratory electron donors and acceptors available to facultative anaerobes.

Mid-point potentials ($E_m$) of redox couples and standard Gibbs free energies ($\Delta G^0$) for the oxidation of NADH by each of three oxidants are indicated.

Abbreviations:  
- TMA, trimethylamine;  
- DHAP, dihydroxacetone phosphate;  
- TMAO, trimethylamine oxide;

After Ingledew and Poole, 1984 {87}. 

\[ \Delta G^0 \]

\[ E_m \]

**Redox Couples**

-400 mV

-200 mV

\(-67 \text{ kJ mol}^{-1}\)

\(-163 \text{ kJ mol}^{-1}\)

\(-218 \text{ kJ mol}^{-1}\)

\(+400 \text{ mV}\)

\(+600 \text{ mV}\)

\(+800 \text{ mV}\)

\(+1000 \text{ mV}\)

- Formate/\(\text{CO}_2\)  \(H_2/2H^+\)

- NADH/NAD\(^+\)

- Glycerol/DHAP  Lactate/pyruvate

- Succinate/fumarate

- TMA/TMAO

- Nitrite/nitrate

- Water/dioxygen
As described above, this proposes that electron transport chains of energy transducing membranes are coupled to ATP synthesis by a proton electrochemical gradient and that electron transport and ATPase activity are each integrated with reversible transmembrane 'proton pumps'. The proton electrochemical gradient generated across the ion-impermeable membrane by electron transport forces the ATPase catalysed reaction in the direction of ATP synthesis. Evidence for the broad distribution of these essential features has been accumulating for a number of years and itself suggests a unified fundamental mechanism when combined with the association of similarly sized proton electrochemical potentials across all functional energy transducing membranes at some 150 mV to 200 mV. Among other pertinent results in support of the chemiosmotic hypothesis is that the majority of 'uncoupler' compounds retain their lipid solubility in both protonated and anionic forms and act by increasing the proton conductance of natural and synthetic membranes — thereby collapsing the proton electrochemical potential — and that of the functional assembly of bacteriorhodopsin plus beef heart mitochondrial ATPase to create a 'coupled' photophosphorylation system. This generalised scheme has been elaborated with many specific hypotheses, notably that of Williams and coworkers that the initial charge separation leading to proton translocation is an intramembrane event and that the translocated protons remain associated with the membrane surface — a localized event in which the energy of hydration of the proton in the lipid phase is responsible for the production of phosphorylated product by the ATPase [209, 210].

(iv) **Structure of respiratory electron transport systems**

Redox potentials of electron transport chains cover a range of about 1250 mV from the redox couples of $\frac{1}{2} \text{H}_2/\text{H}^+$ and $\text{HCOO}^-/\text{CO}_2$ with mid-point potentials at -420 mV and -440 mV respectively, to that of $\text{O}_2/2\text{H}_2\text{O}$ at +820 mV, although the potential spanned by chains present in a functional energy transducing membrane is unlikely to exceed the 1140 mV between the $\text{NAD}^+/\text{NADH}$ (-320 mV) and $\text{O}_2/2\text{H}_2\text{O}$ (+820 mV) redox couples [64, 84, 145]. This latter span is utilized by the electron transport pathway of the eucaryotic mitochondrion, a situation indicative of its efficiency when dioxygen is available as electron acceptor. Broader potential ranges are usually prevented by incompatibility of donor and acceptor for environmental or metabolic reasons — themselves often closely related to the function of procaryotic oxidative and photophosphorylation electron transport systems. For example, the pyruvate-formate lyase generating formate in fermentative *E. coli* is rapidly and irreversibly inactivated in the presence of oxygen [64]. Elements of procaryotic respiratory control are discussed below in the context of the thesis topic.
The molecular components of a respiratory chain must act effectively as redox carriers equilibrating electrons within a particular segment of the chain so that there is a net transfer between the initial donor and terminal acceptor. In order to accomplish this, and maintain the critical reversibility of electron transport associated with oxidative phosphorylation, the various components must operate within the chain at a potential approximating that of their individual mid-point potentials thereby minimising free energy changes. This criterion influences the molecular structures functioning in electron transport chains at different potentials: hence particular types of redox component are generally associated with particular respiratory functions. The initial dehydrogenases function at relatively low potentials, with succinate dehydrogenase at the upper potential limit of these activities, the mid-point potential of the succinate/fumarate redox couple being +30 mV.

The dehydrogenases are generally protein complexes with at least one subunit being a flavoprotein others containing iron-sulphur clusters and, in some dehydrogenases, another may be a cytochrome. Quinones transfer electrons from the dehydrogenases to the higher potential respiratory constituents although the mechanism of their action remains controversial. Cytochromes are components of these higher potential constituents, frequently as type-β cytochromes functioning between lower potential components and the terminal oxidase. Since oxidized quinones and reduced quinones are electrically neutral but the semiquinone, half-reduced state is a highly reactive free radical it is generally assumed that the latter form is not released as a free entity in the hydrophobic energy transducing membrane during respiration. In order to account for the transfer of two electrons from quinol to higher potential cytochromes which can accept only a single electron, Mitchell has proposed a protonmotive quinone cycle or ‘Q-cycle’ mechanism for the mitochondrial system in which an electron is passed from the quinol to each of cytochrome $b_T$ and cytochrome $c_1$ with release of protons at the outer face of the membrane {138}. Electron transfer within the ‘Q-cycle’ is thought to proceed from cytochrome $b_T$ to cytochrome $b_K$ and back to the oxidized quinone at the inner membrane surface in preparation for its complete reduction to quinol (Fig. 1b). The precise role of quinol in reacting with the higher potential cytochrome components has not been determined in many procaryotic respiratory systems, including those of E. coli. Terminal oxidases probably display the greatest structural diversity between the groups of respiratory components, as might be expected from the range of possible electron acceptors. The terminal oxidases naturally have the highest mid-point potentials of any respiratory component within an electron transport chain and all appear to contain multiple cytochromes. This variation between oxidase types is of particular interest for evolutionary studies: an alteration of the efficiency of electron transport may have dramatic effects upon growth rate and oxidase-related proteins are often highly conserved. Moreover,
environmental conditions at a particular evolutionary stage may be reflected directly in the respiratory competence of a particular procaryote. These different types of redox carrier and aspects of their functions will be discussed below with respect to the aerobic respiratory chains of *Escherichia coli*.

(v) **Structural properties of cytochromes**

Lemberg and Barrett have developed a practical definition of cytochromes as:

"haemoproteins whose principal biological function is electron and/or hydrogen transport by virtue of a reversible valency change of their haem iron between ferrous and ferric forms" \[112\]

Reactive properties of the haem moiety are modified by the protein so that certain of them are magnified and others depressed. Haemoglobin and myoglobin are not oxidized following the binding of dioxygen and the bound intermediate is stable but dissociable. Hydroperoxidases decompose hydrogen peroxide extremely rapidly with concomitant oxidation of hydrogen donors in the case of true peroxidases. In comparison cytochromes are generally unreactive with dioxygen or hydrogen peroxide, the oxidation and reduction of the haem iron underlying their metabolic function, a notable exception being the reaction of aerobic terminal oxidases with dioxygen. Consequently the protein environment of the haem is particularly important for maintaining the appropriate chemical properties of a cytochrome as well as for its interactions with other respiratory components and metabolic control of its function.

The initial classification of cytochromes has been based upon the structure of the haem prosthetic group which is generally indicated by the holoprotein's visible absorption spectrum. Structures of the four haem types — *a*, *b*, *c* and *d* — are illustrated in Figure 3. The *b* and *d* cytochromes are characterized by protohaem and dihydroporphyrin (chlorin) prosthetic groups respectively. Other haemoproteins may use these haems to generate very different properties such as the enzymatic activities of catalases and peroxidases or the ligand binding characteristics of haemoglobins and myoglobins.

Cytochromes of type *b* and *d* are of particular relevance to the current work and both true prosthetic groups in that the haem is bound non-covalently. In many of the cytochromes *b* for which information is available both axial coordination sites of the haem iron are occupied by ligation to imidazole nitrogens of histidine residues in the peptide chain, although other amino acids may occupy one or other of these positions, notably the *ε*-amino group of lysine \[112\]. Aerobic terminal oxidases would require one free axial coordination site for binding dioxygen.
**Fig. 3:** Structures of protoporphyrin IX and haem prosthetic groups.

Protoporphyrin IX binds an iron atom to form haem b and is modified as shown at positions 2+8, 2+4 or 5+6 to generate haem a, c, or d respectively.

**a.** Haem a,

**b.** Protoporphyrin IX,

**c.** Haem c, with covalent linkage to peptide methionine residues indicated,

**d.** Haem d.
Because of the presence of multiple type-\(b\) cytochromes in respiratory chains they have been distinguished by the absorption maxima of their reduced \textit{minus} oxidized difference spectra, in particular by the \(\alpha\)-band absorbance maximum which is measured at 77K to increase resolution and which is denoted as a subscript \(112\). These absorption maxima vary with temperature, and as this thesis demonstrates, even the improved resolution obtainable at liquid nitrogen temperatures may be insufficient when used as the sole method of distinguishing between cytochromes with similar spectral properties. Appendix ‘A’ provides an introduction to the spectral characteristics of cytochromes pertinent to this study.

(vi) **Bacterial respiratory chains**

Bacterial respiratory chains may comprise one or more segments of the total complement possible, the induction of particular components depending upon environmental and growth conditions. Additionally, investigation of the possible routes of electron transfer after growth in the presence of multiple terminal electron acceptors suggests that the alternative respiratory electron transport chains induced have components in common. Thus there is the possibility of branching between electron transport chains within the energy transducing membrane. The relatively large amounts of reducible quinone present in many of these bacterial membranes has been suggested to be the common ‘sink’ for reducing equivalents through which multiple electron transport chains may communicate (\textit{v.i.}). During growth in liquid media many enterobacteria produce one aerobic terminal oxidase in response to relatively high dissolved oxygen conditions and another under poor aeration. Intermediate or changeable conditions such as those developing in the later stages of simple aerated batch cultures lead to both oxidases being present and utilized simultaneously.

In spite of these additional complexities the relative ease with which certain bacterial species may be manipulated genetically, their ability to grow rapidly under defined conditions on simple nutrients such that energy sources may be better controlled and the great resource of knowledge about the organization of the genome of \textit{Escherichia coli} in particular has maintained this bacterium as an organism of choice for studying both chemical and physical properties as well as the biological consequences of electron transport and proton translocation.
B. AEROBIC ELECTRON TRANSPORT IN ESCHERICHIA COLI

(i) Energetic diversity

Evolutionary constraints upon the genetic development of a bacterial species dictate the type of energy source it is capable of employing, phototrophs and chemotrophs employing electromagnetic or chemical energy sources respectively. Escherichia coli, a facultative anaerobe, uses only organic substrates for the dual rôle of electron donor and carbon source but may exploit a variety of electron acceptors including dioxygen, oxides of nitrogen and sulphur or several reducible organic compounds including fumarate, dimethylsulphoxide (DMSO) or trimethylamine oxide (TMAO). This Gram negative enterobacterium indulges in mixed acid fermentation in the absence of external electron acceptors, hence its nutritional classification as a C-heterotrophic chemoorganotroph.

(ii) Environmental and ecological factors

E. coli populates the human intestine shortly after birth: indeed, at the time of Keilin’s pioneering work on cytochromes from a variety of biological sources it was known as Bacterium coli communis [93]. Capable of aerobic respiration under low oxygen tensions the bacterium effectively scavenges dioxygen thereby assisting in the creation of the anaerobic conditions necessary for the remainder of the normal flora to become established. After a few weeks the majority of the bacteria in the intestine are strict anaerobes and E. coli constitutes a miniscule proportion of the total population — one thousandth to one millionth of the total cell count. The efficiency with which aerobic culture conditions may be used to recover this minor organism from such a largely anaerobic environment after $10^4$ to $10^5$ bacterial generations demonstrates that its ability to respire aerobically serves a continued purpose throughout this period. It also indicates that that the organism itself, with this capability, functions to the advantage of the bacterial population as a whole within the human intestine — in addition, presumably, to that of the proprietor [21, 87]. A symbiotic aspect of the relationship between E. coli and its human host is that the former may synthesise menaquinone as a component of its respiratory chain. This compound, vitamin K, is required for synthesis of the clotting factor prothrombin in higher animals and may also used by them as a constituent of non-respiratory microsomal electron transport systems.
The ability to consume oxygen and also to be capable of growth in the resulting anaerobic conditions may be particularly useful in the vicinity of epithelial cells with their generous blood supply. Other nutrients available in this environment are obviously variable and their diversity open to conjecture since much of the free carbohydrate and protein is absorbed in the upper digestive tract. Growth rate in the gut is also influenced significantly by competition from other bacterial species for nutrients, especially iron \[87, 146]. The ability of \textit{E. coli} to thrive on a limited supply of simple nutrients has been exploited in the laboratory as well as being of use to the organism in its normal habitat. Active transport systems enable the bacterium to grow on low concentrations of specific nutrients, the energy required being supplied by electron transport or fermentative substrate level phosphorylation depending on the growth conditions. Not only do these properties permit the organism to adapt to fluctuations in its natural environments but they make it particularly useful for studies of the mechanisms and the genetic and metabolic control of these various functions.

(iii) Respiratory control

Control of aerobic respiratory activity is able to respond to environmental alterations very rapidly, as shown by the 35 s to 120 s periodicity of oscillations in fluorescence intensity from flavins and pyridine nucleotides following interruption of the air supply to cultures of \textit{E. coli} in late exponential phase \[87]. Although the rate of electron transport is dependent upon supplies of both donor and acceptor and is subject to the constraint of forming a maximal proton electrochemical potential the mechanisms regulating respiratory activity are largely unknown and their elucidation will require further data regarding the structure and activity of individual respiratory components and the precise nature of proton translocation. It should be noted that under the most frequently employed laboratory culture conditions electron transport is the rate limiting activity for growth whereas this is certainly not the case for \textit{E. coli} in its natural habitat \[87].

Factors affecting longer term, transcriptional control are beginning to be understood as the genomic organisation of operons coding for certain complexes is revealed. The presence of electron acceptors with high mid-point potentials represses the expression of reductases reacting with acceptors of lower potential, each of these latter being able to repress fermentative activity to some degree \[194]. The \textit{fnr} operon is involved in the regulation of anaerobic respiratory chains and also that of purely fermentative enzymes, originally being detected by its control over induction of fumarate and nitrate reductases. The differential stimulation of these activities that was obtained by varying the gene dosage after cloning \textit{fnr} indicates that a complex interaction with other regulatory factors must
occur, while genetic sequence and other evidence implicates the catabolite gene-activator protein CAP and the nucleotide cofactor cyclic AMP in addition to the well established repressive effects of the highest potential electron acceptor, dioxygen \[31, 153, 194\]. Regulation of the two terminal oxidases reacting with dioxygen is much less well understood, partly because identification of their genomic locations has been made more recently: their structural genes being sited in the cyd and cyo operons at 16.7 min and 10.2 min respectively \[6, 61\]. Studies using individual and dual cyd and cyo mutants have shown that *E. coli* aerotaxis responds to alterations generated in the proton electrochemical gradient and hence to changes in the efficiency of aerobic electron transport under low partial pressures of oxygen \[185\].

Respiration in rich media with high oxygen levels is naturally efficient and results in low concentrations of respiratory components per cell, the cytochrome *o* complex being the sole terminal oxidase present in the early stages of growth. A decreased supply of oxygen causes the induction of the cytochrome *d* terminal oxidase and repression of the cytochrome *o* complex, an adaptation reflected in the values of \(K_m\) for oxygen of the two oxidases: 0.38 mM and 2.9 mM for solubilized cytochromes *d* and *o* respectively \[98\]. This changeover is directly attributable to the altered concentration of dioxygen but the stage of growth of the culture may also influence terminal oxidase expression \[56, 87\]. Anaerobic growth with nitrate as terminal electron acceptor causes repression of the cytochrome *d* complex but this effect is less marked if fumarate is used in place of nitrate. It is unclear how these effects are mediated in the light of evidence that the *fnr* gene product is also required for cyd expression, while expression of *fnr* itself is independent of anaerobiosis, positively regulated by cAMP and negatively by its own gene product \[54, 151\]. Furthermore, under semianaerobic conditions cyd expression exhibits no requirement for the *fnr* gene product or CAP although it is transcriptionally regulated and induction occurs when the oxygen tension of a highly aerated culture decreases below a threshold level, as in the later growth phases of batch cultures \[56\]. While cAMP has no direct effect upon cyd induction it has been reported to enhance expression of the cytochrome *d* complex in several earlier studies \[34, 56\]. The nature of the available carbon source may also influence cyd expression quantitatively, or in the case of certain mutants, absolutely \[56, 89\]. Poole has reported the persistence of cytochrome *o* under conditions of limited oxygen, albeit with modified spectral properties \[160\].

The majority of studies measuring synthesis of respiratory chain components through the cell cycle have indicated a continual increase in respiratory capability, although complex results obtained in certain investigations may reflect the influence of particular methods of cell cycle synchronization \[87, 181\].
(iv) **Aerobic respiratory chains — status of research prior to the current study**

The extent of knowledge of aerobic electron transport in *E. coli* has expanded dramatically over the past ten years, and especially in the last five with the advent of certain cloned constituents. Nevertheless there is still controversy over some fundamental properties of these respiratory assemblies including the number and function of participating cytochromes.

Earlier descriptions of the aerobic respiratory chain of *E. coli* were based upon functional analyses and recognised the presence of dehydrogenases which were associated with the flavoproteins and iron-sulphur proteins that had been detected, in addition to ubiquinone-8 (a benzoquinone) plus menaquinone-8 (a naphthoquinone), and four types of cytochrome identified by their spectral properties. Characteristic terminal oxidases were detected by spectral alterations following carbon monoxide binding and were identified as cytochromes $d$, $a_1$ and cytochrome $o$ \(^{17,18,24,71}\). Cytochrome $o$ was the predominant terminal oxidase in cells harvested in early exponential phase while cytochromes $d$ and $a_1$ were found in greater proportion in late exponential and stationary phase cells. Cytochrome $b_1$ was a significant constituent of respiratory chains at all stages of growth \(^{17,71}\). Current views of the composition of the electron transport chains of aerobically grown *E. coli* are given below (section B.v).

Analysis of the dehydrogenases was restricted by difficulties in isolating active complexes following membrane solubilization, the succinate dehydrogenase proving to be particularly refractory to purification in an active form \(^{71}\). The broad range of possible physiological reductants available to cells grown aerobically on rich media has necessitated strict control of the nutrients supplied in the growth medium in order to limit the number and to define the type of dehydrogenases present. It was known from purification studies that $b$-cytochromes were associated with membrane preparations containing certain dehydrogenase activities and that some of these preparations would reduce artificial and natural quinones \(^{71}\). Non-haem iron (iron-sulphur protein) and flavoprotein were associated with these activities.

The quinol component of the aerobic respiratory chains contained ubiquinol-8 but the utility of menaquinol-8 under these growth conditions was equivocal. The latter had been shown to be required for cellular activity of the anaerobic fumarate reductase through the use of auxotrophic mutants, but either of the two quinols appeared to be capable of functioning under aerobic conditions, in which ubiquinol-8 was the predominant form \(^{42,71}\). Not only was the existence of a 'Q-cycle' mechanism unproven for the transfer of electrons from the iron-sulphur and flavoprotein centres to the
cytochromes but the order of interaction between the quinol present and the type-\(b\) cytochromes was unknown and disputed. Indeed, the presence of alternating segments of the respiratory chain transferring hydrogen atoms and electrons had not been proven in \(E.\ coli\) and so the method of creating a proton electrochemical potential in these aerobically grown procaryotes was not know to be equivalent to that in mitochondria, although other bacteria such as \(Paracoccus\ denitrificans\) are believed to possess a similar aerobic respiratory chain to that found in eucaryotes \[88\].

Spectral analyses of the cytochromes of the aerobic respiratory chains had permitted an initial classification used to identify the presumed haem content of each cytochrome: cytochromes \(a_1\) and \(a_2\) apparently containing haem-\(a\) as prosthetic group, cytochrome \(b_1\) containing haem-\(b\), and minor quantities of a cytochrome \(c\) with type-\(c\) haem as covalently-bound prosthetic group \[49, 93\]. Cytochrome \(a_2\) was subsequently termed cytochrome \(d\) when shown to contain a chlorin or \(d\)-type haem and more recently cytochrome \(a_1\) has been renamed cytochrome \(b_{595}\) since its ‘\(a\)-type’ spectrum has been found to be caused by a type-\(b\) haem containing a high-spin iron atom \[18, 71, 112\]. This high-spin \(b\)-haem was responsible for the spectral characteristics previously attributed to the minor amount of cytochrome \(c\), it having been shown that there is no cytochrome \(c\) in aerobically grown \(Escherichia\ coli\) \[4, 87, 118\]. Cytochrome \(o\) had been named for its oxidase activity since it was thought to have minimal absorbance properties in the redox \(\alpha\)-band — the major spectral characteristic used for cytochrome identification — but had later been shown to be a \(b\)-type cytochrome since \(hema^+\) mutants required incubation with haematin and ATP to generate a functional electron transport system incorporating cytochrome \(o\) \[71\].

Apart from uncertainty as to the true arrangement of respiratory components in the aerobic electron transport pathways of \(E.\ coli\) the characteristics, function, and even the number of cytochromes present in aerobically grown cells was subject to dispute. Improvement in the sensitivity of spectrophotometric techniques had led to renewed interest in the multiple rôles of cytochrome \(b_1\) which had been determined to be a composite of several cytochromes \(b\) with barely resolvable spectra, each being identified by the wavelength of its low temperature \(\alpha\)-band absorption maximum. Thus cytochrome \(b_{558}\) was known to be induced in proportion and simultaneously with cytochrome \(d\) as a component of the low aeration pathway, but the function of cytochrome \(a_1\) was reported variously as that of a terminal oxidase, of a non-oxidase cytochrome and of an oxidase with low oxidase activity \[17, 71\]. The identity of cytochromes formed under conditions of high aeration was even more contentious. Since cytochrome \(o\) could not be identified directly by spectrophotometric means, its \(\alpha\)-band absorbance being obscured by that of the other type-\(b\) cytochromes, the identities and sequence of cytochromes in the aerobic respiratory chains had been
suggested in virtually every possible combination. Thus with three major type-\(b\) cytochromes identified by their reduced minus oxidized (redox) low temperature \(\alpha\)-absorption bands as cytochromes \(b_{555}\), \(b_{556}\) and \(b_{562}\), cytochrome \(o\) had been variously identified as cytochrome \(b_{555}\) \([95, 97]\), as cytochrome \(b_{556}\) \([162, 174]\), as cytochrome \(b_{557}\) \([173]\), as cytochrome \(b_{562}\) \([69]\) and as a cytochrome with a split \(\alpha\)-absorbance band identified as cytochrome \(b_{555-b_{562}}\) \([117]\). The possibility remained that cytochrome \(o\) was actually a high-spin \(b\)-type cytochrome with minimal absorbance in the \(\alpha\)-band region of the spectrum — \textit{i.e.} it was none of the above candidates — as was suggested by the spectrum of its CO complex, but countered by electron paramagnetic resonance (EPR) evidence which had failed to detect the presence of high-spin iron in aerobically grown cell membrane preparations \([159]\).

It should be noted that variations in spectrophotometric precision and accuracy may cause problems in interpreting results from different laboratories when the analyses are restricted to such a narrow wavelength range. That the spectral resolution required to distinguish between these cytochromes can only be obtained at liquid nitrogen temperatures precludes straightforward kinetic analyses and the methods used for these investigations included inhibitor studies, potentiometric techniques and spectral perturbation with carbon monoxide, each coupled with low temperature spectrophotometry. As the identity of cytochrome \(o\) was strongly disputed, so too was the sequence of electron transfer between the remaining cytochrome components of this respiratory chain. The isolation of a cytochrome \(o\) preparation solubilized with the ionic detergent Sarkosyl and identified by its quinol oxidase activity had been shown to contain both cytochromes \(b_{555}\) and \(b_{562}\) suggesting that cytochrome \(b_{556}\) was located at an earlier site in the pathway, at lower potential than either of these 'oxidase-associated' cytochromes \([97, 103]\).

Early potentiometric studies not only suffered from the complicating effects of the presence of variable quantities of the cytochromes associated with each of the two aerobic terminal oxidases, but were also carried out in isolation from low temperature spectrophotometric analyses, so that the results obtained could not be attributed confidently to specific cytochromes.

Thus the nature of the aerobic respiratory chains of \textit{E. coli} was uncertain at the time the current studies were initiated. Indeed, the situation was accurately summarized some time earlier in a description of similar efforts relating to molecular analyses of a rather more fundamental nature:

"But when I took the pains to impartially examine the Bodies themselves that are said to result from the blended Elements, and to torture them into a confession of their constituent Principles, I was quickly induc'd to think that the number of the Elements has been contended about by Philosophers with more Earnestness, than Success." \([16]\)
Moreover, there were suggestions that the efficiency of electron transport was less than that observed in the mitochondrial system, and that of just two coupling sites, one was associated with the dehydrogenase activities and one with the terminal oxidases (71, 112). Determination of the proton translocation stoicheiometry of this respiratory system, the definition of the chemiosmotic redox loops active in these respiratory membranes and their organisation required that the overall architecture of the chains should be determined, in addition to the characterization of the individual respiratory components and their functions.

(v) Aerobic respiratory cytochromes — progress of the present investigation

The elucidation of the number of individual cytochromes functioning in the electron transport chains of aerobically grown *Escherichia coli* plus the determination of their individual properties was of importance to addressing many of the uncertainties described above. In consequence the purpose of the present study was to employ analytical techniques as combined procedures in an attempt to dissect the characteristics of the cytochrome components in membrane preparations. Performing combined spectral and potentiometric analyses was already being used with some success in studying anaerobic respiratory chains in the host laboratory, although it became apparent that these would be insufficient to delineate the properties of each of the aerobic cytochromes clearly. A program of refining the available methodologies was undertaken, large improvements being made in spectrophotometric and potentiometric measurements, partly through procedural modifications and partly through instrument upgrading. In addition the technique of poised potential trapping was investigated and mutant strains were used to minimise interference from alternate electron transport paths. In spite of the combination of these approaches data acquired from membrane preparations showed that definitive characterization of individual b-cytochrome components *in situ* would not be possible, although useful information was obtained regarding minimum numbers of resolvable type-b cytochromes present in the cytoplasmic membrane under specific growth conditions, induction and spectral properties of cytochrome *d*, and the existence of cytochrome pools in these resuspended membranes with different electronic transfer rates.

Solubilization of the respiratory cytochromes of *E. coli* was known to modify their properties and activity, and many attempts at fractionation of such solubilized preparations in the host laboratory had been unsuccessful (P. D. Bragg, personal communications). Nevertheless solubilization of the respiratory cytochromes was required to separate them sufficiently to allow adequate characterization, and as a preliminary step in the purification of selected cytochromes.
Techniques of cytochrome solubilization, fractionation and purification were devised and developed for further investigation of several individual cytochromes or cytochrome complexes, including the cytochrome o complex, a cytochrome b_{556} thought to be a component of the succinate dehydrogenase complex and another cytochrome b_{556} possessing hydroperoxidase activity. The fractionation procedure was refined to permit routine resolution of cytochrome constituents solubilized from membrane preparations so that extracts of membranes from cells grown under different conditions could be screened for cytochromes b_{556} associated with growth on specific substrates. Spectral, electrochemical and certain kinetic properties of the purified cytochrome o were characterized and compared to those of cytochrome o from a cloned source developed by R. B. Gennis.

Continual adaptations have had to be made to the project's aims and research emphasis in order to prevent duplication of other groups' research interests and to maintain its relevance in an area of rapidly progressing knowledge. Consequently the Results and Discussion sections of this thesis have been combined, thereby enabling the various sections of the work to be placed in context with greater clarity. The Conclusion of this thesis provides a summation and overall discussion of the project's achievements in the light of current understanding.

C. EXPERIMENTAL PROGRESS CONCURRENT WITH THIS INVESTIGATION

(i) Overview

The vibrant interest in bacterial respiratory cytochromes over the past few years has dramatically expanded the extent of knowledge of procaryotic electron transport systems, especially those of aerobically-grown E. coli. Yet more interest has been stimulated as physiological parameters have gradually been defined and purer biochemical systems isolated to enable structural, kinetic and mechanistic properties of certain of these cytochromes and their reactions to be studied. Genetic manipulation of the respiratory cytochromes has been central to the advances in this field following location of their structural genes on the genome as have attempts to solubilize individual cytochromes or groups of cytochromes for investigations of their respective properties and functions in isolation from other components. Successes with these latter experiments have led to reconstitution attempts and the formation of model systems in which certain purified constituents of the electron transport chains have been reassembled in artificial liposomes for studying the conditions
required to generate and maintain a proton electrochemical potential. The regulatory processes governing expression of the different respiratory cytochromes are now coming under scrutiny as molecular biological techniques are used to investigate the organisation of genes coding for the terminal oxidase complexes.

At the commencement of these studies the arrangement of cytochromes in the aerobic respiratory chains of *E. coli* was disputed, as described above. A common factor among the various models proposed was that cytochromes closely associated with each terminal oxidase would interact with ubiquinol-8 in the membrane to provide quinol oxidase activity in the presence of oxygen. The models generally incorporated a split electron transport chain with the quinol component receiving electrons (or hydrogen atoms) from the dehydrogenases and delivering them to either of the two terminal oxidase complexes. Many models attempted to arrange the known cytochromes with one or more pools of quinol linking them in one or other sequence before the proposed 'branch point' to the oxidase complexes (see, for example, [42, 95]). Thus the bacterium's energetic adaptability could be explained at a simplistic level by the interaction of multiple electron transfer routes which could be mobilised to the extent dictated by the concentrations of whichever electron donors and acceptors were available and yet also subject to exclusive transcriptional controls, such as that of oxygen over the anaerobic oxidases. Multiple electron transport routes connected by branch points suggested that a less rigid organisation of respiratory components might exist in the bacterial membrane than in the more specialised energy transducing membrane of the mitochondrion. Thus the bacterial system might require communication between cytochrome pools with different functional and/or topographical properties. The fifty-fold molar excess of ubiquinol over terminal oxidase in *E. coli* membranes, compared to seven-fold in the mitochondrion, made the small, lipophilic and therefore mobile quinol an excellent candidate for the redox mediator connecting segments of the respiratory chains [4]. However, this itself raised questions of whether the bacterial system could carry out 'Q-cycling' in such circumstances and if not, how might the quinols deliver two electrons to a cytochrome that would accept only one? The complexity of the oxidases, each associated with several distinct cytochromes, might illustrate a mechanism for solving this dilemma.

(ii) **Haem synthesis and incorporation into apocytochrome**

Recently published reports indicate that *hemA* mutants, which are characterized by 5-aminolaevulinic acid auxotrophy, are due to lesions in the C$_5$ pathway from the intact five carbon chain of glutamate via glutamyl-tRNA dehydrogenase [9, 114]. This pathway was
previously thought to exist only in plants, algae and anaerobic bacteria whereas animals, fungi and facultative bacteria were believed to utilise the ALA synthase pathway which is now known to be absent from *E. coli* [114]. The incorporation of iron into protoporphyrin IX synthesised from 5-aminolaevulinic acid results in the formation of haem b and is catalysed by ferrochelatase in the mitochondria of eucaryotes. The procaryotic system probably requires enzymatic catalysis also, in view of the iron-limiting conditions in which the organism frequently exists and the extensive siderophore mechanisms developed to transport iron into the cell (section B.ii.) [146].

If *hemA* cells are grown aerobically without 5-aminolaevulinic acid no cytochromes are detectable by spectroscopic methods and oxidase activities are minimal [70]. When membrane preparations from these cells were incubated with haematin (haem b) plus ATP spectra typical of type-b cytochromes were observed and NADH oxidase activity was detected. Since reconstitution of these properties was independent of protein synthesis the apocytochromes must have been synthesised in the cells and inserted into the membrane in the absence of haem, and the membrane associated apocytochromes' haem pockets must have been accessible for the haem insertion process, which required ATP [70, 173].

More recently the expression of the two subunits of the cytochrome d complex has been shown to be dependent on the *cydC* gene, located separately from the *cydAB* operon containing the structural genes for cytochromes $b_{558}$, $b_{595}$ and d. The polar mutation *cydA* prevents the production of either subunit of the oxidase complex [60, 62]. Growth of *cydA* strains carrying the *cydA* gene on multicopy plasmids has shown that subunit I may be synthesised and inserted into the membrane independently of subunit II, as cytochrome $b_{558}$ [63]. Cells containing a *cydC* lesion lack cytochrome d terminal oxidase activity but synthesise low, possibly constitutive, levels of both subunit apoproteins, while haem d is completely absent [57]. The *cydC* strain carrying *cydAB* genes on a multicopy plasmid overproduces both subunit apoproteins and haem b is inserted at two sites to create cytochrome $b_{558}$ as subunit I and cytochrome $b_{595}$ which is thought to be formed by positioning of haem-b between the two subunits [57, 62, 63]. This result implies that synthesis of haem b must be coordinated with apoprotein overproduced by the multicopy plasmid and that the cytochrome $b_{595}$ haem-binding site, previously found to be particularly sensitive to denaturing reagents, is reconstituted by the association of holosubunit I with aposubunit II which has sites for two haem d moieties [57, 62, 118].

The chlorin which forms haem d is thought to be derived from protoporphyrin IX although the biosynthetic pathway is unknown [57]. Found only as the oxygen and carbon monoxide binding moiety in the cytochrome d terminal oxidase complexes of certain bacteria the iron containing form of haem d is more labile than the unstable metal-free form [205].
suppositions that haem $d$ was similar to haem $d_1$ from bacterial dissimilatory nitrite reductases, in which the haem is a derivative of bacteriochlorin, and that haem $d$ contained a spirolactone substituent have been modified to propose the structure shown in Figure 3 \cite{112, 200, 205}. In the case of these type-$d$ haems the visible spectra show many similarities in spite of substantial structural differences in their porphyrin substituents \cite{200, 201, 205}.

(iii) Immunologically based cytochrome studies

The function of the small, soluble cytochrome $b_{562}$ of *E. coli* remains unknown although it has been purified and studied extensively to reveal a wealth of structural information. This haemoprotein was shown to be unrelated to any of the accessible domains of the membrane bound cytochromes by polyclonal antibody binding studies which also showed that the 'aerobic cytochrome $b_{556}$' was not formed in a constant ratio to cytochrome $o$, with which it had been consistently associated in previous studies, and was unrelated to the cytochrome $b_{556}$ of the anaerobic nitrate reductase (cytochrome $b_{nr}$) \cite{106}. These immunological studies by R. B. Gennis and coworkers were continued with the generation of antibodies to the two aerobic terminal oxidase complexes following the isolation of mutant strains failing to produce one or other complex. These monoclonal and polyclonal antibodies were subsequently used to identify and characterize components of the oxidases during and after purification and to show that oxidases cross reacting with the cytochrome $o$ complex or the cytochrome $d$ complex exist in many other Gram-negative bacteria, oxidases closely related to the *E. coli* cytochrome $d$ complex being particularly widely distributed \cite{106, 107, 109}.

Immunochemical studies of succinate dehydrogenase were used to characterise this unstable enzyme and later to aid its isolation as an active complex as discussed in relation to results from the current investigation under Results & Discussion \cite{32, 91}. A cytochrome $b_{556}$ of $M_r = 17,500$ isolated by Kita *et al.* following solubilization of cytoplasmic membranes with a Sarkosyl/cholate mixture was thought to be the cytochrome $b_{556}$ associated with the respiratory chain terminating in cytochrome $o$ \cite{102}. It was reduced by D-lactate dehydrogenase from *E. coli* in the presence of menadione, as expected for a respiratory intermediate connecting the dehydrogenase and oxidase segments of the electron transport chain \cite{102}. However, amino acid analyses and genetic mapping studies later identified it as the cytochrome $b$ of the succinate dehydrogenase complex which had been investigated by means of protein chemistry by P. Owen and coworkers and by molecular biology in the laboratory of J. Guest \cite{32, 143, 144, 215}. A very recent collaboration has analysed the cloned complex after overexpression and solubilization in Lubrol PX which resulted in a high
succinate dehydrogenase activity and a mid-point potential of the cytochrome $b_{556}$ similar to that obtained in the current study (+36 mV and +20 mV respectively) [101]. This preparation was able to generate succinate oxidase activity when reconstituted into phospholipid vesicles with ubiquinone-8 and purified cytochrome $o$ terminal oxidase which has been proposed as evidence for a cellular electron transport chain of minimal complexity [101].

(iv) Genetically based studies of terminal oxidases

The development of cyd$^-$ strains of the bacterium by R. B. Gennis and coworkers provided a major advance for such investigations just as the current study was undertaken. These strains were isolated by their failure to oxidize N,N,N',N'-tetramethyl-p-phenylenediamine and are unable to induce the cytochromes of the 'low aeration' electron transport pathway terminating in cytochrome $d$ [60]. Therefore they enable investigators to concentrate exclusively on those cytochromes associated with the alternative, 'high aeration' pathway which had previously been available only in low yield from early exponential phase cells. In the current study these strains have also been exploited in this way, enabling spectral and potentiometric analyses of greater resolution to be achieved and permitting fourth order finite difference spectra to be calculated with reproducible results. In the latter case, the qualitative response to the analytical method from samples of wild-type strains results in interference from disproportionate contributions to the analysis from constituents of 'minor' respiratory chains which are induced under imperfectly controlled conditions of growth and harvesting. One of the original cyd$^-$ mutants was subsequently shown to possess both regulatory and structural lesions, features of which are discussed in relation to results from this investigation (Results & Discussion) [69].

Subsequently the Gennis laboratory generated cyo$^-$ strains which fail to produce the cytochrome $o$ complex, but grow adequately in aerobic conditions by expressing cytochrome $d$ [8]. Potentiometric analyses of membrane suspensions from these oxidase deficient strains have indicated that cytochrome $o$ and the cytochrome $b_{556}$ associated with it have mid-point potentials of +165 mV and +35 mV respectively, the values for cytochromes of the cytochrome $d$ complex — which are spectrally distinct — being +260 mV ($d$), +180 mV ($b_{558}$) and +150 mV ($b_{595}$) [117]. By combining the techniques of potentiometric titration and spectrum deconvolution Stouthamer and coworkers analysed cytochromes $b$ in membrane preparations of aerobically grown wild-type cells. Unfortunately no indication of the quantity of cytochrome $d$ (and hence of cytochrome $b_{558}$) was given, although poised potential low temperature spectrophotometry provided values of +187 mV.
and +46 mV for cytochromes b with 77 K redox α-band absorbance maxima at 563.5 nm and 555.7 nm which may have corresponded to cytochrome o and cytochrome b₅₅₆ respectively \[203\]. Other in situ potentiometric titrations of type-b cytochromes from wild-type cells grown under high aeration had also indicated that the two major species had \( E_m \) values of +175 mV to +200 mV and of +30 mV to +70 mV \[66, 80, 170\].

\( \text{(v) Purification and characterization of detergent solubilized terminal oxidases} \)

Procedures used for the isolation of detergent-solubilized terminal oxidase complexes incorporating either cytochrome o or cytochrome d have now been reported by the laboratories of Y. Anraku, R. B. Gennis and H. R. Kaback as well as from the current study \[99, 130, 135, 214\]. Purification and characterization of the cytochrome d complex in Zwittergent solution showed that it comprised two peptides with \( M_r = 57,000 \) (subunit I) and 43,000 (subunit II) with the three cytochrome constituents \( b_{558}, b_{595} \) and d \[118, 135\]. These two subunits could not be separated without denaturing the complex which possessed quinol oxidase activity as isolated or when reconstituted into membranes \[104, 135\]. There are two moles of cytochrome d and one mole of each type-b cytochrome associated with each mole of the complex \[4\]. Subunit I corresponds to cytochrome \( b_{558} \), which is transcribed from the cydA gene, is a transmembrane protein and has been overexpressed and characterized in isolation from subunit II \[4, 62, 63\]. Oxidation of quinol substrates by the complex is inhibited by antibodies to subunit I which has been shown to possess the quinol binding site and to monitor the redox steady state of the ubiquinol-8 pool in the aerobic respiratory chain \[115, 219\]. Subunit II contains two type-d haems or chlorins which EPR studies indicate to be located close to the cytochrome \( b_{595} \) \[4, 74\]. The cytochrome \( b_{595} \) was renamed from cytochrome \( a_1 \) when the cytochrome d complex was shown to lack haem a and when spectral deconvolution analyses revealed a ‘peroxidase type’ cytochrome b with high-spin haem iron and a small α-band absorption at 595 nm \[118\]. This cytochrome binds carbon monoxide weakly and is present in the purified complex but is not isolated with either subunit, suggesting that the haem may be inserted between the two peptides and therefore that it may be particularly susceptible to protein denaturation: this latter characteristic may account for earlier observations attributing CO binding properties of this cytochrome to oxidase activity \[163\]. Potentiometry of the purified cytochrome d complex yielded mid-point potentials of +232 mV (d), +55 mV to +150 mV (\( b_{558} \)) and +113 mV (\( b_{595} \)), with each \( E_m \) value being sensitive to pH and that of cytochrome \( b_{558} \) particularly sensitive to the solubilizing detergent \[105, 119\]. Recent electron
paramagnetic resonance and spectrophotometric studies have indicated that the cytochrome \( d \) is extremely stable in the oxygenated state which forms spontaneously when the membrane bound or solubilized forms are exposed to air, and which affects the EPR signal of cytochrome \( b_{595} \): the EPR studies also showed that oxidized cytochrome \( b_{558} \) contains high-spin haem iron \( \{4, 74, 116\} \).

Purification of the cytochrome \( o \) terminal oxidase complex in the laboratories of Y. Anraku and H. R. Kaback has resulted in preparations containing two copper (II) atoms and comprising either two \( \{97\} \) or four subunits \( \{129\} \). The current study demonstrates that there are at least four subunits, none of which stains strongly for haem and one showing split bands in SDS polyacrylamide gel electrophoresis. The physical and spectral properties of this solubilized complex are described in detail with the high resolution results obtained for this thesis, low temperature redox difference spectroscopy exhibiting a complex absorption pattern with at least three absorption maxima in the \( \alpha \)-band \( \{214\} \). Three type-\( b \) cytochrome components were observed in potentiometric titrations, the high potential component undergoing radical mid-point potential shift upon complexing with carbon monoxide and the two lower potential components being susceptible to moderate perturbations in \( E_m \) when the reduced forms interacted with potassium ferricyanide. The complex has been shown to be a quinol oxidase, reducible by duroquinol, its activity inhibited by the quinol analogue HOQNO during both oxidative and reductive phases. The high potential cytochrome has a split redox \( \alpha \)-band at \( 77 \) \( K \) which absorbs most strongly at \( 563.5 \) \( \text{nm} \) and also at \( 555 \) \( \text{nm} \) and the other two components absorb at \( 555.0 \) \( \text{nm} \) and \( 557.0 \) \( \text{nm} \). Other published spectra have demonstrated a split \( \alpha \)-absorption band but at much lower resolution, work from the present laboratory suggesting that a cytochrome \( b \) component with an \( \alpha \)-absorption maximum at \( 562 \) \( \text{nm} \) had a high mid-point potential \( \{69, 97\} \). The spectral identity of cytochrome \( o \) is still being disputed in the literature with Kaback and colleagues suggesting that of two type-\( b \) cytochromes in the complex one is cytochrome \( o \) with a redox absorption maximum at either \( 558 \) \( \text{nm} \) or \( 562 \) \( \text{nm} \), Anraku and coworkers identifying cytochrome \( o \) as cytochrome \( b_{555} \) and the Gennis laboratory proposing that it is a split absorption cytochrome \( b_{555/562} \) \( \{3\} \). The results of the current study indicate possible reasons for this confusion, for there appears to be synchronous reduction of component cytochromes within the complex. Moreover, the current analysis of the complex expressed from the \( cyo \) operon cloned by R. B. Gennis shows spectral and potentiometric distinctions in the absence of gross structural differences from the cytochrome \( o \) complex prepared from wild-type cells. Evidence is presented suggesting that the complex may exist in different stable states which may have functional relevance.

The EPR and resonance Raman data of Anraku which suggest that cytochrome \( b_{555} \) reacts
with carbon monoxide are in direct opposition with the titration and poised potential results from the current study which show the CO binding species to be cytochrome $b_{562}$, also termed cytochrome $b_{563}$ as a result of a shift in $\alpha$-absorption maximum upon solubilization \cite{3, 202, 214}. Anraku’s group has also suggested that the cytochrome $c$ complex in both reduced and air-oxidized states contains a haem with a high-spin iron atom, in contrast to the earlier investigations which found no evidence for such a constituent of this terminal oxidase \cite{159, 202}. Working with intact, wild-type cells Poole and Chance have investigated the kinetics of photolysis of the CO adduct of cytochrome $c$ in the presence of dioxygen at various subzero temperatures \cite{160, 164}. The CO binding reaction is slower and more rapidly reversible by photolysis than that with dioxygen and the latter produces oxygen-bound intermediates with spectral characteristics similar to those of the CO adduct which may be trapped below $180 \, K$ (-98°C) for further analysis \cite{164, 165}.

\textbf{(vi) Molecular biological analyses of terminal oxidase genes}

Although genes for both terminal oxidases have now been cloned by the laboratory of R. B. Gennis and their products overexpressed in growing cells, no data has been published providing sequences for any of the components, nor for the number or arrangement of components in the cyo operon \cite{6, 61}.

The \textit{cybB} gene, encoding another aerobic respiratory cytochrome $b$, has also been cloned and its product isolated by solubilization in Sarkosyl or Triton X-100 followed by HPLC fractionation or standard ion-exchange chromatography respectively \cite{100, 141}. Sequence data for this cytochrome also remains unpublished although purification studies have shown it to be a dihaem cytochrome $b_{561}$ with redox $\alpha$-absorption maxima at 555 nm and 561 nm \cite{100, 142}. The mid-point potential of cytochrome $b_{561}$ is reported as +20 mV, although the value of $n$ is not provided and the titration profile may constitute two individual haem responses that overlap substantially as has been found and discussed in the current study and in the cytochrome $b^{NT}$ of the \textit{E. coli} respiratory nitrate reductase \cite{67, 142}. Membrane preparations from strains producing amplified quantities of cytochrome $b_{561}$ demonstrated aerobic steady state reduction of this cytochrome with substrates of NADH or D-lactate, indicating that it is a respiratory component preceding ubiquinone-8 in the electron transfer chain, although wild-type strains are reported to produce minor quantities of cytochrome $b_{561}$ \cite{142}.

The product of the \textit{cybA} gene is a cytochrome $b_{556}$, which has been cloned as the \textit{sdhC} gene and is described above in relation to succinate dehydrogenase activity (section C.ii) \cite{215}. 
Both the DNA sequence and the amino acid sequence have been determined, but to date no indication has been provided of which residues bind the haem iron (144, 215). Evidence is provided in this thesis to suggest that there are multiple cytochromes $b_{556}$ in the membranes of aerobically grown $E. coli$ although many other workers, notably those of the Anraku and Gennis laboratories have assumed that the succinate dehydrogenase cytochrome $b_{556}$ is the sole cytochrome present with these spectral characteristics (3,4). Consequently earlier data indicating that a respiratory cytochrome $b_{556}$ is associated with the cytochrome $o$ branch of aerobic electron transport has been ignored when schemes have been proposed for the overall architecture of the respiratory systems. These topics are addressed in the Conclusion.

(vii) Reconstitution experiments and models of aerobic respiratory architecture

Since the current studies were initiated both of the two aerobic terminal oxidases have been purified by other laboratories and each oxidase complex has subsequently been reconstituted into liposomes in order to examine its mechanistic properties and interaction with other respiratory components. Initial experiments by Kaback and coworkers using membrane vesicles from mutants lacking D-amino acid dehydrogenase or D-lactate dehydrogenase demonstrated that an intact respiratory chain, functioning with the appropriate substrate, was reconstructed when one or other dehydrogenase had been reconstituted into either side of the membrane (72,148). That the same effect was obtained with simultaneous reconstitution of the dehydrogenases into such vesicles suggested that they bound at dissimilar sites. As both activities generated a proton translocating step which preceded the oxidases it was proposed that this exists as a common reducible intermediate between the flavin-linked dehydrogenases and the cytochromes and that the standard chemiosmotic model of redox loops, as initially described for mitochondrial systems by Mitchell, is inappropriate for describing the first coupling site of the $E. coli$ respiratory system (72,148).

Reconstitution of purified cytochrome $d$ terminal oxidase complex plus purified pyruvate oxidase complex and ubiquinone-8 into pure phospholipid vesicles catalysed electron transport between pyruvate and oxygen with the generation of a 180 mV transmembrane potential which was sensitive to uncouplers (104). When reconstituted into vesicles without the flavoprotein pyruvate oxidase the cytochrome $d$ complex was shown to serve as a respiratory coupling site and to function as a quinol oxidase with specificity for ubiquinol-8, whereas it will also oxidize menaquinol-8 in detergent solubilized solutions (87,104).

Several reconstitution studies have been performed with the purified cytochrome $o$ terminal
oxidase complex in spite of the lack of consensus on the identity of cytochrome o itself. When sufficient phospholipid is present with the purified oxidase complex to form proteoliposomes, the fluorescence of the lipophilic probe N-phenyl-1-naphthylamine (NPN) will reflect the redox state of the complex {116}. This constitutes the uncoupler insensitive component of NPN fluorescence which requires sufficient lipid for the oxidase complex to be incorporated into vesicles and which occurs in addition to that observed with intact cells of E. coli in which the probe responds to energization of the cytoplasmic membrane {182}. Other experiments in which the cytochrome o complex was reconstituted into phospholipid vesicles or planar bilayers demonstrated that it would generate a membrane potential when supplied with artificial electron donors {73, 96}. These potentials were dissipated by protonophore uncouplers and inhibited by applied voltages of up to +150 mV on the substrate side of a bilayer or by the oxidase inhibitors KCN and HOQNO, a quinol analogue. The cytochrome o oxidase was thus identified as a coupling site for oxidative phosphorylation.

The earliest demonstration that membrane potentials generated in vitro could be used as models for cellular processes was provided by reconstitution of two purified complexes into phospholipid vesicles, one being the cytochrome o complex and the other the lac carrier protein from E. coli. By adding ubiquinol as electron donor a proton electrochemical potential was set up across the membrane, its magnitude being dependent upon the concentration of cytochrome o. The proteoliposomes would then transport a variable amount of lactose, dependent upon the potential created, against a lactose concentration gradient {128}. Construction of artificial electron transport chains has been attempted by reconstituting the purified cytochrome o complex into phospholipid vesicles in the presence of a purified dehydrogenase or bacterial photosynthetic reaction centres. In the former case electron transfer between D-lactate dehydrogenase and oxygen via cytochrome o was obtained in right-side-out and in everted membrane vesicles from aerobically grown cydF cells and also in vesicles reconstituted from phospholipid, D-lactate dehydrogenase and ubiquinone-1. The proton electrochemical potential generated by limited addition of either D-lactate or quinol indicated the existence of a single site of generation with a 1H+/e− stoichiometry, whereas that obtained on addition of NADH suggested the presence of two sites, one before and one after the quinone step {126}. Hybrid proteoliposomes containing the photosynthetic reaction centre plus the cytochrome o complex have been used to generate a proton electrochemical potential with associated oxygen consumption by means of steady illumination {140}. Flash illumination caused emission of single electron from each reaction centre, creating a pulse of quinol and transitory reduction of the oxidase. This reduction was sensitive to inhibition by quinol analogues and the reoxidation by dioxygen was
sensitive to cyanide inhibition (140). No spectral distinction could be made between the oxidase haems, even when a ‘kinetic potentiometric titration system’ was created by flashing at poised potentials.

Thus the cytochrome o terminal oxidase appears to function through vectorial electron translocation and scalar proton transfer, creating a proton electrochemical potential when reduced within the lipid bilayer by ubiquinol-8 and reoxidized at the inner surface of the cytoplasmic membrane by dioxygen. These concepts will be addressed further in the Conclusion in the light of results from the current study.

D. SYNOPSIS OF RESEARCH AIMS AND STRATEGY

This work describes the resolution of *E. coli* aerobic cytochrome properties by combinations of several methodologies which had previously been employed independently. By determining the number of cytochrome species and elucidating their individual characteristics their precise rôle and their interaction with other electron carriers may be better understood.

The initial results described relate to studies in which reference cytochromes were utilized to refine the spectrophotometric and potentiometric techniques for optimal sensitivity. Data produced by both of these methodologies now resolve the properties of individual cytochromes to a greater degree than those reported in the literature, whether the cytochromes are in solubilized preparations or beside in membrane vesicles. Although there should be less artifactual perturbation of spectral and potentiometric properties of membrane proteins in vesicle preparations than in solubilized solutions the complexity of the results obtained from the multiple type-6 cytochromes present in membranes prevented reliable interpretation. Attempts were made to generate mutant strains with aberrant or missing type-6 cytochromes, including those of the cytochrome o complex of the ‘high-aeration’ respiratory chain. Several mutants were isolated and determined to contain an altered complement of respiratory cytochromes-b when grown aerobically. Nevertheless their characterization remains unclear despite extended investigation.

Protocols were developed for the solubilization and fractionation of *E. coli* aerobic respiratory cytochromes. The resolving power of these techniques exceeds that of related liquid chromatographic procedures published elsewhere. The fractionated cytochromes were subsequently investigated individually by the modified analytical techniques described above. The combination of
improved techniques for fractionation and analysis permitted investigations of detergent-solubilized fractions of cytochrome $b_{556}$ and cytochrome $o$. The former comprised several similar cytochromes, each induced independently. Cytochrome $o$ was isolated and its spectral, electrochemical and certain kinetic properties characterized. Nevertheless the details of the interaction of cytochrome $o$ with components preceding it in the aerobic respiratory chain remains open to speculation as indicated by the conflicting reports continuing to be published. Comparison of these proposals' predictions with the current data is provided in the Conclusion.
MATERIALS & METHODS

(a) Chemicals

All reagents used in this study were of the highest quality available from commercial sources. Components of growth media were of ACS grade or higher. The cytochrome c used for reference studies was 'type VI' from equine heart, and the catalase was from bovine liver: both proteins were obtained from Sigma Chemical Co., St. Louis, MO, USA. Purified bovine hepatic microsomal cytochrome b₅ (soluble trypsinized fragment) and triphenanthroline cobalt were prepared and generously provided by Dr. A. G. Mauk, Department of Biochemistry, U.B.C..

(b) Cell types

The various strains of Escherichia coli K-12 used during this study are indicated in the text and listed in Appendix ‘B’ with their genotypes and sources. The constituents of bacterial growth media and of buffer solutions are described in Appendices ‘C’ & ‘D’ respectively.

(c) Growth of cells

Cells were grown in batch culture at 37°C to the desired phase of growth on one of several minimal media as indicated in the text (see list in Appendix ‘C’). Additions of nutritional supplements and antibiotics required to maintain the expression of desired phenotypic characters are described in detail in the text. Growth phase was estimated by optical density readings measured with a Perkin-Elmer 124 double beam spectrophotometer at 600 nm (OD₆₀₀). Two standard incubation techniques were used, batches of up to 8 L being grown in 500 mL aliquots within 2 L Erlenmeyer flasks rotated at 300 rpm in a New Brunswick incubator/shaker and larger batches (to 22 L) being grown in a static vessel vigorously sparged with compressed air or a mixture of nitrogen/carbon dioxide (95:5 w/w) as indicated in Appendix ‘C’. Sparged cultures also received 200-500 μL Antifoam Reagent A (Dow Corning Silicones Ltd., Downsview, ON.) which was added to the bulk medium with the final inoculum. Conditions were altered in some experiments to modify the degree of aeration of the cultures: details of these techniques are given in the text. Inoculum size was 10% of the fresh medium volume.
(d) Crude membrane preparation

Cells ready for harvesting were cooled rapidly on ice and centrifuged at 10,000 x g for 25 minutes at 4°C in Beckman JA10 rotors, washed twice with TM buffer, then weighed and either used immediately or stored at 0°C for a maximum of 12 hours before use. All subsequent procedures were carried out at or below 4°C. After being resuspended in 1.5 volumes TM buffer the cells were disrupted by two passages through a French press (AMINCO, Inc; Silver Spring, MD, USA) at 1400 kg cm$^{-2}$ (20 000 lb in$^{-2}$) in the presence of a few crystals of calf thymus deoxyribonuclease type 1 (Sigma Chemical Co., St. Louis, MO, USA). Unbroken cells were removed by centrifugation at 10,000 x g for 20 minutes in a Beckman JA20 rotor and the supernatant diluted three-fold before being centrifuged for 2.5 hours at 250,000 x g in Beckman Type 60Ti or 45Ti ultracentrifuge rotors. The resulting membrane pellets were washed by resuspension in TM buffer using a Thomas Teflon™-glass homogenizer and sedimentation as before to yield the ‘crude membrane fraction’. Crude membranes were placed on ice as pellets and resuspended in the appropriate buffer immediately prior to further processing.

(e) Inner membrane preparation

Inner membranes were purified from crude membrane preparations by one of three methods as indicated in the text:

1. Crude membranes were resuspended in TDGA buffer, pH 7.5, and sedimented by ultracentrifugation as described above. The pellet was then resuspended in TDGB buffer, pH 7.5, at a membrane protein concentration of 20.0 mg mL$^{-1}$ and dialysed against 3 L of the same buffer for 16 hours. After further ultracentrifugation the soft, pigmented upper layer of the resulting pellet was readily removed with a spatula to provide the inner membrane preparation.

2. Crude membranes were resuspended in TM buffer containing 0.5 mM dithiothreitol to a final protein concentration of 10.0 mg mL$^{-1}$. An equal volume of fresh 10 M urea containing 1.0 mM PMSF (pH 7.8, 20°C) was added slowly and the solution stirred for 15 min at 0°C. The urea-washed membranes were centrifuged for 3.0 hours at 250,000 x g causing the inner membrane to separate as a soft brown upper layer of the resulting pellet, distinct from the hard, white outer membrane layer below. The inner membrane layer was removed with a spatula and immediately washed by resuspension in TM buffer and recentrifugation to remove traces of urea.

3. Crude membranes were prepared in the presence of 0.1 mM dithiothreitol, then resuspended and centrifuged into a sucrose cushion as described below in section ‘k’.
(f) Solubilization of membranes

Crude membranes and inner membranes were solubilized by resuspending in TX buffer to a protein concentration of 10.0 mg mL\(^{-1}\) using a Thomas Teflon-glass homogenizer. The homogenate was then stirred slowly at 0°C while an equal volume of TTX buffer was added one drop at a time, generating final extraction concentrations of 5.0 mg mL\(^{-1}\) membrane protein and 5.0% Triton X-114. The mixture was stirred gently for 30 min at 0°C, pH 7.8 and centrifuged for 1.0 hour at 250 000 x g in Beckman Type 60Ti or 45Ti ultracentrifuge rotors to sediment unextracted material. The supernatant constituted the ‘Triton-solubilized’ crude membrane or inner membrane preparations used in this study.

(g) Fractionation of solubilized cytochromes

Solubilized membranes were used as a source of membrane cytochromes suitable for fractionation by a variety of liquid chromatography techniques. On a routine basis Triton solubilized membranes were diluted with an equal volume of TTE buffer to yield final concentrations of 2.5 mg mL\(^{-1}\) membrane protein, 10.0 mM Tris-HCl, 5.0 mM MgCl\(_2\), 0.5 mM EDTA, 2.5% (w/v) Triton X-114 and 0.5% (w/v) Triton X-100 at pH 7.8. This solution was stirred at 0°C for 45 min to permit equilibration of the detergents with the sample. Conductivity of the solution was checked to ensure that it was below 2.0 mS cm\(^{-1}\) before being loaded onto a (12.0 x 1.5) cm column of DEAE-BioGel.A (Bio-Rad Laboratories, Richmond, CA, USA) previously equilibrated with TTE buffer. The loaded column was washed with two column volumes of TTE buffer in order to remove significant quantities of unbound cytochrome and elution was achieved by a linear gradient of (0-400) mM KCl in ten column volumes of TTE buffer. Flow rates were approximately 20 mL hr\(^{-1}\) during loading and washing but a constant flow of less than or equal to 2.0 mL hr\(^{-1}\) was required during elution to ensure consistent resolution of cytochromes, the latter rate being maintained with a peristaltic pump utilizing a stepping motor. The protein content of each 2 mL fraction was estimated by a modified Lowry assay (v.i., section ‘r’), the elution of visible chromophores was monitored at 412 nm in a Perkin-Elmer Lambda 3A double beam spectrophotometer (A\(_{412}\)), and the relative ionic strength recorded from a Markson Model 10 conductivity meter. Those fractions potentially containing significant quantities of cytochrome, as shown by the A\(_{412}\) measurements, were analysed by low-temperature reduced minus oxidized difference spectra as described below in section ‘I’. Resolution and recovery of cytochromes from a single DEAE-BioGel.A column operated under the stated conditions for 72 hours were superior to those obtained by fractionating samples upon multiple ion-exchange and gel filtration matrices over a similar period at higher flow
rates. Significant variations of these techniques are described at appropriate junctures in the text.

(h) Purification of cytochrome o

The cells used for routine cytochrome o preparations were cyd-cells which had been grown to stationary phase although certain experiments, indicated in the text, required w+ cells grown to mid-exponential phase. Inner membranes were prepared from crude membranes by the urea-wash technique ('(e) 2' above). This preparation was then resuspended in TM buffer and a solution of 10.0 % (w/v) sodium cholate in TM buffer was slowly added to yield final concentrations of 4.0 mg mL\(^{-1}\) membrane protein and 6.0 % (w/v) sodium cholate. The solution was stirred gently on ice for 30 min and then centrifuged for 60 min at 250 000 \(\times\) g in a Beckman Type 60Ti or 45Ti ultracentrifuge rotor. After resuspending the pellets in TM buffer the washed, urea+cholate-stripped membranes were sedimented by recentrifuging for 2.5 hours at 250 000 \(\times\) g. These membranes were then subjected to Triton X-114 extraction using TX and TTX buffers as described above (section 'f'). Fractionation of cytochromes to purify cytochrome o was accomplished by modification and extension of the liquid chromatographic techniques described earlier, the fractions of each stage being monitored for cytochrome, protein and conductivity as indicated in section 'g' above. An initial DEAE-BioGel.A column of (12.0 x 2.4)cm or (12.0 x 1.5)cm, depending upon the quantity of material being purified, was equilibrated with TTE buffer at pH 7.8, loaded with the sample, washed and then eluted with a (0-400)mM KCl gradient with a constant 2.0 mL hr\(^{-1}\) flow-rate as previously described (section 'g'). The fractions recognised as 'Peak II' were pooled and the cytochrome precipitated by adding slowly, with stirring, 361.0 mg (NH\(_4\))\(_2\)SO\(_4\) per millilitre of pooled sample: the solution was stirred slowly, on ice, for 10 min. The cytochrome was recovered as a floating precipitate when centrifuged at 12 000 \(\times\) g in a Sorvall HB-4 swinging-bucket rotor and was resuspended in a minimal volume of TTE buffer (typically 500 \(\mu\)L) before loading onto a (37.5 x 1.5)cm column of Sephacryl S-300 'Superfine' (Pharmacia Inc., Uppsala, Sweden) equilibrated with TTE buffer, pH 7.8. The column was eluted with TTE buffer at a constant 2.0 mL hr\(^{-1}\). The major cytochrome peak was then pooled, checked to ensure that its ionic strength resulted in a conductivity value below 2.0 mS cm\(^{-1}\) and loaded onto a (12.0 x 1.5)cm or (8.0 x 0.8)cm DEAE-BioGel.A column equilibrated with TTE buffer. After washing the sample on the column with TTE buffer the cytochrome was eluted at a slow rate from the column by a pH-gradient created by pumping from the second of two linked reservoirs containing:

(i) four column volumes of 1.0 % (w/v) Triton X-100, 7.5 mM citric acid, 1.0 mM
EDTA (both of the latter being in free acid form), pH 4.0 with KOH, and

(ii) four column volumes of TTE buffer, pH 7.8.

Cytochrome remaining on the column at the end of the pH gradient was eluted with 200 mM KCl in TTE buffer, pH 7.8. Both the pH gradient and salt-eluted fractions were shown to contain purified cytochrome \( c \) by spectrophotometric and gel electrophoretic techniques.

Exchange of buffer and detergent in preparation for potentiometric titration was achieved by a three-fold dilution of the cytochrome \( c \) sample with DTE buffer, pH 7.8, and loading it onto a (7.5 x 50.0)mm column of DEAE-BioGel.A equilibrated with DTE buffer, pH 7.8. The cytochrome was washed with ten column volumes of DTE buffer, pH 7.8 and then eluted in a concentrated fraction of approximately 2.5 mL with DTE buffer, pH 7.8, containing 300 mM KCl. This fraction was concentrated to approximately 500 \( \mu \)L in an Amicon ultrafiltration cell using a PM-10 ultrafiltration membrane (Amicon Corp., Danvers, MA, USA) thereby restoring the Triton X-100 concentration to circa 1% (w/v). This 500 \( \mu \)L sample was loaded onto a (7.5 x 420)mm column of Sephacryl S-300 'Superfine' (Pharmacia Inc., Uppsala, Sweden) and eluted into 600 \( \mu \)L fractions with a running buffer of 0.2% Triton X-100, 100 mM potassium phosphate, pH 7.0 at a flow rate of 1.0 mL hr\(^{-1}\). The Sephacryl column was calibrated under the standardized running conditions with 'molecular weight protein standards'.

(i) Partial purification of the 'Peak III' respiratory cytochrome

The liquid chromatographic procedure for fractionating solubilized cytochromes described above in section (g) resulted in the preparation of a series of major cytochrome 'peaks', several of which were purified further. Recoveries of 'Peak I' cytochromes were adequate for subsequent analytical procedures but insufficient for preparative techniques. Fractions contributing to 'Peak II' were pooled and used for the isolation of cytochrome \( o \) (section (h)) and to separate 'Peak III' cytochromes when present (v.i.). The 'Peak IV' fractions were pooled for further investigations of their cytochrome content and the associated hydroperoxidase activity.

The 'Peak III' respiratory cytochrome was observed as a component of a fused 'Peak II/III' when cells grown aerobically on L-proline were subjected to the standard procedures of membrane cytochrome extraction and fractionation on DEAE-BioGel.A. Fractions making up this fused peak were pooled and loaded onto an hydroxylapatite column of BioGel HTP (2.5 cm diameter, 3.5 cm in length) (Bio-Rad Laboratories, Richmond, CA, USA) which had previously been equilibrated with TTE buffer, pH 7.8. The sample was washed with TTE buffer and eluted with a 200 mL gradient
of (0-400) mM potassium phosphates, pH 7.8, in TTE buffer at pH 7.8.

**Partial purification of the ‘Peak IV’ hydperoxidase**

After the initial DEAE-BioGel.A separation of cytochromes from Triton-solubilized membranes (section ‘g’) the fractions recognised as ‘Peak IV’ were pooled and either used directly for analyses or were prepared for potentiometric titration by buffer and detergent exchange procedures similar to those employed for the ‘Peak II’ fraction (cytochrome o) described in section ‘h’. In the latter case pooled ‘Peak IV’ fractions were diluted with an equal volume of DTE buffer, pH 7.8, and loaded onto a (5.0 x 30.0) mm column of DEAE-BioGel.A previously equilibrated with DTE buffer, pH 7.8. The cytochrome was washed with ten column volumes of DTE buffer, pH 7.8 and then eluted in a concentrated fraction of approximately 2.5 mL with DTE buffer, pH 7.8, containing 400 mM KCl. This fraction was concentrated to approximately 500 µL in an Amicon ultrafiltration cell using a PM-10 ultrafiltration membrane thereby restoring the Triton X-100 concentration to circa 1% (w/v). The 500 µL sample was loaded onto a (7.5 x 420) mm column of Sephacryl S-200 ‘Superfine’ using TTE buffer, pH 7.8 as running buffer and eluting at a rate of 1.0 mL hr⁻¹ into 600 µL fractions which were monitored at 412 nm for cytochromes and for protein by the modified Lowry technique described below in section ‘s’. An analogous ion-exchange plus ultrafiltration procedure was used to concentrate the major cytochrome peak from this first gel filtration column after which it was loaded onto a (7.5 x 420) mm column of Sephacryl S-300 ‘Superfine’ using a running buffer of 0.2% (w/v) Triton X-100, 100 mM potassium phosphate, pH 7.0 in preparation for subsequent potentiometric titration: fractions were collected and monitored as for the previous gel filtration step. Both Sephacryl columns were calibrated under the standardized running conditions with ‘molecular weight protein standards’ (Pharmacia Inc., Uppsala, Sweden).

**Preparation of nitrate reductase**

Large batches of cells of strain RK4353 were grown on NR medium (Appendix ‘C’) at 37°C in static culture and sparged with the (N₂ + CO₂) gas mixture as described in ‘c’, above. When the cells had reached stationary phase the culture was cooled on ice for 45 min and harvested in the standard manner. The sample temperature was maintained at (0-4)°C during all subsequent steps. Crude membranes were prepared as in ‘d’, with the exception that all buffers were degassed and contained 0.1 mM dithiothreitol [28]. The upper, dark brown layer enriched in inner membranes was resuspended in 400 mL TM buffer containing 0.1 mM DTT and 25 mL aliquots were overlaid
upon 2.5 mL cushions of 60 % (w/v) sucrose in TD buffer in preparation for ultracentrifugation for 2.5 hours at 250,000 x g in Beckman Type 60Ti or 45Ti ultracentrifuge rotors. The resulting dark brown sucrose solution was diluted to 11.0 mg mL$^{-1}$ protein with TD buffer and a 20 % (w/v) solution of Triton X-100 in TD buffer was added dropwise as the preparation was stirred slowly on ice. The Triton extraction was continued for 60 min after which it was centrifuged at 250,000 x g for 1.5 hours. The supernatant was loaded onto a (2.5 x 31)cm column of DEAE-BioGel.A previously equilibrated with TD buffer containing 0.1% (w/v) Triton X-100. Fractions of 12.0 mL were collected as the column was washed with 2.5 volumes of equilibration buffer and eluted with a gradient of (0-300) mM NaCl in six column volumes of the equilibration buffer [67].

(I) Spectrophotometric analysis of cytochromes

Two double beam, dual wavelength analytical spectrophotometers were available: an Hitachi/Perkin-Elmer model 356 and an SLM/Aminco model DW2c with accompanying Midan II data processor and plotter. Both instruments are capable of measuring small optical absorbances in highly opaque samples such as aqueous suspensions of crude membranes. The DW2c is capable of spectral resolution of such samples to within 0.5 nm when adjusted optimally (v.i.).

Pyridine haemochromogen estimation was performed by the method of Falk [49], with 'reduced minus oxidized' difference spectra and absolute reduced spectra being measured at ambient temperature on either of the two analytical spectrophotometers.

Ambient temperature reduced minus oxidized difference spectra of cytochromes, membrane suspensions and solubilized material were each collected with a 10 mm pathlength and a bandwidth of 2.0 nm (PE-356) or 2.2 nm (DW2c) to cover the (400-700) nm spectral range: in many cases the (380-400) nm range also provided useful information. Potassium phosphate buffer, 100 mM, pH 7.0, was used to suspend and dilute samples before analysis. Electrochemical reduction and oxidation of the sample and reference solutions was achieved with a variety of reagents depending on the requirements of the experiment, but a few grains of Na$_2$S$_2$O$_4$ and fresh 3.0 % (v/v) H$_2$O$_2$ were routinely employed as reductant and oxidant respectively. Extended equilibration times at ambient temperatures are required for spectrophotometry of preparations containing Triton X-114 due to the 20°C cloud-point of this detergent; low temperature difference spectra are more suitable for such samples.

Low temperature reduced minus oxidized difference spectra were obtained at liquid nitrogen temperature (-196°C, 77 K) in specialized sample chambers available as accessories for both PE-356 and DW2c spectrophotometers to provide increased resolution of the absorption bands of cytochrome
spectra. Standard conditions include dilution of sample to double strength in 100 mM potassium phosphate buffer, pH 7.0 followed by dilution with an equal volume of the buffer containing 2.0 M sucrose \(^{214}\). The sample was split into two 1.0 mL aliquots, one being oxidized as the reference (normally with a drop of 3.0 % (v/v) H\(_2\)O\(_2\)) and the other being reduced (usually with a few grains of Na\(_2\)S\(_2\)O\(_4\)) before placing each into the appropriate locations in the brass (PE-356) or aluminium (DW2c) sample holder. The holder was then carefully immersed in liquid nitrogen and left submerged to equilibrate for a minimum of 20 min after which it was positioned in the cryogenic chamber’s Dewar containing the maximum volume of liquid nitrogen compatible with a stable signal \(^{66,90,206,212}\). Sample pathlengths were 1.0, 2.0 or 3.0 mm as indicated in the text; standard conditions are indicated in Table I. While the use of sucrose at 1.0 M dramatically increased both sensitivity and spectral resolution obtainable with the instruments devitrification was unnecessary with these samples and is generally reserved for cryogenic spectroscopy of glycerol solutions \(^{206,212}\).

Samples were poised at a variety of selected ‘low temperatures’ for studies of the photon induced relaxation of the complex formed between carbon monoxide and certain cytochromes in their reduced state. These distinct temperature conditions were produced in the Dewar of the PE-356 cryogenic sample chamber by combinations of dry ice with ethanol (-75°C, 198 K), or wet ice with either an equal volume of ethanol (-30°C, 243 K) or with NaCl to 2.0 M (-12°C, 261 K) \(^{83}\), the latter conditions being monitored with the remote temperature probe.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Data rendition</th>
<th>Optimal scan speed</th>
<th>Path Length</th>
<th>Bandwidth</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE-356</td>
<td>Electromechanical</td>
<td>0.5 nm s(^{-1})</td>
<td>Ambient: 10.0 mm</td>
<td>Cryogenic: 3.0 mm</td>
</tr>
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<td></td>
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<td>2.0 nm</td>
<td>2.0 nm</td>
</tr>
<tr>
<td>DW2c</td>
<td>Electronic</td>
<td>immaterial</td>
<td>Ambient: 10.0 mm</td>
<td>Cryogenic: 2.0 mm</td>
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<tr>
<td></td>
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<td></td>
<td>2.2 nm</td>
<td>0.8 nm</td>
</tr>
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</table>

**Table I:** Spectrophotometer settings for optimal spectral resolution.

Resolution of the \(\alpha\)-bands of ambient and low-temperature difference spectra was optimized by adjusting the bandwidth and scan speed of the instrument performing the spectral analysis and by averaging the results of up to nine successive spectral scans. These parameters varied between instruments and the critical values are indicated in Table I. Spectral calibration of the
spectrophotometers was achieved by reference to the absorption spectrum of a standard holmium oxide filter (SLM/Aminco #A-0724) and to the visible emission maxima of each instrument’s deuterium lamp at (486.0, 656.1 & 972.0) nm.

Reduced plus carbon monoxide minus reduced difference spectra, or ‘CO-binding’ difference spectra — commonly referred to as ‘carbon monoxide difference spectra’, were routinely measured at ambient temperature. Instances of cryogenic measurement of such spectra for detailed analysis of their α-bands are indicated in the text. The sample was diluted to the required concentration in 100 mM potassium phosphate buffer, pH 7.0, reduced by adding a few crystals of Na₂S₂O₄ and carefully separated into two cuvettes without aeration. One of these reduced fractions was placed in the spectrophotometer’s reference beam and the other was transferred to a fume-hood and had carbon monoxide bubbled through it gently from a fine nozzle for 90 s after which it was sealed with Parafilm™ and placed in the instrument’s sample beam. The CO-treated sample was maintained under dark conditions throughout the gassing and loading procedures and exposure to the analytical light beams was kept to a minimum before spectral data were collected. Similar precautions were taken with low temperature measurements, the reduced, gassed samples being transferred to the pre-cooled cryogenic sample holder with a CO-filled Pasteur pipette, and frozen to the required temperature under a slow stream of carbon monoxide. Subsequent replacement of the cooling solution in the Dewar enabled temperature modulated relaxation studies to be performed as indicated above.

(m) Derivative analysis of spectrophotometric data

Derivative spectra were plotted after calculation of the fourth-order finite difference spectra of original data gathered by the DW2c. Data storage and manipulation was achieved with the Midan II processor installed with the spectrophotometer by the manufacturer. Interpretation of these derivative spectra is described in section I.i.d of the Results & Discussion {180, 186}.

(n) Redox kinetics of cytochromes

Dual-wavelength spectrophotometry was used to monitor the kinetics of cytochrome oxidation and reduction by means of the net absorption of the sample at (559.0-580.0) nm. Membrane suspensions were prepared at a protein concentration of 5.0 mg mL⁻¹ in Hepes buffer and preoxidized in a standard cuvette with 1.0 μL of 3.0 % (w/v) aqueous H₂O₂ [123]. The addition of microlitre volumes of concentrated chemical or biological reductants plus rapid mixing initiated reduction of
sample components, including dissolved oxygen, and established dynamic redox equilibria. The reduction state of the population of cytochromes was then followed spectrophotometrically. The effect of respiratory inhibitors and amphipathic electrochemical mediators was also tested as were the kinetic responses to reoxidation by chemical and biochemical reagents [214].

(o) Potentiometric titrations

Procedures described in this section are modifications of those developed by several investigators in this laboratory over an extended period [66, 69, 167, 170].

The potentiometric titrations were carried out using the PE-356 spectrophotometer in split beam mode and with its secondary sample chamber modified to accept a lateral stirring motor and a Dutton-style side-arm cuvette accommodating a top-mounted combination platinum electrode with internal reference (Fisher #13-639-82), remote temperature probe (YSI Tele-Thermometer Model 42SC, Yellow Springs Instrument Co., Yellow Springs, OH, USA) and nitrogen flushing lines [6, 66]. A single side-arm sealed with a serum stopper provided a port for the addition of chemical oxidants and reductants. Nitrogen gas of ‘prepurified’ grade (minimal CO content) was passed through a two stage scrubbing system to remove oxygen and in which it was also water-saturated and brought to neutral pH. Stage one comprised sparging from a fritted glass inlet filter through a 500mL volume of zinc amalgam plus ammonium vanadate in HCl prepared as described by Meites and Meites [67, 131]. This was followed by passage through two successive Fisher-Milligan gas-exchange chambers each containing 300 mL of the phosphate buffered solution of methyl viologen, proflavin and EDTA developed by Sweetser [67, 198]. The treated gas was passed at a slow rate across the surface of the sample in the modified Dutton cuvette during the equilibration period and throughout the titration. The exhaust gas was passed through a small bubble chamber in order to monitor the exit flow rate and chamber pressure and to check that all seals were gas-tight.

The sample was prepared by diluting it with potassium phosphate buffer (100 mM, pH 7.00) to a known concentration of protein in 25 mL such that, optimally, the α-band of the ambient temperature redox difference spectrum provided approximately 70% deflection on the PE-356 at a full scale range setting of 0.1 A. This corresponded to a protein concentration of 5-10 mg mL⁻¹ protein in typical membrane preparations and provided adequate sensitivity while minimizing interference by baseline shifts resulting from changes in mediator oxidation states. Moreover, by operating the spectrophotometer in split beam rather than in dual beam mode as traditionally used by other investigators [8, 81] a convenient yet accurate estimation was readily obtained of changes occurring in the spectral baseline as the potential of the sample altered [66, 105]
117, 187}. A 2.0 mL fraction was removed from the sample preparation, oxidized in a cuvette with several crystals of $K_3Fe(CN)_6$, sealed with Parafilm™ and placed in the reference beam of the spectrophotometer. Mediators were immediately added to the remaining sample which was mixed by inversion and sealed in the Dutton-style cuvette (5.0 μL each of 5.0 mg mL$^{-1}$ fresh mediator solutions as indicated in Table II, yielding final concentrations of approximately 20 μM). The sample was allowed to equilibrate under scrubbed nitrogen, with stirring, for at least 60 minutes during which the temperature stabilized at 305 K.

Monitoring of the potentiometric titrations was achieved by scanning the α-band of the ambient temperature reduced minus oxidized difference spectra at 1.0 nm s$^{-1}$ from 580.0 nm to 530.0 nm (650.0 nm to 530.0 nm for titrations of the cytochrome $d$ complex) at approximately 5 mV intervals throughout the potential range of the sample. As the monochrometer scanned through the wavelength of maximum absorbance ($\lambda_{\text{max}}$ approximately 560 nm) the electrochemical potential was read from the millivoltmeter (Fisher ‘Accumet’ Model 325) connected to the platinum electrode. Collecting spectral data over the wavelength range indicated permitted an accurate baseline to be drawn on the plotted output and the peak height to be calculated at various wavelengths of interest; generally $\lambda = 558.0$ nm, $\lambda_{\text{max}}$ and $\lambda = 563.0$ nm for type-$b$ cytochromes.

Monitoring was initiated during the autoreduction that was observed after the initial equilibration and continued throughout the subsequent reduction which was achieved at an extremely slow rate by injecting microlitre quantities of fresh phosphate-buffered NADH through the rubber septum sealing the side-arm of the Dutton cuvette. This slow reduction rate ensured full redox equilibration within the sample suspension. Monitoring of non-membranous sample solutions began with the NADH reduction step. Subsequent oxidation was achieved by injecting a fresh, phosphate-buffered, concentrated solution of either $K_3Fe(CN)_6$ or $H_2O_2$. Spectral and potentiometric data were gathered during the reductive phases of several reduction and oxidation cycles. Samples of membrane suspensions occasionally generated hysteretic and non-reproducible results when monitored during oxidation, presumably due to differences in the redox equilibration rates of certain sample components in comparison with those of the electrode. Consequently each oxidative phase was followed by an extended equilibration period of at least 30 minutes. The final measurements of each titration were taken at potentials below -300 mV following full reduction of the sample with $Na_2S_2O_4$ in order to observe the presence of any low potential cytochromes and to check that no significant sample degradation had occurred during the protracted experimental procedure.

Titrations carried out in the presence of carbon monoxide were accomplished under forced ventilation with direct venting to the exterior of the building of the $N_2$ and CO gas supply systems,
### Materials & Methods

**Table II:** Electrochemical mediators for potentiometric titrations.

These reagents were used collectively as electrochemical mediators in potentiometric titrations. The buffer used to dissolve the hydrophilic reagents was 100 mM potassium phosphate, pH 7.0. Fresh stock solutions were made up immediately before each titration.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>( E_m )</th>
<th>n</th>
<th>solvent</th>
<th>stock concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-monocarboxyferrocene</td>
<td>+530</td>
<td>1</td>
<td>ethanol</td>
<td>5.0 mg mL(^{-1})</td>
</tr>
<tr>
<td>triphenanthroline cobalt III</td>
<td>+370</td>
<td>1</td>
<td>buffer</td>
<td>5.0 mg mL(^{-1})</td>
</tr>
<tr>
<td>quinhydrone</td>
<td>+270</td>
<td>1</td>
<td>buffer(^a)</td>
<td>5.0 mg mL(^{-1})</td>
</tr>
<tr>
<td>2,6-dichlorophenolindophenol</td>
<td>+224</td>
<td>2</td>
<td>buffer(^a)</td>
<td>5.0 mg mL(^{-1})</td>
</tr>
<tr>
<td>1,2-naphthoquinone</td>
<td>+157</td>
<td>2</td>
<td>ethanol(^a)</td>
<td>5.0 mg mL(^{-1})</td>
</tr>
<tr>
<td>phenazine methosulphate</td>
<td>+ 92</td>
<td>2</td>
<td>buffer(^b,c)</td>
<td>5.0 mg mL(^{-1})</td>
</tr>
<tr>
<td>phenazine ethosulphate</td>
<td>+ 55</td>
<td>2</td>
<td>buffer(^b,c)</td>
<td>5.0 mg mL(^{-1})</td>
</tr>
<tr>
<td>duroquinone</td>
<td>± 0</td>
<td>2</td>
<td>ethanol(^a)</td>
<td>5.0 mg mL(^{-1})</td>
</tr>
<tr>
<td>menadione</td>
<td>- 50 (?)</td>
<td>2</td>
<td>ethanol(^a)</td>
<td>5.0 mg mL(^{-1})</td>
</tr>
<tr>
<td>2-hydroxy-1,4-naphthoquinone</td>
<td>-139</td>
<td>2</td>
<td>ethanol(^a)</td>
<td>5.0 mg mL(^{-1})</td>
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<tr>
<td>anthraquinone-2-sulphonate</td>
<td>-225</td>
<td>2</td>
<td>water(^a)</td>
<td>5.0 mg mL(^{-1})</td>
</tr>
</tbody>
</table>

\(^a\) Warming is required to dissolve the reagent at the indicated concentration.

\(^b\) Extremely light-sensitive in aqueous solution.

\(^c\) Possible neoplast.
the spectrophotometer sample chamber and the titration vessel exhaust gases. These titrations followed a standard reductive titration of the sample. A moderate flow of carbon monoxide was passed into the fully reduced sample through the stoppered side-arm of the titration vessel such that it bubbled slowly through the stirred liquid and was emitted from the nitrogen outlet. Nitrogen was not flushed over the sample surface during the fifteen minutes of carbon monoxide treatment, but was restarted immediately afterward at a minimal flow rate in order to maintain a positive internal gas pressure and prevent sample interaction with oxygen. Carbon monoxide was introduced through the side-arm periodically during the remainder of the procedure. Exposure of the sample to light was minimized throughout the treatment.

Potentiometric data were modified by a simple FORTRAN program to generate data tables of standardized results and derived values in formats suitable for input to the BMD: P3R software for non-linear regression analyses (BMDP Statistical Software, Inc., Los Angeles, CA, USA) and into the TELL-A-GRAF two-dimensional graphics software (Computer Associates International, Inc., Garden City, NY, USA) running under the Michigan Terminal System on the University of British Columbia’s Amdahl mainframe network (MTS-G, UBCnet). FORTRAN subroutines had been developed for one- to four-component curve fitting using BMD: P3R [40, 66]: additional subroutines were written to permit data-fitting analyses with up to six distinct theoretical components although it was determined empirically that the program was unable to resolve components differing in mid-point potential by less than 50 mV. Following tabulation, data from BMD: P3R analyses were converted to a format acceptable to TELL-A-GRAF by *TELLABANK software provided by the U.B.C. Computing Centre.

(p) Spectrophotometry at selected electrochemical potentials

Two methods of electrochemical poising were employed, one achieved kinetically and the other through potentiometric titration: both employed rapid freezing at 77 K as the method of voltage clamping during the extended spectrophotometric analyses.

Kinetic poising of samples at specific potentials was either achieved by exploiting the temporary ‘steady states’ of reduction induced in the electron transport components of membrane suspensions by the addition of chemical oxidants or biological substrates to an aerated sample or by utilizing the partial reduction equilibria created during substrate reduction of solubilized cytochrome preparations. In both cases a preliminary experiment was required using timed dual-wavelength spectrophotometric studies to determine the duration of each phase of reduction at a specific temperature. Subsequent potentiometric poising could then be accomplished under equivalent
conditions on the bench-top in the spectrophotometer's cryogenic sample holder which would be frozen rapidly in liquid nitrogen at the prescribed time. The frozen sample could then be subjected to standard low temperature spectrophotometry. Alternatively one spectrophotometer could be used to monitor a sample's reduction kinetics in dual-wavelength mode (PE-356) while the other (DW2c) was set for low temperature split-wavelength mode analyses of fractions withdrawn from the sample chamber of the first instrument and rapidly frozen under an inert atmosphere as described above. Thus the freezing to 77 K enabled the poised electrochemical potential of the sample to be maintained throughout the extended time period required for spectral determination as well as enabling accurate spectrophotometric analyses to be performed.

During potentiometric titrations aliquots could be withdrawn through the septum sealing the side arm of the Dutton cuvette using a nitrogen-flushed 1.0 mL syringe fitted with a 2” 18-gauge stainless steel needle. The withdrawn sample could then be deposited into the sample holder of the cryogenic accessory under a stream of nitrogen in preparation for rapid freezing and low temperature spectrophotometry. The Results section describes control experiments in which the titrations were carried out in the presence and absence of 1.0 M sucrose in order to obviate the physical problems associated with mixing sucrose into the sample after the latter had been removed from the titration vessel. In these experiments the cryogenic accessory would be precooled with the reference chamber containing the frozen reference sample. It was possible to undertake such experiments with a single spectrophotometer by carrying out the titration itself in the Dutton cuvette within a light-proof box (thus protecting the light-sensitive mediators), withdrawing the sample in the manner described above and using the spectrophotometer exclusively for gathering cryogenic spectra. The decision of sampling time would be based upon the electrochemical potential values provided by the millivoltmeter. A superior technique was to monitor the titration spectrophotometrically at ambient temperature as well as potentiometrically, withdrawing samples from the 'titration' spectrophotometer (PE-356) as described above, and to transfer these under nitrogen to precooled cryogenic sample holders in preparation for immediate analysis in the second spectrophotometer (DW2c).

(q) Stopped-flow spectrophotometry

An Aminco/Morrow stopped-flow accessory (SLM/Aminco, Urbana, WI, U.S.A.) was utilized in conjunction with the SLM/Aminco DW2c spectrophotometer operated in dual wavelength mode for stopped-flow rapid kinetic analysis of the reduction of Triton-solubilized cytochrome \( \alpha \) preparations. A fully oxidized sample of cytochrome in TTE buffer at a membrane protein
concentration of 5.0 mg mL\(^{-1}\) was injected into the reaction chamber with an equal volume of fresh duroquinol (maintained under nitrogen) or Na\(_2\)S\(_2\)O\(_4\), each at one of several concentrations. The ensuing reaction was monitored over times extending to several seconds at sample and reference wavelengths of 560 nm minus 575 nm.

(r) Genetic manipulation of *E. coli* cells

Transduction between *E. coli* strains with the generalized transducing bacteriophage P\(_{1\text{vir}}\) was performed following standard procedures \(^{134}\).

Mutagenesis of *E. coli* strains was achieved using the nitrosoguanidine techniques described by Miller \(^{134}\). Survival curves were generated and used to determine exposure times necessary to produce 50 % killing under standard conditions: fresh mutagen at 50.0 mg mL\(^{-1}\) in 100 mM sodium citrate buffer, pH 5.5, 37°C containing 5 \(\times\)10\(^{8}\) freshly suspended exponential phase cells per millilitre. Treatments of 46 min and 15 min duration were required for strains PLJ01 and PLJ04 respectively. Exposure was terminated by sedimentation of the cells for 1.0 min in a microfuge followed by two washes in sterile 100 mM potassium phosphate buffer, pH 7.0, resuspension in the same buffer and immediate dilution for plating or for enrichment and selection procedures.

Generally, P\(_{1\text{vir}}\) transduction was followed by straightforward enrichment and selection procedures exploiting the constructs' anticipated auxotrophic or antibiotic resistance characteristics \(^{134}\). Identification was achieved by screening for multiple phenotypic markers on additional selective media and, for cytochrome variants, by redox spectrophotometry \(^{89}\).

When manipulating cytochrome genes the transduction or mutagenesis of cultures was followed by ‘enrichment’ growth under anaerobic conditions for 16-24 hours on minimal medium M9-K supplemented with glucose, a fermentable carbon-energy source. Although this shift to minimal medium caused these cultures to experience a significant lag phase it ensured that recovery of nutritional auxotrophs was minimized while providing the least growth disadvantage to cells undergoing alterations of genes governing the expression of aerobic respiratory components.

Modifications of loci within the *cyd* operon were carried out by P\(_{1\text{vir}}\) cotransduction of the neighbouring *nadA* or *sdh* genes from *Escherichia coli* strains carrying the required characteristics of *cyd* gene expression, and thereby either modifying the recipient’s auxotrophic status with respect to nicotinic acid or changing its ability to utilize succinate as sole carbon source \(^{89}\). Cells experiencing a positive change of status in either of these marker genes were obtained by direct selection on minimal agar plates containing the appropriate nutrient. Isolation of transductants carrying negative markers was more efficient after enrichment using penicillin-G at 50 mg mL\(^{-1}\) for
several hours in minimal medium M9-K followed by sedimentation plus sterile washing as described above and replica plating onto minimal agar plates with and without the relevant nutritional supplement.

Cells containing mutations associated with the cytochrome o respiratory pathway were obtained from strain PLJ01 in which the expression of the cyd operon is nutritionally dependent and from strain PLJ04 which is cyd" [89]. Mutagenised cultures of PLJ01 were exposed to two rounds of penicillin enrichment. Following 16 hours’ anaerobic growth in 5.0 mL minimal medium M9-K with glucose they were sedimented in a Beckman JA-20 rotor at 12,000 x g for 10 min at 22°C, resuspended in 5.0 mL glucose supplemented M9-K, partially degassed in a partly evacuated, uncharged desiccator and grown to stationary phase in standing culture over approximately nine hours. Glucose causes induction of the cyd operon under these conditions of low oxygen tension, consequently cyd" mutants and those unable to produce any functional cytochromes were rapidly outgrown by cyd+ cells. The cells were sedimented as before, and resuspended in 5.0 mL M9-K with lactate and succinate as carbon-energy sources. This culture was vigorously aerated with water-saturated, filter-sterilized air and after four hours another 5.0 mL M9-K was added, containing lactate plus succinate and also sufficient penicillin-G to yield a final concentration of 50 mg mL⁻¹.

The airflow through this initial penicillin-enrichment culture was maintained for two more hours in order to kill cells using cytochrome o for energy production; cyd expression would have been repressed under these highly aerobic conditions rich in lactate and succinate, thereby preventing the growth of cyd+cyo cells and promoting their survival. Since lactate and succinate are non-fermentable carbon sources cells unable to synthesise both aerobic terminal oxidases would also survive this enrichment procedure, as would all obligatorily fermentative cells: hence the importance of the primary outgrowth step described above. After two hours the cells were spun down as before and washed in 10.0 mL sterile 100 mM potassium phosphate buffer, pH 7.0 for twenty minutes in order to complete the lysis of penicillin-weakened cells. The cells were washed again with the buffer and resuspended in 5.0 mL M9-K medium with glucose as carbon-energy source. The culture was partially degassed as before and grown to stationary phase at 37°C for 15 hours in order to enrich or cyd+ cells. Samples were removed for plating after this stage and also after the entire penicillin enrichment procedure had been repeated. Plating was carried out onto M9-K medium plus glucose followed by growth in a gas jar under low but significant oxygen tensions so that cyd+ cells would grow rapidly, whether or not active cytochrome o was present. Colonies from these plates were subsequently picked onto gridded M9-K plates containing either glucose or a combination of lactate plus succinate and screened for ability to grow solely on the former under normal aerobic conditions at
37°C. Thus cyo⁻ cells were selected from wild type and from obligatorily fermentative mutants including those which are unable to produce any functional cytochromes, *eg.* unc⁻ and hem⁻ strains, respectively.

Another method used to generate cyo⁻ strains from mutagenized cells of both PLJ01 and PLJ04 was that of Au et al.. This technique uses anaerobic conditions and a growth medium incorporating glycerol as sole carbon/energy source with nitrate as electron acceptor in order to select against obligatorily fermentative mutants [8]. Well aerated growth medium containing lactate plus succinate and supplemented with ampicillin at 40 mg mL⁻¹ enables enrichment for cyo⁻ cells. Survivors are grown under the established anaerobic conditions and tested for inability to grow under aerobic conditions on lactate and succinate. Under these environments both PLJ01 and PLJ04 would behave with a cyd⁻ phenotype.

Selection of *Tet*⁵ mutants from strains carrying *Tn10* was accomplished by the Davis implementation [37] of the technique of Bochner [13] in which induction of tetracycline resistance by non-toxic autoclaved chlorotetracycline results in a sensitivity to growth inhibition by the ion chelating activities of fusaric and quinaldic acids.

Plasmid DNA 'mini-preps' and full-scale CsCl purifications of plasmid DNA were carried out by the standard methods described in Maniatis' manual [123].

**(s)** **Biochemical assays**

Protein concentrations were estimated by the method of Lowry et al. [29, 120] as modified for greater sensitivity [23] and the presence of membrane lipid [125]. Bovine serum albumin fraction V was used as the reference (Sigma Chemical Corp., St. Louis, MO, U.S.A.). The Triton family of detergents interferes with several common methods of protein determination, including those of Barrett [178], Bartlett [11, 52] and Lowry *et al.* [120]. Consequently protein measurements of samples containing such detergents were executed in the presence of an equivalent concentration (w/v) of SDS in the assay reagent 'mix' as there was Triton added with the sample [125, 175]; this technique minimizes the errors due to the Triton but lowers the sensitivity of the of the assay and requires centrifugation of fractions high in potassium salts in order to remove finely suspended precipitates of potassium dodecyl sulphate. Colour development in the presence of SDS required approximately one hour and was stable for 20 hours in the dark at room temperature, during which time the fine precipitates settled to form a loosely packed pellet.

Fumarate, nitrate and TMAO reductase activities were assayed in anaerobic cuvettes by the method of Jones & Garland [92] The oxidation of reduced benzyl viologen in the presence of an
aliquot of sample membrane suspension by stock solutions of the appropriate substrate was monitored at 660 nm [66].

Oxidase activities were determined by dual wavelength kinetic analyses of cytochrome reduction (section ‘n’) indicating the rate at which dissolved oxygen was depleted from a freshly mixed sample and assuming an aqueous dioxygen saturation concentration of 260 μM.

(1) Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Protein electrophoresis was carried out on denaturing polyacrylamide gels of either 10 % or 13 % (w/v) with a 4 % (w/v) stacking gel using protocols developed by Laemmli [110]. It was found that gel penetration and component resolution was superior if solubilized cytochrome o samples were not boiled in the SDS solution before loading onto the gels. When loading samples derived from fractions eluted off of ion-exchange columns with potassium salt gradients it was necessary to prevent precipitation of potassium dodecyl sulphate in the gel’s loading well. This was accomplished by one of two techniques: either warm water at 50°C was circulated through the cooling system of the gel apparatus during the loading procedure and the initial ten minutes of electrophoresis or the sample was mixed into a sample buffer containing 0.5 % (w/v) SDS instead of the standard 2.3 % (w/v) SDS. The efficacy of each approach and the resulting resolution of peptide components depended upon the concentrations of potassium ions and protein in the samples.

Fairbanks stain, incorporating Coomassie Brilliant Blue R250 was used for routine staining of SDS-PAGE gels [47] which was followed by destaining and fixing in 10 % (v/v) aqueous acetic acid. Photochemical silver staining of SDS-PAGE gels was also carried out when extra sensitivity was required [132]. SDS-PAGE of protein samples to be stained for haem was carried out with gels which had been ‘pre-electrophoresed’ in order to remove excess ammonium persulphate [199]. Staining for haemoproteins separated on these gels under reducing or non-reducing conditions was accomplished using these proteins’ residual peroxidase activity in conjunction with the addition of hydrogen peroxide and either dimethylbenzidine [53] or 3,3′,5,5′-tetramethylbenzidine [90, 133, 199].
RESULTS & DISCUSSION

II: REFERENCE STUDIES OF SOLUBLE CYTOCHROMES

This initial section provides an overview of the techniques that were employed for the analysis of *E. coli* respiratory cytochromes.

Three soluble cytochrome preparations were used as standard protein solutions when developing and calibrating the procedures and instruments utilized in this study: mitochondrial cytochrome *c* (equine heart), catalase (bovine liver) and the soluble fragment of trypsinized microsomal cytochrome *b*$_5$ (bovine liver), each being obtained from the sources described under Materials & Methods.

(i) **Redox Difference Spectroscopy**

Appendix A provides a brief description of those spectral properties of cytochromes that are pertinent to the current investigation.

Difference spectra were collected at ambient and liquid nitrogen temperatures in order to compare values obtained from standard cytochrome solutions against those recorded in the literature. These spectra included reduced minus oxidized (redox) difference spectra, reduced plus carbon monoxide minus reduced (CO-binding) difference spectra and reduced minus oxidized difference spectra of the pyridine haemochromogen derivatives of selected cytochrome samples. Analytical redox difference spectrophotometry was routinely performed at 77 K in order to exploit the greater resolution of cytochrome α-absorption bands obtainable under these conditions as is illustrated below.
Fig. 4: Visible range redox difference spectra of soluble reference cytochromes.

Ambient temperature (295 K) [reduced minus oxidized] difference spectra of soluble reference cytochromes in 100 mM potassium phosphate buffer, pH 7.0. Cytochrome c refers to equine heart cytochrome c, cytochrome b$_5$ refers to the soluble tryptic fragment of bovine hepatic microsomal cytochrome b$_5$. $\Delta A = 0.20$.

a. Cytochrome c, 12.5 $\mu$g mL$^{-1}$ (Sigma-Aldrich, St. Louis, MO) plus cytochrome b$_5$, 10.0 $\mu$g mL$^{-1}$ (Dr. A. G. Mauk, U.B.C.).

b. Cytochrome c, 25.0 $\mu$g mL$^{-1}$.

c. Cytochrome b$_5$, 10.0 $\mu$g mL$^{-1}$.

d. Baseline.
Cryogenic accessories each incorporating a Dewar flask holding liquid nitrogen within the modular sample chamber of the respective high-resolution spectrophotometer were employed for these analyses as were the signal:noise enhancement techniques described under Materials & Methods.

(a) **Ambient temperature redox difference spectra**

Visible redox absorption spectra of standard mammalian cytochromes are shown in Figure 4. The major spectral features of each are the Soret, or \( \gamma \)-band absorption peak at \((420-430)\)nm, the \( \beta \)-band absorbance at \((520-535)\)nm and that of the \( \alpha \)-bands at \((545-565)\)nm. Precise values for the absorption maxima are given in Table III. While each of the absorption peaks of the type-c cytochrome was at a lower wavelength than the corresponding peak of cytochrome \( b \) the combined solution showed a fused Soret peak without distinguishable characteristics of the individual components (Fig. 4). Although absorbance in the \( \alpha \)-band region was less intense than that of the Soret (typically having a relative intensity at 295 K of one eighth the Soret size, and approximately one fifth at 77 K) this region of the combined spectrum displayed features of both individual spectra as is illustrated in greater detail in Figure 5. Analyses of \( \beta \)-band absorption patterns offered reduced intensity and resolution in comparison to those of the \( \alpha \)-absorbance region.

Although the cytochrome \( b \) \( \alpha \)-band spectrum was clearly asymmetrical at ambient temperature, this was not the case for that of cytochrome \( c \) although the location of the type-c \( \alpha \)-band absorption maximum at a shorter wavelength than that of the type-\( b \) cytochrome was recognisable (Fig. 5a,c). Some characteristics of the \( \alpha \)-bands of both component cytochromes were displayed by the ambient temperature \( \alpha \)-band spectrum of the combined solution (Fig. 5e).

(b) **Low temperature redox difference spectra**

The improvement in resolution of spectral features of the reduced *minus* oxidized cytochrome \( \alpha \)-bands when observed at low temperatures was demonstrated by the expanded mid-range spectra of Figure 5 (curves b, d & f). This technique provided narrower absorbance peaks with a shift of the maximal absorption to shorter wavelength and an increase in absorbance, although for practical purposes the increase in the extinction coefficient was offset by the requirement for a shorter pathlength due to the opacity of the sample below freezing temperatures and the need for a uniform sample cooling rate (90). Both standard, soluble cytochromes display a pronounced biphasic
### Reduced minus Oxidized Difference Spectra

<table>
<thead>
<tr>
<th>Temp. (K)</th>
<th>λ_\text{γ max} (nm)</th>
<th>λ_\text{β max} (nm)</th>
<th>λ_\text{α max} (nm)</th>
<th>Reference #</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytochrome bs</strong> (bovine liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>295</td>
<td>423</td>
<td>526</td>
<td>556</td>
<td>(112)</td>
</tr>
<tr>
<td>295</td>
<td>(171)</td>
<td>527</td>
<td>556.3+560.0</td>
<td>(hinc)</td>
</tr>
<tr>
<td>77</td>
<td>—</td>
<td>—</td>
<td>552+559</td>
<td>(112)</td>
</tr>
<tr>
<td>77</td>
<td>422.5</td>
<td>525.5+532.0</td>
<td>551.8+557.3</td>
<td>(hinc)</td>
</tr>
<tr>
<td><strong>Cytochrome c</strong> (equine heart)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>295</td>
<td>416</td>
<td>520.5</td>
<td>550.0</td>
<td>(112)</td>
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<td>77</td>
<td>417+434</td>
<td>519.0</td>
<td>538+546+548.5</td>
<td>(hinc)</td>
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### Pyridine Haemochromogen Derivatives

<table>
<thead>
<tr>
<th>Reduced minus oxidized difference spectra</th>
<th>Absolute Spectra (reduced form)</th>
</tr>
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<tbody>
<tr>
<td><strong>Haem b</strong></td>
<td></td>
</tr>
<tr>
<td>295</td>
<td>557</td>
</tr>
<tr>
<td>(20.7)</td>
<td>{112}</td>
</tr>
<tr>
<td>295</td>
<td>556.5</td>
</tr>
<tr>
<td><strong>Haem c</strong></td>
<td>—</td>
</tr>
<tr>
<td>(18.6)</td>
<td>{48,49}</td>
</tr>
<tr>
<td>295</td>
<td>414</td>
</tr>
</tbody>
</table>

**Table III:** Spectral characteristics of standard cytochromes c, bs and their pyridine haemochromogen derivatives.

Absorption maxima and extinction coefficients are provided for the salient features of the dithionite reduced minus peroxide oxidized difference spectra of these cytochromes when observed at ambient (295 K) and liquid nitrogen (77 K) temperatures. Extinction coefficients are given in parentheses below the wavelength to which they apply; units = mM^{-1}; 'hinc' refers to the current study.
Fig. 5: α-absorption bands from ambient and low temperature redox difference spectra of soluble reference cytochromes.

Ambient temperature (295 K) [reduced - oxidized] difference spectra of soluble reference cytochromes in 100 mM potassium phosphate buffer, pH 7.00 and low temperature (77 K) reduced minus oxidized difference spectra of soluble reference cytochromes in 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0. Cytochrome c refers to equine heart cytochrome c, cytochrome b$_5$ refers to the soluble tryptic fragment of bovine hepatic microsomal cytochrome b$_5$.

a. Cytochrome c, $25.0 \mu g \text{ mL}^{-1}$; 295 K; $\Delta A = 0.025$.

b. Cytochrome c, $12.5 \mu g \text{ mL}^{-1}$; 77 K; $\Delta A = 0.050$.

c. Cytochrome b$_5$, $20.0 \mu g \text{ mL}^{-1}$; 295 K; $\Delta A = 0.025$.

d. Cytochrome b$_5$, $10.0 \mu g \text{ mL}^{-1}$; 77 K; $\Delta A = 0.050$.

e. Mixed cytochromes c, $25.0 \mu g \text{ mL}^{-1}$, and b$_5$, $20.0 \mu g \text{ mL}^{-1}$; 295 K; $\Delta A = 0.025$.

f. Mixed cytochromes c, $6.75 \mu g \text{ mL}^{-1}$, and b$_5$, $10.0 \mu g \text{ mL}^{-1}$; 77 K; $\Delta A = 0.050$. 
spectrum at 77 K with contributions from each spectral discontinuity being clearly observable in the mixed solution. These 77 K measurements provided clear evidence of the multiple features of the \( \alpha \)-band spectra in each case.

As described in Appendix A the majority of individual cytochromes possess symmetrical \( \alpha \)-band absorption peaks which undergo a blue shift at the low temperatures associated with high resolution spectrophotometry of these compounds \cite{90, 112, 220}. Thus the chosen cytochrome standards were atypical in that they displayed biphasic absorbance characteristics. Although there have been no type-\( c \) cytochromes definitively associated with the respiratory chains of aerobically-grown \textit{Escherichia coli} the distinct spectral properties of these standard solutions facilitated optimization and calibration of the analytical equipment (Table I) \cite{section II.A.i.b.1, 4, 87}.

An example of a haemoprotein with a haem containing high-spin iron was provided by catalase, illustrated in Figure 6a;1, the \( \alpha \)-band region being expanded in Figure 6b;1. The minimal \( \alpha \)-band absorbance in relation to that of the Soret region is apparent, the spectrum of cytochrome \( c \) being shown for comparison (Fig. 6a;3, 6b;3).

(c) \textbf{Pyridine haemochromogen redox difference spectra}

The pyridine haemochromes are haem complexes formed by coordination with two molecules of a base. They may be prepared by denaturation of haemoproteins and solubilization of the haem in alkaline pyridine, their preparation from \( c \)-type cytochromes requiring cleavage of the covalent bond between haem and peptide (Materials & Methods) \cite{48, 49}. Curves 2 + 4 of Figures 6a and 6b show the reduced \textit{minus} oxidized pyridine haemochrome spectra of samples prepared from catalase and cytochrome \( c \) respectively. It can be seen from Figure 6b that the iron atom in the the type-\( b \) haem of catalase relaxed into a low-spin form resulting in greater \( \alpha \)-band absorbance by the haem than occurred in the original cytochrome with its 'high-spin' iron \cite{49, 158, 216}. Since they result from solubilized free haem the redox spectra of pyridine haemochromogens of all type-\( b \) haems are equivalent. Consequently this technique provided a means of detecting the types of haem present in a complex mixture of cytochromes (Fig. 6b) even when the cytochrome samples were constituents of membrane complexes \cite{48}.
**Fig. 6:** Redox difference spectra of pyridine haemochromogen from types b and c soluble reference cytochromes.

Ambient temperature (295 K) [reduced - oxidized] difference spectra of soluble reference cytochromes in 100 mM potassium phosphate buffer, pH 7.0. Catalase refers to bovine hepatic catalase (Sigma-Aldrich, St. Louis, MO), cytochrome c refers to equine heart cytochrome c.

**a.** Broad visible range spectra:
1. Catalase, 1000 µg mL\(^{-1}\); \(\Delta A = 0.200\).
2. Pyridine haemochromogen derivative of catalase at 1000 µg mL\(^{-1}\); \(\Delta A = 0.200\).
3. Cytochrome c, 10.0 µg mL\(^{-1}\); \(\Delta A = 0.040\).
4. Pyridine haemochromogen derivative of cytochrome c at 10.0 µg mL\(^{-1}\); \(\Delta A = 0.040\).

**b.** Expanded \(\alpha\)- and \(\beta\)-bands of spectra in (a):
1. Expanded \(\alpha\)- and \(\beta\)-band region of (a,1); \(\Delta A = 0.040\).
2. Expanded \(\alpha\)- and \(\beta\)-band region of (a,2); \(\Delta A = 0.016\).
3. Expanded \(\alpha\)- and \(\beta\)-band region of (a,3); \(\Delta A = 0.004\).
4. Expanded \(\alpha\)- and \(\beta\)-band region of (a,4); \(\Delta A = 0.004\).
Fourth order derivatives of redox difference spectra

Fourth order finite difference spectra were calculated as 'fourth order derivatives' from the α-band region of 77 K reduced minus oxidized difference spectra using upgraded firmware routines resident in the Midan II data processor interfaced with the SLM/Aminco DW-2c spectrophotometer. Excessive electronic noise prevented useful fourth order derivative analyses being obtained from the Soret region of the spectra when the instrument was adjusted for maximal resolution and sensitivity in the α-band range, a property of the optical design of this instrument. Figure 7 shows examples of fourth order derivative spectra calculated from redox spectrum α-bands of a combined preparation of mammalian cytochromes c and b5 measured at both 77 K and 295 K. Four major peaks may be observed in either case, these peaks having maximal absorbance at the same wavelengths as the dual peaks observable in individual preparations of these cytochromes at each temperature (Table III). Although the four distinct features of the combined spectrum are clearly apparent in the redox difference spectrum measured at 77 K the utility of determining component features from complex spectra is revealed by the ability of the fourth order derivative to distinguish four constituent peaks from the 295 K redox difference spectrum which displays a fused spectral aggregate in the α-absorption region (Fig. 7a,b). The individual contributing absorption maxima from the 77 K spectra correlated closely with values provided in the literature although such comparative analyses have not been presented for ambient temperature spectra (Table III) (48, 49).

Fourth order derivative studies of the reduced absolute ultraviolet absorption spectrum of equine heart cytochrome c by Dufnach and coworkers have been used to determine the influence of pH upon intramolecular movement of aromatic amino acid residues (46). Thus a comparative calibration could be carried out to ensure the validity of wavelength values determined from derivative analyses in the current investigation (Fig. 8). It should be noted that in the current study the amplitude of peaks in the fourth order derivatizations provide only qualitative data since the height of each derivative peak is dependent upon the curvature of the original spectrum over the wavelength range described by that particular derivative peak. Consequently the amplitude of any peak corresponding to a specific discontinuity in the original spectrum is influenced by the proximity (in terms of wavelength) of neighbouring spectral features (22). Duńach exploited this characteristic of the technique to investigate small spectral shifts due to changes of environment undergone by specific amino acid residues which resulted in the fourth order derivatives of cytochrome c displaying significant alterations in amplitude in addition to small shifts in wavelength of their maxima (46). The interpretation of variable peak height was not feasible for fourth order derivative analysis in the current
Fig. 7: Fourth-order finite difference spectra of $\alpha$-absorption bands from soluble reference cytochromes.

Ambient temperature (295 K) reduced minus oxidized difference spectra of soluble reference cytochromes in 100 mM potassium phosphate buffer at pH 7.0, low temperature (77 K) reduced minus oxidized difference spectra of soluble reference cytochromes in 100 mM potassium phosphate buffer at pH 7.0 containing 1.0 M sucrose, and fourth-order derivatives of these spectra. Cytchrome $c$ refers to equine heart cytochrome $c$, cytochrome $b_5$ refers to the soluble tryptic fragment of bovine hepatic microsomal cytochrome $b_5$.

a. Mixed cytochromes $c$, 25.0 $\mu$g mL$^{-1}$, and $b_5$, 20.0 $\mu$g mL$^{-1}$; 295 K; $\Delta A = 0.025$.

b. Fourth-order finite difference spectrum calculated from $\alpha$-band of curve 'a'.

c. Mixed cytochromes $c$, 6.75 $\mu$g mL$^{-1}$, and $b_5$, 10.0 $\mu$g mL$^{-1}$; 77 K; $\Delta A = 0.050$.

d. Fourth-order finite difference spectrum calculated from $\alpha$-band of curve 'c'.

Fig. 8: Absolute ultra-violet absorption spectrum and fourth-order finite difference spectra of soluble reference ferrocyanochrome c.

Equine heart cytochrome c at 250 μg mL⁻¹ in 100 mM potassium phosphate buffer, pH 7.1, reduced by the procedure of Duñach et al. (46).

a. Absolute absorption spectrum, \( \Delta A = 0.100 \).

b. Fourth-order finite difference spectrum calculated from curve 'a' by four successive first-order derivatizations, \( \Delta A = 0.100 \).

c. Fourth-order finite difference spectrum calculated from curve 'a' by two successive second-order derivatizations, \( \Delta A = 0.020 \).
study because of the unknown number and nature of cytochromes present in the biological materials being investigated.

Nevertheless, determining the wavelengths of maximal intensity of fourth order derivatives from low temperature redox difference spectra greatly facilitated the identification of individual components contributing to partially resolved α-bands in those spectra. Also relevant to these analyses was the requirement of optimizing the signal:noise ratio which degrades with each derivatization. This was achieved by means of moving average procedures accompanied by compensation for associated wavelength shifts. Two firmware procedures were provided for calculating the fourth order derivative spectra, one incorporating more substantial smoothing than the other, as illustrated in Figure 8. Although moving-average smoothing of spectra might be expected to lower the sensitivity of the technique the reproducibility of fine detail in derivative spectra obtained from diverse experimental samples over extended periods of time confirmed the validity of these analyses. Generally the option providing less noise has been illustrated for clarity, exceptions being noted in the figure legends. The possibility that the results might be influenced by instrument-induced spectral abberations was eliminated by the observation of similar spectral features in samples analysed by both types of high-resolution spectrophotometer that were available: DW2c and PE-356 (data not shown).

Cautions relating to limitations of the technique and overinterpretation of fourth order derivative data have been presented by other investigators {90, 22}. The degree of confidence which can be placed in a single fourth order derivative trace is highly dependent upon the amount of noise associated with both the original signal and the final trace. For this reason most of the spectra illustrated in the manuscript are derived from summed and averaged scans and shown in the damped form, in which the salient features are readily apparent. Where less obvious features are to be discussed, they are demonstrated with representative traces plotted at higher sensitivity, the interpretation being based upon analyses of independent spectra collected in multiple experiments. When similar minor features have appeared reproducibly in samples prepared from different sources and treatments they have ultimately been recognised as significant. The repetitive nature of such features was a requirement for them to be distinguished from random noise and to be regarded as significant. As has been noted by others, with some experience fourth order derivative bands can usually be observed as peaks or small shoulders in the absorption spectrum {22}. That discontinuities in the original difference spectra causing minor fourth order derivative features as described above should be distinguishable in results from both types of high resolution, low temperature spectrophotometer supports this contention (data not shown). In sum, with the difficulty of determining from the parent
spectra the precise wavelengths of those components causing such spectral features the advice of Butler & Hopkins is pertinent to spectral analysis by fourth order finite difference spectroscopy \cite{22}:

1. "It is apparent that higher derivatives can extract information from spectral data that is not readily apparent in the original curves."

2. "Whenever possible additional evidence should be sought to confirm the validity of higher derivative bands."

Appropriate optical and mechanical adjustments enabled reproducibility and accuracy of routine DW2c spectral wavelength determinations to be maintained within 0.5 nm and version 2.02 of the Midan II firmware enabled fourth order finite difference analyses to conform to these criteria. It was from the combined results of these spectrophotometric techniques, yielding mutually compatible data and calibrated against values published in the literature for the various standard cytochromes, that confidence could be expressed in the refined procedures that were instrumental in producing the highly detailed spectral data that were obtained from experimental samples. Thus overlapping spectral features could be distinguished and values estimated for their individual absorption maxima.

(ii) Potentiometric Titrations

(a) Standard titrations and electrode calibration

Spectroelectrochemical (potentiometric) titrations of standard solutions of equine heart cytochrome \textit{c} were used to develop modifications and improvements to the procedures of Hackett \cite{66, 69} as described under Materials & Methods and elsewhere \cite{214}. In addition these standard titrations were used to calibrate the combination platinum electrodes incorporated into the apparatus. Data from typical standard titrations are shown in Figures 9 and 10, in each case a theoretical one-component curve being fitted to the data. Figure 9 illustrates the result of the BMD.P3R non-linear regression analysis of the original data and Figure 10 the corresponding Nernst plots with associated linear regression analyses. Figures 9b and 10b show the result of titrating the standard cytochrome \textit{c} in the presence of 1.0 M sucrose in preparation for poised potential low temperature spectrophotometric analysis (described under Materials & Methods) in which no significant deviation from standard behaviour could be detected. Routine standard titrations provided duplicate mid-point potential values for cytochrome \textit{c} that were within a 5 mV range for a particular
Fig. 9: Potentiometric titrations of standard cytochrome c in presence and absence of sucrose: direct plots.

The curves illustrate theoretical values for single electron transfer, one component fits.

a. Equine heart cytochrome c at 30.0 μg mL⁻¹ titrated in 100 mM sodium phosphate buffer, pH 7.0,

b. Equine heart cytochrome c at 30.0 μg mL⁻¹ titrated in 100 mM sodium phosphate buffer, 1.0 M sucrose, pH 7.0.
Results & Discussion

(a) Reduced b cytochrome (% total)

(b) Reduced b cytochrome (% total)

$E_h$ (mV vs NHE)
Fig. 10: Potentiometric titrations of standard cytochrome c in the presence and absence of sucrose: Nernst plots.

Linear regression curves indicate least squares fit:

a. Nernst plot of data in Fig. 9 'a'

\[ E_m = 260.0 \text{ mV vs. NHE}, \text{ slope } = 56.0 \text{ mV}, \text{ n } = 1.05 \],

b. Nernst plot of data in Fig. 9 'b'

\[ E_m = 260.5 \text{ mV vs. NHE}, \text{ slope } = 59.0 \text{ mV}, \text{ n } = 1.00 \].
platinum combination electrode, individual electrodes providing values within 10 mV of each other. All absolute potential values are given relative to the normal hydrogen electrode (mV vs. NHE).

Potentiometric titration and electrochemical characterization of the components of combined solutions of cytochromes was more complex, Figure 11 providing an example in which the standard cytochromes \( c \) and \( b_5 \) are resolved into separately reducible species, described by a theoretical two-component fit of the data in panel 'a' and by Nernst curves in panel 'b'. It can be seen from Figure 11b that interpretation of multicomponent Nernst curves in order to derive mid-point potentials and proportions of total reducible cytochrome becomes progressively more complex with an increasing number of components. Figure 11b also illustrates a theoretical linear regression analysis of the data assuming that the cytochrome \( b_5 \) is fully oxidized and the cytochrome \( c \) fully reduced at the 'plateau' potential of +100 mV. Although in this case the values for mid-point potentials derived from the Nernst analyses agree with published values {112, 176, 193} the technique is impractical for samples with greater numbers of cytochrome components and for those in which the reduction profiles overlap. In these cases, as illustrated in Figure 12a+b, computerized non-linear regression analyses of the original absorbance data was required to provide mid-point potential values for each component and the magnitude of its contribution to the total cytochrome \( \alpha \)-band redox absorption {69, 214}.

(b) Comparison of experimental data with published membrane cytochrome values

Figure 12 presents examples of potentiometric titrations of biological samples. Curve 12a was obtained from the titration of a sample from an homogenized suspension of washed membrane vesicles. A detergent solubilized preparation of nitrate reductase was partially purified from this membrane suspension and titrated to yield curve 12 b. Curve 12a illustrates a five-component best fit of data obtained from titrating cytochromes present in a washed membrane preparation of \( E. coli \) wild-type cells of strain RK4353 grown anaerobically with nitrate as terminal electron acceptor. Mid-point potentials and the proportion of total cytochrome corresponding to each component are provided in the figure legend. These data and the derived values correlate well with those from the literature for similar analyses on such preparations, as indicated in Table IV {66, 67, 69}. Analysis of the data from the solubilized nitrate reductase preparation revealed the presence of three cytochrome components: equal quantities of two major cytochromes (43 % of total \( \Delta A \) each, \( E_m = +34 \text{ mV} \) and \( +118 \text{ mV} \)) and a minor, high potential species (\emph{vide infra}).

Nernst plots derived from the data of Figure 12b are presented in Figure 13. Panel 13a illustrates the treatment of the complete set of redox data with the slope, derived from equation (4), of
Fig. 11: Potentiometric titrations of soluble reference cytochromes.

Cytochrome c refers to equine heart cytochrome c, cytochrome b$_5$ refers to the soluble tryptic fragment of bovine hepatic microsomal cytochrome b$_5$.

a. Cytochrome c at 30.0 µg mL$^{-1}$ and cytochrome b$_5$ at 30.0 µg mL$^{-1}$ titrated in 100 mM sodium phosphate buffer, pH 7.0,

b. Nernst plot of data in ‘a’:

0--0, total cytochrome.

♦-♦, cytochrome c; $E_m$ = +257.0 mV vs. NHE, slope=63.5 mV, n=0.93.

•-•, cytochrome b$_5$; $E_m$ = +3.0 mV vs. NHE, slope=62.0 mV, n=0.95.
Fig. 12: Potentiometric titration of membrane cytochromes and nitrate reductase preparation from *E. coli* grown anaerobically in the presence of nitrate: direct plots.

a. Membranes of *E. coli* strain RK4353 grown anaerobically in the presence of nitrate were resuspended in degassed 100 mM potassium phosphate buffer, pH 7.0 at a protein concentration of 10.0 mg mL$^{-1}$. The theoretical curve for a five component fit is illustrated. The mid-point potentials of these components and their individual contributions to the total type-$b$ cytochrome are indicated:

+249.5 mV vs. NHE : 5.5 % total $\alpha$-band $\lambda_{max}$ absorbance,
+117.0 mV vs. NHE : 24.5 % total $\alpha$-band $\lambda_{max}$ absorbance,
+ 27.0 mV vs. NHE : 23.0 % total $\alpha$-band $\lambda_{max}$ absorbance,
- 41.0 mV vs. NHE : 26.0 % total $\alpha$-band $\lambda_{max}$ absorbance,
-134.0 mV vs. NHE : 20.5 % total $\alpha$-band $\lambda_{max}$ absorbance,

b. Nitrate reductase was solubilized and partially purified from membranes of *E. coli* strain RK4353 grown anaerobically in the presence of nitrate as described in Materials & Methods. The preparation was titrated in a solution of 100 mM potassium phosphate buffer, 0.2 mM diethiothreitol, 0.1% (w/v) Triton X-100 at a protein concentration of 0.88 mg mL$^{-1}$ and at pH 7.0. An arrow indicates the potential below which the cytochrome of highest potential is fully reduced. The theoretical curve for a three component fit is illustrated. The mid-point potentials of these components and their individual contributions to the total type-$b$ cytochrome are indicated:

+231.5 mV vs. NHE : 14.0 % total $\alpha$-band $\lambda_{max}$ absorbance,
+118.0 mV vs. NHE : 43.0 % total $\alpha$-band $\lambda_{max}$ absorbance,
+ 34.0 mV vs. NHE : 43.0 % total $\alpha$-band $\lambda_{max}$ absorbance.
[2.303 RT/nF = 120 mV] yielding the improbable value of 0.5 for ‘n’, the number of electrons involved in the transfer (section I.ii.c). Since the redox mediators effect electrochemical equilibration within the titration system this result indicates the presence of two contiguously titrating single electron components, and on close inspection a discontinuity at near 100 mV divides the data into two linear sets of points. Redox absorption spectrophotometry detected a sole, symmetrical cytochrome α-band in the sample, this being of type-β with an absorption maximum of 556.0 nm at 77 K (data not shown) [67]. As described above, non-linear regression analysis of the titration data, utilizing an algorithm assuming single electron transfer, had provided theoretical values for two cytochromes of equal intensity with mid-point potentials approximately 90 mV apart: a separation that would provide a virtually contiguous titration curve. By choosing the half-reduction point of this curve, and treating each half independently the Nernst plots charted in Figure 13b were obtained. The value of ‘n’ approximates to unity for each of these curves confirming that results from these two methods of analysis were equivalent and suggesting that the solubilized nitrate reductase preparation contained two major cytochrome components of equal magnitude. This concept was originally proposed by Hackett & Bragg [67] as an interpretation of potentiometric titration data, for no spectroscopic, chromatographic or genetic evidence for more than a single form of cytochrome $b^{NR}$ had been found [14, 67, 69]. Chaudhry & MacGregor erroneously challenged this view on the basis of spectrophotometry alone, actually confirming the proposition in the earlier publication [28]. The current study supports all the evidence put forward to date, suggesting that the nitrate reductase cytochrome $b^{NR}$ appears as a single type-β cytochrome with a single absorption maximum of 556 nm in reduced minus oxidized spectrophotometry at 77 K but that it may be resolved into two distinct cytochrome species by potentiometric analyses.

More recently cytochromes $c_4$ from several sources have been studied by spectroelectrochemical redox titration and demonstrated a range of spectral and potentiometric responses with biphasic titration curves and either split or single α-bands (measured at ambient temperature) [111]. The dihaem cytochromes $c_4$ are found in *Pseudomonads* and other procaryotes where they appear to have a respiratory rôle in conjunction with cytochrome $o$. The authors suggest models in which the two haems may either function with individually defined mid-point potentials or in which they are equivalent in the fully oxidized state, the addition of a first electron occurring at either haem and causing a conformational modification such that the addition of the second electron is a less favourable process and takes place at a lower redox potential [111]. The spectral similarity of the two components has been attributed in each case to the dihaem cytochrome $c_4$ having undergone gene duplication, as supported by amino acid analyses, each of the two resulting domains having similar
{A} Cytochrome b5: (tryptic fragment of bovine hepatic cytochrome b5):

<table>
<thead>
<tr>
<th></th>
<th>Current Study</th>
<th>Reference (112)</th>
<th>Reference (176)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E_h ) (mV vs. NHE)</td>
<td>+3.0</td>
<td>±0, +20</td>
<td>+6</td>
</tr>
</tbody>
</table>

(Under equivalent conditions)

{B} Washed Membranes: nitrate-grown cells of strain RK4353:

<table>
<thead>
<tr>
<th></th>
<th>Current Study</th>
<th>Reference (204)</th>
</tr>
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<tbody>
<tr>
<td>( E_h ) (mV vs. NHE)</td>
<td>cytochrome b</td>
<td>cytochrome b</td>
</tr>
<tr>
<td>(mV vs. NHE)</td>
<td>(mV vs. NHE)</td>
<td>(mV vs. NHE)</td>
</tr>
<tr>
<td>cytochrome b (% total)</td>
<td>cytochrome b (% total)</td>
<td>cytochrome b (% total)</td>
</tr>
<tr>
<td>+249.5</td>
<td>5.5 %</td>
<td>+149.5</td>
</tr>
<tr>
<td>+117.0</td>
<td>24.5 %</td>
<td>+ 59.0</td>
</tr>
<tr>
<td>+ 27.0</td>
<td>23.0 %</td>
<td>+ 26.0</td>
</tr>
<tr>
<td>- 41.0</td>
<td>26.0 %</td>
<td>-109.0</td>
</tr>
<tr>
<td>-134.0</td>
<td>20.5 %</td>
<td></td>
</tr>
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{C} Solubilized, partially purified nitrate reductase preparation:

<table>
<thead>
<tr>
<th></th>
<th>Current Study</th>
<th>Reference (69)</th>
</tr>
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<tbody>
<tr>
<td>( E_h ) (mV vs. NHE)</td>
<td>cytochrome b</td>
<td>cytochrome b</td>
</tr>
<tr>
<td>(mV vs. NHE)</td>
<td>(mV vs. NHE)</td>
<td>(mV vs. NHE)</td>
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<tr>
<td>cytochrome b (% total)</td>
<td>cytochrome b (% total)</td>
<td>cytochrome b (% total)</td>
</tr>
<tr>
<td>+231.5</td>
<td>14.0 %</td>
<td>+122.0</td>
</tr>
<tr>
<td>+118.0</td>
<td>43.0 %</td>
<td>+ 17.0</td>
</tr>
<tr>
<td>+ 34.0</td>
<td>43.0 %</td>
<td></td>
</tr>
</tbody>
</table>

Table IV: Comparison of potentiometric analyses of biological samples with previously published values.

Electrodes were calibrated by titration of standard solutions of equine heart cytochrome c, as described under Materials & Methods. Cell type, growth conditions and the number of components resolved will affect determinations of membrane cytochrome complement [B] although these factors are minimized in analyses of solubilized cytochrome preparations subjected to purification techniques [C].
Fig. 13: Potentiometric titration of partially purified *E. coli* nitrate reductase: Nernst plots.

(a) Nernst plot of data from Fig. 13 'b'. An arrow indicates the potential below which the cytochrome of highest potential is fully reduced.

O—O; total cytochrome: apparent $E_m = +95 \text{ mV vs. NHE}$; slope = 120 mV; $n = 0.5$.

(b) Nernst plots of data at potentials below discontinuity indicated by the arrow in Fig. 13 'b':

•—•; $E_m = +120.0 \text{ mV vs. NHE}$; slope = 52.5 mV; $n = 1.12$,

•—•; $E_m = +28.0 \text{ mV vs. NHE}$; slope = 48.5 mV; $n = 1.22$. 
Further validation and calibration of the improved titration and curve fitting techniques was obtained by comparing mid-point potentials of cytochromes in membrane preparations from several bacterial strains with published values, including those for the temperature sensitive polar chlC mutant TS9A at permissive and at non-permissive temperatures, governing formation of the chlI gene product: a type-\textit{b} cytochrome implicated in nitrate reduction \cite{39, 66, 67, 204}. Nevertheless, an empirically determined resolution limit of 50 mV between mid-point potentials was observed when using the BMD.P3R non-linear regression analyses: although this limited the number of possible components that could be fitted to an experimental curve, a practical threshold of 10\% of total sample cytochrome was used as a minimum significant quantity.

(c) Electrochemically poised high resolution spectrophotometric analysis

The coupling of analytical techniques promised to provide a correlation between the limited information available when each procedure was used in isolation. Such an approach has been attempted for some years in this laboratory, coupling high-resolution redox spectrophotometry with dual wavelength kinetic studies or with potentiometric poising and the latter combination has also been used in similar investigations by R. B. Gennis \cite{69, 117, 214}. Withdrawal of aliquots from a sample undergoing potentiometric titration and the subsequent manipulation and freezing of these aliquots under strictly anaerobic conditions in order that their electrochemical potential remained constant presented significant mechanical and electrochemical problems. It has already been shown that there was no effect upon the potentiometric titration itself when the 1.0 M sucrose necessary for optimizing the 77 K redox spectra \cite{188, 206} was included in the titration buffer (Fig. 9+10). Figure 14 demonstrates that the refined procedure was capable of producing high resolution low temperature redox difference spectra that reflected the progression of the potentiometric titration from which the samples were taken. Alteration of the slope of the baseline as the potential of the sample was modified was due to differential redox absorbance by the electrochemical mediators in the titration buffer.

Comparison of the proportion of cytochrome reduced as indicated by the poised potential low temperature redox difference spectra and those redox difference spectra collected at ambient temperature in the process of performing the titration shows clearly that the redox behaviour of the samples providing these spectra at the two temperatures was different (Figure 15). Curve 15a is the theoretical curve for a single component, single electron transfer reaction at the temperature of the
Fig. 14: Potentiometric titrations of standard cytochrome c: low temperature poising and spectrophotometry at 77 K.

The technique was performed on a sample of equine heart cytochrome c at 30.0 µg mL\(^{-1}\) titrated in 100 mM sodium phosphate buffer, 1.0 M sucrose, pH 7.0 as described in Materials & Methods. The α-bands of the cytochrome c reduced minus oxidized difference spectra measured with the PE-356 at 77 K are shown after sampling and poising at the following potentials:

\begin{align*}
\text{a. } & +254.0 \text{ mV vs. NHE,} \\
\text{b. } & +237.0 \text{ mV vs. NHE,} \\
\text{c. } & +233.0 \text{ mV vs. NHE,} \\
\text{d. } & +225.5 \text{ mV vs. NHE,} \\
\text{e. } & +212.5 \text{ mV vs. NHE,} \\
\text{f. } & +207.5 \text{ mV vs. NHE,} \\
\text{g. } & +198.0 \text{ mV vs. NHE.}
\end{align*}

Equivalent reduction estimates were obtained from absorbance changes measured at 550.5 nm (\(\lambda_{\text{max}}\)) or at 548.0 nm, the wavelength of the α-band 'shoulder'.
$\Delta A = 0.10$

\begin{align*}
&\lambda (\text{nm}) \\
&530 \quad 550 \quad 570
\end{align*}
Fig. 15: Potentiometric titrations of standard cytochrome c: effects of poising at low temperature.

Nernst plots of data collected at 296 K and 77 K; each data point is the average of two or more measurements.

a. O--O ; Equine heart cytochrome c at 30.0 μg mL⁻¹ titrated and analysed in 100 mM sodium phosphate buffer, 1.0 M sucrose, pH 7.0 at 296 K. The curve illustrates theoretical values for single electron transfer, one component fit. ($E_m = +260.0 \text{ mV vs. NHE}; \text{ slope } = 59.0 \text{ mV}; n=1.00$).

b. Recalculation of theoretical curve ‘a’ using a temperature coefficient of $T = 270$ K (freezing point of sample) and arbitrarily assigned $E_m = +260.0 \text{ mV vs. NHE}$. (Slope = 15.3 mV; n=1.00).

c. •—• ; Equine heart cytochrome c at 30.0 μg mL⁻¹ titrated in 100 mM sodium phosphate buffer, 1.0 M sucrose, pH 7.0 at 296 K and poised samples analysed spectrophotometrically at 77 K. The linear regression curve is illustrated and indicates theoretical values of $E_m = +213.0 \text{ mV vs. NHE}; \text{ slope } = 42.6 \text{ mV}$. The value of ‘n’ corresponds to unity at a temperature of 215.5 K (−57.5°C).

d. Recalculation of theoretical curve ‘c’ using a temperature coefficient of $T = 77$ K and arbitrarily assigned $E_m = +213.0 \text{ mV vs. NHE}$. (Slope = 15.3 mV; n=1.00).
titration and it correlates with reduction values obtained from the ambient temperature reduced minus oxidized difference spectra collected during titration. The intercept has been positioned at 260 mV, the value obtained by linear regression analysis of these data. Curve 15c demonstrates similar data obtained from high resolution redox difference spectrophotometry at 77 K of samples poised at specific potentials during the titration. Theoretical curves corresponding to single component, single electron transfer at 270 K (the freezing point of the buffered sucrose solution) and at 77 K (the temperature of spectral measurement) are illustrated as curves 15b and 15d respectively. The intercepts for curves 15b and 15d have been chosen arbitrarily since insufficient data is available to describe the behaviour of cytochrome mid-point potentials at freezing temperatures.

Electron equilibration between half cells such as a redox couple and the normal hydrogen electrode may be described by equation (3), first published by Peters and generally referred to as the Nernst Equation \{43, 213, 217\}.

\[
E_h = E_o + \frac{RT}{nF} \ln \frac{[A_{ox}]}{[A_{red}]} \tag{3}
\]

where \(E_h\) is the redox potential difference between the sample and reference half cells at the ambient temperature with the subscript ‘\(h\)’ denoting the reference half cell to be the standard hydrogen half cell; \(E_o\) is the standard redox potential of redox couple A (the \(E_h\) at which \([A_{ox}] = [A_{red}]\) with both factors maintained at unit activities at pH = 0); \(R\) is the gas constant (8.31 J K\(^{-1}\) mol\(^{-1}\)); \(T\) is the ambient temperature in Kelvin; \(n\) is the number of electrons transferred in the reaction and \(F\) the Faraday constant (96 493 J V\(^{-1}\)). Modifications of these standard conditions to those appropriate for biological samples, where activities and often concentrations are unknown and pH values may have to be restricted to physiological ranges, are represented by a similar equation, (4), in which \(E_o\) is replaced by the mid-point potential at the ambient pH of the determination, \(E_m\) \{43, 45, 213\}.

\[
E_h = E_m + 2.303 \frac{RT}{nF} \log_{10} \frac{[A_{ox}]}{[A_{red}]} \tag{4}
\]

In the current study all potentiometric titrations were buffered with 100 mM potassium phosphate buffer at pH 7.0 and \(E_h\) was derived from the potential measured at the calibrated
platinum combination electrode as each spectrum was recorded during the course of the titration. The values of $R$ and $F$ are constant and that of $T$ is governed by the conditions of the titration and poising procedures. It is assumed that for type-$b$ cytochromes $n = 1$ since the haem iron alternates between Fe$^{II}$ and Fe$^{III}$ redox states (43, 45).

From the equations it is apparent that the the $E_m$ value of the molecule under study will dictate the ordinate intercepts of the Nernst curves in Figure 15 — the mid-point potential being a characteristic molecular property under a defined set of applied conditions. The apparent mid-point potential exhibited by those samples which were rapidly cooled and then analysed at liquid nitrogen temperatures was 47 mV lower than that derived from those samples measured by the standard procedure at ambient temperature (curves 15 a+c). Consequently the redox phenomena being observed must have been more complex than simple poising of the low temperature samples at the potential of the system at which they were withdrawn. Although this phenomenon might have been caused by a small degree of oxidation during the transfer and freezing steps, and this might also explain the change in slope of the Nernst plot, the regularity of the data argues against such an arbitrary cause for either of these effects. Mid-point potential values for type-$b$ cytochromes are known to be particularly susceptible to environmental changes in terms of proton activity (response to pH), specific interactions (especially preferential ligand binding to one redox form), ionic strength, temperature and intramolecular modification (45, 156, 176). In addition to the effects of the temperature drop the consequences of freezing upon the $E_m$ are unknown, although it has been reported that freezing phosphate buffered solutions may cause pH shifts as large as -3 pH units in sodium phosphate buffers and -1 pH unit in potassium phosphate buffers (149). Acidification of metahaemoglobin has been observed upon cooling to cryogenic temperatures, although in the current investigation the presence of 1.0 M sucrose in the titration buffer — added in order to optimise the signal:noise ratio and reproducibility of the high resolution spectra — would be expected to decrease pH changes and aberrations due to the formation of large ice crystals (90, 94, 149). (It has been shown earlier, section I.ii.a, that the sucrose had no effect upon the results of standard titrations.) Factors relevant to the alteration of redox characteristics in the frozen samples may include localised concentration effects brought about by the physical phase change (this may cause preferential precipitation of one of the buffer salts) or disruption of the equilibrium with the redox mediators in the sample.

The change in redox state of the cytochrome as the applied potential was modified was also different between the two sets of data, and is reflected in the difference of the slopes of Figure 15, curves a+c. As defined in equation (1) (see Introduction), if $R$, $F$ and $n$ are assumed to be
constants and the spectrophotometric measurements are indicative of the proportion of sample reduced (as is supported by the linearity of the data) then a sample that was not truly 'poised' at the potential of the system during withdrawal and rapid freezing would be expected to reflect a dependence upon the temperature, $T$. Yet the low temperature samples did not behave in the manner expected of samples 'poised' at the temperature of the titration itself, at the temperature of the physical phase change of the sample (measured independently at $-3^\circ\text{C}$), or at their temperature during analysis ($77\ \text{K}$), but as if they were responding to a specific applied temperature of $215.5\ \text{K}$ ($-57.5^\circ\text{C}$). The consistency of the data with respect to this temperature suggests that while the factors described above may be responsible, these observations may also correspond to the energetic trapping of a mechanistic intermediate in the transfer of electrons between sample and mediators, the original equilibrium being modified upon cooling. In practical terms these results imply that one should not assume that low temperature spectra recorded from 'potentiometrically poised' samples are representative of their precise sampling potentials, as has previously been the case [69, 117] but that if a series of such poised potential, low temperature spectra is analysed it will provide a representative, high-resolution display of spectral phenomena occurring in sequence over a broad, albeit currently ill-defined range of electrochemical potentials. Thus the technique, performed with all the appropriate precautions, may be deemed as qualitatively valid, accompanied by the reservations stated above. These observations merit further investigation.

Steady state poising has been employed in dual wavelength investigations of cytochrome kinetics in studies associated with the current and other investigations [42, 69, 95, 214]. Not only may these reactions be carried out within the cryogenic sample holder with a minimum of manipulation and very rapidly frozen at the appropriate time but the stability of the steady states due to the presence of relatively large pools of oxidant or reductant create a system in which the gross electrochemical potential is electrochemically buffered, although trapping of particular intermediates reflecting specific intramolecular modifications may still be important phenomena at these low temperatures [25]. A similar situation is described by Chance's investigations of mammalian cytochrome oxidase in which low temperatures were used to trap intermediates in the binding and release of oxygen and carbon monoxide within the 'oxygen pocket' of the oxidase [38].
II: MEMBRANE STUDIES

{A} Aerobic Respiratory Type-b Cytochromes

(i) Redox Difference Spectroscopy

(a) Visible range spectrophotometry of E. coli membrane cytochromes

Absolute reduced and absolute oxidized spectra of washed membranes from E. coli wild-type strain GR17N, grown aerobically on a defined medium to stationary phase, are shown in Figure 16; a+b. One function of recording redox difference spectra from biological samples was to eliminate background absorption from all material failing to undergo spectral changes with an alteration of oxidation state: the reduced minus oxidized difference spectrum from curves 16(a-b) is shown in curve 16c, all three spectra having been collected at 295 K (ambient temperature). As described in the Introduction stationary phase cells contain the components of both aerobic respiratory chains and Figure 16c is marked with the approximate locations of the standard absorption patterns caused by α-, β- and γ-bands of the b- and d-type cytochromes. The absorption bands of cytochrome b₅₉₅, which contains a high-spin type-b haem (118) are not marked and will be discussed in detail below; nevertheless a minor absorption band is visible at 595 nm in Figure 16c as is the Soret band shoulder at 440 nm which is associated with the cytochrome d complex containing cytochrome b₅₉₅. Mutants expressing a cytochrome d complex deficient in cytochrome b₅₉₅ have only recently been isolated (communication from R.B. Gennis) and at present it is still uncertain whether the Soret shoulder is caused by the cytochrome b₅₉₅ or by cytochrome d itself.

This question is of importance in determining the identity of the terminal oxidase of the cytochrome d respiratory chain, since carbon monoxide binding to the terminal oxidases causes perturbations of the reduced spectrum as shown in Figure 17. Recent publications have affirmed that cytochrome d is the terminal oxidase and that it is responsible for the Soret shoulder and for spectral shifts upon CO binding (87, 105, 135). Absolute reduced spectra, measured at ambient temperature,
Fig. 16: *Escherichia coli* respiratory cytochromes: visible range spectra of cell membrane suspensions at ambient temperature.

Wild-type (*w*+) strain GR17N grown on glucose to stationary phase: cells were grown to stationary phase on CYD minimal medium containing glucose; crude membranes were prepared from them and washed as described in Materials & Methods. These membranes were resuspended in 100 mM potassium phosphate buffer, pH 7.0 to a protein concentration of 5.0 mg mL\(^{-1}\).

a. Dithionite reduced absolute spectrum obtained at ambient temperature, \(\Delta A = 0.40\),

b. Hydrogen peroxide oxidized absolute spectrum at ambient temperature, \(\Delta A = 0.40\),

c. Dithionite reduced minus peroxide oxidized difference spectrum obtained at 295 K.
**Fig. 17:** Spectral identification of *E. coli* terminal oxidases following exposure of membranes to carbon monoxide.

Cells were grown on CYD minimal medium containing glucose; crude membranes were prepared from them and washed as described in *Materials & Methods*. These membranes were resuspended in 100 mM potassium phosphate buffer, pH 7.0 to a protein concentration of 5.0 mg mL⁻¹.

a. Dithionite reduced absolute spectrum obtained at ambient temperature, $\Delta A = 0.40$.
   Strain GR17N $(w^+)$ grown on glucose to stationary phase.

b. Dithionite reduced absolute spectrum obtained at ambient temperature, $\Delta A = 0.40$.
   Strain GR17N $(w^+)$ grown on glucose to early exponential phase.

c. Dithionite reduced plus carbon monoxide *minus* dithionite reduced difference spectrum obtained at 295 K. Strain GR17N $(w^+)$ grown on glucose to stationary phase.

d. Dithionite reduced plus carbon monoxide *minus* dithionite reduced difference spectrum obtained at 295 K. Strain GR17N $(w^+)$ grown on glucose to early exponential phase.
were obtained from washed membranes of cells grown to stationary and early exponential phases (Fig. 17 a+b). Ambient temperature 'reduced plus carbon monoxide minus reduced difference spectra' ('carbon monoxide spectra') for each of these samples are illustrated in curves 17c and 17d respectively, demonstrating the alterations in the spectral features of these reduced samples. Carbon monoxide has been shown to bind to the free coordination site of several terminal oxidases, including the mitochondrial terminal oxidase cytochrome $a_a^3$ [38, 112] and it acts similarly in those procaryotic terminal oxidases that react with oxygen [87, 112, 216]. Thus the major absorbance troughs in the Soret region of Figure 17c+d indicate that in stationary phase cells carbon monoxide binds to terminal oxidases with Soret absorbances at 430 nm and 440 nm whereas in early exponential phase only the 430 nm oxidase is present. In addition curve 17c demonstrates the major effect of carbon monoxide upon the isolated $\alpha$-band of cytochrome $d$ with a shift from 623 nm to 645 nm. No spectral features of the cytochrome $d$ complex are visible in absorption spectra of membranes from early exponential phase cells, curve 17d. Table V provides precise data for these various spectral parameters.

High resolution, low temperature redox difference spectra of membranes from stationary and early exponential phase cells are shown in Figure 18, curves a and b respectively. These spectra provide the extra detail and sharpness of absorption bands noted earlier in those from the standard soluble cytochromes (Fig. 5, 7). Inclusion of 1.0 M sucrose in the low temperature sample buffer was found to increase the extinction coefficient of the total membrane cytochrome preparations by a factor of five (data not shown) as predicted and discussed by Jones & Poole [90]. As the cells progressed from exponential to stationary phase the Soret absorbance indicated that the total cytochrome complement per unit of membrane protein doubled. There was also an increase from 4.0 to 6.4 in relative maximal absorbance between the type-6 Soret and $\alpha$-bands suggesting that cytochromes with distinct Soret bands and superimposed $\alpha$-bands had been replaced with those exhibiting the reverse characteristics.

Examination of the $\alpha$-band regions of curves 18a and 18b reveals that there was a complete absence of cytochrome $d$ in the early exponential phase cell membranes (and, by inference, the other components of the cytochrome $d$ complex with less clearly defined spectra were also absent: cytochromes $b_558$ and $b_595$) whereas a pronounced type-$b$ $\alpha$-band shoulder is visible at (560-565)nm, indicative of the cytochrome $o$ respiratory chain. The characteristics of the cytochrome $d$ complex described above were more distinct at 77 K than at ambient temperature (Fig.18a and Fig.16c). Moreover the stationary phase membranes displayed greater absorbance due to iron-sulphur protein and flavoprotein, as may be seen from the absorbance trough between 400 nm
### (A) Reduced minus oxidized difference spectra:

<table>
<thead>
<tr>
<th>Growth Phase</th>
<th>Temperature (K)</th>
<th>cyt. b $\lambda_{\text{max}}^\gamma$ (nm)</th>
<th>cyt. b $\lambda_{\text{max}}^\beta$ (nm)</th>
<th>cyt. b $\lambda_{\text{max}}^\alpha$ (nm)</th>
<th>cytochrome $d$ $\lambda_{\text{max}}^\alpha - \lambda_{\text{min}}^\alpha$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exponential</td>
<td>295</td>
<td>430</td>
<td>529</td>
<td>562</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>428</td>
<td>526+532</td>
<td>556, (563)</td>
<td>—</td>
</tr>
<tr>
<td>Stationary</td>
<td>295</td>
<td>430, (440)</td>
<td>528</td>
<td>560</td>
<td>626</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>428, (438)</td>
<td>526</td>
<td>558</td>
<td>624-626</td>
</tr>
</tbody>
</table>

### (B) Reduced plus carbon monoxide minus reduced difference spectra:

<table>
<thead>
<tr>
<th>Growth Phase</th>
<th>Temperature (K)</th>
<th>oxidase $\lambda_{\text{max}}^\gamma$ (nm)</th>
<th>cytochrome $o$ $\lambda_{\text{min}}^\gamma$ (nm)</th>
<th>cytochrome $d$ $\lambda_{\text{min}}^\gamma$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exponential</td>
<td>295</td>
<td>416</td>
<td>430</td>
<td>—</td>
</tr>
<tr>
<td>Stationary</td>
<td>295</td>
<td>416</td>
<td>428-432</td>
<td>440</td>
</tr>
</tbody>
</table>

### (C) Extinction coefficients at 295 K:

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Spectrum</th>
<th>Wavelength Pair</th>
<th>$\epsilon$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total membrane cyt. $b$</td>
<td>Reduced minus oxidized</td>
<td>560/580</td>
<td>17.5</td>
<td>[104]</td>
</tr>
<tr>
<td>Purified cyt. $d$ complex (using cyt. $b_{558}$)</td>
<td>Reduced minus oxidized</td>
<td>562/580</td>
<td>10.5</td>
<td>[104,135]</td>
</tr>
<tr>
<td>Purified cyt. $o$ complex</td>
<td>Reduced minus oxidized</td>
<td>560/580</td>
<td>18.7</td>
<td>[97]</td>
</tr>
<tr>
<td>Purified cyt. $o$ complex</td>
<td>Reduced+CO minus reduced</td>
<td>416/430</td>
<td>145.0</td>
<td>[97]</td>
</tr>
<tr>
<td>Purified cyt. $o$ complex</td>
<td>Reduced+CO minus reduced</td>
<td>416/430</td>
<td>80.0</td>
<td>[129]</td>
</tr>
</tbody>
</table>

### Table V:

Absorption maxima and extinction coefficients for respiratory cytochromes of cells grown to early exponential and stationary phases.

Values in parentheses indicate incompletely resolved absorption peak 'shoulders'.
**Fig. 18:** *Escherichia coli* respiratory cytochromes: high resolution visible range redox difference spectra of cell membrane suspensions.

Cells were grown on CYD minimal medium containing glucose; crude membranes were prepared from them and washed as described in Materials & Methods. Membrane samples were resuspended in 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0 to a protein concentration of 5.0 mg mL\(^{-1}\).

a. **77K[R-O]DS**: Dithionite reduced minus peroxide oxidized difference spectrum obtained at 77 K. Strain GR17N (w\(^+\)) grown on glucose to stationary phase, \(\Delta A = 0.40\).

b. **77K[R-O]DS**: Dithionite reduced minus peroxide oxidized difference spectrum obtained at 77 K. Strain GR17N (w\(^+\)) grown on glucose to early exponential phase, \(\Delta A = 0.20\).

c. Fourth-order finite difference spectrum calculated from curve ‘a’ by four successive first-order derivatizations, \(\Delta A = 0.08\).

d. Fourth-order finite difference spectrum calculated from curve ‘b’ by four successive first-order derivatizations, \(\Delta A = 0.08\).
and 500 nm.

In both exponential and stationary phase membrane preparations the \( b \)-type cytochrome \( \alpha \)-bands were asymmetrical and shown to contain at least five distinguishable features by their fourth order derivatives, curves 18c+d. Distinct features of the cytochrome \( \beta \)-bands are also visible in both redox spectra and their derivatives and at least nine fourth order derivative bands are observed in the Soret region. Because of the complexity and incomplete resolution of these \( \beta \)- and \( \gamma \)-band derivatives they will not be considered further. Of particular interest was the lack of response of the fourth order derivative analysis to the complex \( \alpha \)-bands of cytochrome \( d \), suggesting that absorbance features of both the oxidized and the reduced forms (which overlap to some extent at approximately 630 nm) are neither Gaussian nor Laurentzian in nature but comprise absorption bands displaying a constant fourth order rate of change of absorbance with unit change in wavelength.

Unless stated otherwise, further discussion of reduced minus oxidized difference spectra of cytochromes from membranes or solubilized preparations will be restricted to those collected at 77 K.

(b) High resolution \( \alpha \)-band cytochrome studies of \textit{E. coli} membrane cytochromes

1. \( \alpha \)-bands of cell membrane type-\( b \) cytochromes

Four type-\( b \) \( \alpha \)-band profiles from redox difference spectra of membranes prepared from wild-type cells grown to stationary, early exponential or late exponential phase are illustrated in Figure 19a, curves 1, 2 and 3, respectively. A progressive change of form of the \( \alpha \)-band profile occurred as the cell population matured and their respiratory cytochrome complement altered. The stationary phase cell membranes contained two predominant type-\( b \) cytochromes with absorption maxima at 556 nm and 559 nm, corresponding to a cytochrome \( b_{556} \) and to the cytochrome \( b_{558} \) of the cytochrome \( d \) complex respectively. Minor shoulders at 548 nm and 565 nm may also be observed in these spectra. The cause of the former has been suggested to be the broad \( \beta \)-band absorption of cytochrome \( b_{595} \) while the latter is probably associated with the cytochrome \( o \) produced during exponential phase \cite{97, 118, 129}. Late exponential phase cell membranes clearly contained a smaller proportion of cytochrome \( b_{558} \) and the spectral shoulder at 565 nm is slightly more pronounced than in spectra from stationary phase membranes, suggesting that significantly less cytochrome \( d \) complex was present and somewhat more of the cytochrome \( o \) complex. This situation was more extreme in the early exponential phase membranes where a broad cytochrome \( b_{556} \) band was a major spectral component and the 'red' shoulder associated with cytochrome \( o \) was large
Fig. 19: Absorbance shifts and improved spectral resolution of type-b membrane cytochrome α-bands at low temperatures.

Cells were grown on CYD minimal medium containing glucose; crude membranes were prepared from them and washed as described in Materials & Methods. Membrane samples were resuspended in 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0 to a protein concentration of 5.0 mg mL\(^{-1}\).

a. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.
   1. Strain GR17N \((w^+\)) grown to stationary phase, \(\Delta A = 0.20\),
   2. Strain GR17N \((w^+\)) grown to late exponential phase, \(\Delta A = 0.20\),
   3. Strain GR17N \((w^+\)) grown to mid exponential phase, \(\Delta A = 0.16\),
   4. Strain PLJ01 \((cyd^+\)) grown to stationary phase, \(\Delta A = 0.16\).

b. Fourth-order finite difference spectra calculated from corresponding curves ‘a’ by four successive first-order derivatizations.
   1. Strain GR17N \((w^+\)) grown to stationary phase,
   2. Strain GR17N \((w^+\)) grown to late exponential phase,
   3. Strain GR17N \((w^+\)) grown to mid exponential phase,
   4. Strain PLJ01 \((cyd^+\)) grown to stationary phase.
enough to indicate the presence of three possible features and to obscure the contribution from any cytochrome $b_{558}$ that may have been present. These features at the various growth stages correspond well to the postulated alterations in cytochrome complement of aerobically grown *Escherichia coli* cells during the maturation of the culture, as described in the Introduction. In Figure 19a curve 4 shows the $\alpha$-band profile of membranes from the cyd$^{-}$ strain PLJ01 grown to stationary phase on the same defined medium as the other examples in Figure 19. This sample displayed $\alpha$-band characteristics which closely resemble those of the exponential phase wild-type cell membranes.

Thus there was partial resolution of type-$b$ membrane cytochrome $\alpha$-bands at 77 K and this was capable of indicating a variation of $b$-cytochromes with the change of respiratory chain constituents expressed at different phases of aerobic growth. Further analyses of these spectra will be discussed after illustrating the types of phenomena that affected the cytochrome $b$ $\alpha$-band profiles and, by implication, the cytochrome $b$ complement of the cell membranes.

Figures 20 and 21 illustrate variations in respiratory cytochrome $b$ complement under different growth conditions and between various strains of cells each grown under identical conditions. Panel 18a, curves 3 and 4 show that wild type strain MR43L grown to stationary phase on defined media with succinate as sole carbon/energy source generated $b$ cytochrome $\alpha$-bands indicating a greater proportion of cytochrome $b_{556}$ to $b_{558}$ under conditions of high aeration than when grown in poorly aerated medium. The proportion of cytochrome $d$ $\alpha$-band absorbance to maximal cytochrome $b$ $\alpha$-band absorbance reflected the increased proportion of cytochrome $d$ complex to total cytochrome $b$ content indicated by the partial resolution of the cytochrome $b_{558}$ $\alpha$-band (data not shown). Earlier studies have presented evidence to suggest that the cause of the cytochrome variations with growth phase (Fig. 19) is the reduction in the dissolved oxygen concentration of the liquid medium during the later phases of growth [87, 172, 204]. This would provide an explanation for the similarity of cytochrome $b$ $\alpha$-bands when cells were grown upon a defined medium with glucose as sole carbon/energy source under either high or low aeration conditions (Fig.20a;1+2), since the greater rapidity of growth on CYD medium supplemented with glucose rather than succinate would enable cultures of moderate to high population density to deplete the oxygen supplied by either aeration rate ($\mu_{g}{}^{}_{lc}{}^{}^{1.5}; \mu_{suc}{}^{}^{0.5}$) [172]. It is noteworthy that the total yield of type-$b$ cytochrome per mg of membrane protein was approximately equal under the two sets of aerated conditions although when grown to stationary phase the succinate-nourished cells produced 2.4 times the total quantity of type-$b$ cytochrome (measured at 556 nm) compared to those
**Fig. 20:** High resolution α-band spectra: variation of wild-type cells’ type-β membrane cytochromes with aeration and carbon source.

Cells of $w^+$ strain GR17N were grown on CYD minimal medium supplemented with the carbon-energy sources stipulated below; crude membranes were prepared from each culture and washed as described in Materials & Methods. Membrane samples were resuspended in 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0 to a protein concentration of 5.0 mg mL$^{-1}$.

**a.** Aeration effects indicated by dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.

1. Strain MR43L ($w^+$) grown to stationary phase on glucose under low aeration, $\Delta A = 0.10$,
2. Strain MR43L ($w^+$) grown to stationary phase on glucose under high aeration, $\Delta A = 0.10$,
3. Strain MR43L ($w^+$) grown to stationary phase on succinate under low aeration, $\Delta A = 0.24$,
4. Strain MR43L ($w^+$) grown to stationary phase on succinate under high aeration, $\Delta A = 0.24$.

**b.** Substrate effects indicated by dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.

5. Strain GR17N ($w^+$) grown to mid-exponential phase on glucose, $\Delta A = 0.10$,
6. Strain GR17N ($w^+$) grown to mid-exponential phase on succinate, $\Delta A = 0.12$,
7. Strain GR17N ($w^+$) grown to mid-exponential phase on lactate, $\Delta A = 0.40$. 
grown on glucose.

Figure 20b displays membrane cytochrome type-b α-bands from cells of wild-type strain GR17N grown to mid-exponential phase under standardized conditions on defined media containing sole carbon/energy sources of glucose, succinate and lactate (curves 20b; 5,6 & 7 respectively). In all three spectra the pronounced ‘red’ shoulder is indicative of the presence of the cytochrome o complex in the membranes although both total and relative amounts of cytochrome o associated type-b cytochrome and the b cytochromes absorbing at (555-556)nm vary with the carbon/energy source supplied. Growth on glucose stimulated the greatest production of cytochrome o associated cytochromes b in these mid-exponential phase cell membranes. Growth on lactate produced by far the lowest complement of total cytochrome b (one third of the concentration produced by growth on glucose). Growth to mid-exponential phase on succinate resulted in membranes with a total cytochrome content similar to that from membranes of cells grown on glucose although there was much greater absorbance in the (555-556)nm region of the α-band in the succinate-grown samples with much less absorbance corresponding to material associated with the cytochrome o complex.

Figure 21 shows the variety of cytochrome b α-band profiles exhibited by membranes from a selection of wild-type cells grown to mid-exponential phase under standard conditions. The major feature of each spectrum is the maximal absorption at (555-556)nm although it is clear that there are a number of features contributing to the shape of the ‘red’ shoulder, and that the relative proportion of each of these features is variable.

The partially resolved α-band spectra of type-b cytochromes illustrated in Figures 19, 20 and 21 indicate that the complement of these cytochromes in cell membranes was dependent upon the cell strain, the carbon/energy source upon which the cells were grown, the abundance of dioxygen provided during aerobic growth and the phase of growth in which the cells were harvested. Although curve deconvolution techniques for quantitating these partially resolved bands were not available (203,204), fourth order derivative analyses were used to aid component peak identification and will be discussed in relation to the phenomena described above.

2. Fourth order derivative spectra of cell membrane cytochrome b α-bands

Curves 1 to 4 of Figure 19b depict the fourth order finite difference spectra corresponding to the redox difference spectra illustrated in panel ‘a’ of Figure 19. These fourth order derivatives illustrate that there was a progression in the change of membrane cytochrome α-band features as the harvesting-time of the cells is moved forward from stationary phase to early exponential phase. The
**Fig. 21:** High resolution $\alpha$-band spectra: type-$b$ cytochrome content of membranes from a selection of wild-type cell strains.

Cells of several $w^+$ strains were grown to mid exponential phase on CYD minimal medium containing glucose; crude membranes were prepared from each culture and washed as described in Materials & Methods. Membrane samples were resuspended in 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0 to a protein concentration of 5.0 mg mL$^{-1}$.

**a.** Dithionite reduced *minus* peroxide oxidized difference spectra obtained at 77 K.

1. Strain GR17N ($w^+$) $\Delta A = 0.20,$
2. Strain PA2-18 ($w^+$) $\Delta A = 0.20,$
3. Strain MR43L ($w^+$) $\Delta A = 0.16,$
4. Strain ML308-225 ($w^+$) $\Delta A = 0.16.$

**b.** Fourth-order finite difference spectra calculated from corresponding curves ‘a’ by four successive first-order derivatizations.

1. Strain GR17N ($w^+$),
2. Strain PA2-18 ($w^+$),
3. Strain MR43L ($w^+$),
4. Strain ML308-225 ($w^+$).
major features of the spectra from stationary phase cell membranes are absorption maxima at 556.0 nm and 559.0 nm, the latter corresponding to cytochrome $b_{558}$ of the cytochrome $d$ complex (Fig. 19b:1). Curve 2 shows that late exponential phase cell membranes contain $b$-type cytochromes with absorption maxima at 556.0 nm, (559-560) nm and 565.0 nm. There is an indication that there may have been other components present also, these less-well resolved constituents being illustrated more clearly by curves 3 & 4, representing spectra from membranes of early exponential phase cells and from membranes of cyd$^+$ cells respectively. In these samples there are contributions to the type-$b$ cytochrome $\alpha$-band profile from components with absorption maxima at 554.5 nm, (556-557.5) nm, 562.5 nm and 565.0 nm. Although the maxima of the cytochromes $b_{556}$ and $b_{558}$ (at 556.0 nm and 559.0 nm) associated with the cytochrome $d$ complex are visible in the early exponential phase sample (Fig. 19b:3) they are clearly absent from membranes of the cyd$^+$ strain which is known to be incapable of expressing components of the cytochrome $d$ complex. All the membranes appear to contain a cytochrome component with an absorption maximum at approximately 552 nm, although reference to the original spectra in panel 'a' shows that this makes only a minor contribution to the total cytochrome spectrum, and it may be caused by a secondary feature of one or more of the cytochromes discussed above.

Table VI shows the absorbance maxima of these membrane preparations and compares them to previously published estimates. The type-$b$ cytochromes associated with each of the two aerobic respiratory pathways are distinct with the electron transport chain terminating in cytochrome $o$ containing a larger number of $b$-cytochrome species. Many dehydrogenases incorporate type-$b$ cytochromes and, depending on the growth conditions, these may be expressed in association with either of the aerobic respiratory chains [4, 87]. By growing cells on minimal media supplemented with different carbon/energy sources it was anticipated that these might be distinguished in the spectrophotometric and electrochemical analyses. Evidence will be provided in subsequent sections linking certain dehydrogenases with multiple cytochromes $b_{556}$. The identification of absorption peaks in the range 555 nm to 556 nm is therefore complicated and the significance of the participation of one or more cytochromes $b_{556}$ in either respiratory chain, not directly associated with dehydrogenase activity, was unclear from these studies. Partial purification of the cytochrome $o$ complex and various inhibition studies have linked a cytochrome $b_{556}$ with the cytochrome $o$ respiratory chain: experiments relevant to the conclusions drawn from these investigations will be described below [95, 117, 128, 143, 180].

The traces in panel 'b' of Figure 21 provide greater detail of the components associated with the cytochrome $o$ complex having absorption maxima above 560 nm and were derived from
<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth Substrate/Phase</th>
<th>(\alpha)-Band Absorption Maxima</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GR17N</strong> (glucose-grown)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exponential</td>
<td>554.5 556.0</td>
<td>560.5 562.0 565.0</td>
</tr>
<tr>
<td>Stationary</td>
<td>556.0 557.5 559.0</td>
<td>——</td>
</tr>
<tr>
<td><strong>PLJ01</strong> (glucose-grown)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exponential</td>
<td>554.5 556.0</td>
<td>560.5 562.0 565.0</td>
</tr>
<tr>
<td>Stationary</td>
<td>554.5 556.0</td>
<td>560.5 562.0 565.0</td>
</tr>
<tr>
<td><strong>GR17N</strong> (succinate-grown)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exponential</td>
<td>554.5 556.0</td>
<td>560.5 562.0 565.0</td>
</tr>
<tr>
<td>Stationary</td>
<td>554.5 556.0 557.5 559.0</td>
<td>560.5 562.0 565.0</td>
</tr>
<tr>
<td><strong>PLJ01</strong> (succinate-grown)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exponential</td>
<td>554.5 556.0</td>
<td>560.5 562.0 565.0</td>
</tr>
<tr>
<td>Stationary</td>
<td>554.5 556.0</td>
<td>560.5 562.0 565.0</td>
</tr>
</tbody>
</table>

**Table VI:** Wavelengths of absorption maxima of cytochrome b components in exponential and stationary phase cell membranes.

Low temperature absorption maxima of \(b\)-cytochrome \(\alpha\)-absorption bands from reduced minus oxidized spectra of membranes from cells grown to exponential or stationary phase on D-glucose or disodium succinate. Wavelength values are expressed in nanometres and derived from fourth order derivative analyses of the original partially resolved high resolution \(\alpha\)-band spectra (Fig. 19).
membranes of four wild-type strains harvested in exponential phase. Although the 77 K redox difference spectra of these cells' membranes displayed markedly different profiles in the 'red' shoulder region of the \( \alpha \)-band, between 560 nm and 565 nm (panel 'a') the fourth order derivative spectra revealed that components with the same absorption maxima are creating the profile in each case.

As illustrated in Figure 19b;3, the sensitivity of the fourth order derivative analysis resulted in contributions from the cytochrome \( b_{558} \) associated with cytochrome \( d \) being observed even when it was present in very low concentrations in exponential phase wild-type cells (Fig. 21b). Further spectrophotometric analysis of this central region of the \( \alpha \)-band required removal of interference by cytochrome \( b_{558} \). A series of \( cyd^- \) strains was developed for this purpose from strain GR19N which fails to express any of the components of the cytochrome \( d \) complex (vide infra) \cite{60, 61}.

3. Multiple cytochrome \( b_{560}^+ \) \( \alpha \)-band components

Demonstration by fourth order derivative analyses that the three 'cytochrome \( b_{560}^+ \)' cytochromes were present in each of the several strains portrayed in Figure 21, coupled with the observation that the original \( \alpha \)-band profile between 560 nm and 565 nm was of radically different shape in each sample indicates that these \( \alpha \)-band discontinuities cannot be the result of a single cytochrome with multiple absorption maxima. If the latter situation were the case, the intensity of the three observable features of the 'red' shoulder would be expected to have differed between samples in constant mutual proportion rather than with the spectral fluctuations that are observed in relative absorbance at 561.0 nm, 562.5 nm and 565.0 nm. Fluctuations of relative absorbance between the cytochromes '\( b_{560}^+ \)' were also observed when single strains of cells were grown on a range of media supplemented with different carbon/energy sources (Fig. 20b). Since these three features always appeared together and their presence was associated with those conditions known to induce cytochrome \( o \), and as they were observed through high resolution analysis of the 'cytochrome \( b_{563} \)' component of the type-\( b \) cytochrome \( \alpha \)-band spectrum it is proposed that that these three components are associated with the cytochrome \( o \) complex itself. It is possible that they may be the result of a single cytochrome being exposed to three distinct environments within the respiratory system, the proportions of cytochrome in each environment, or the availability of each environment, varying in each of the strains illustrated. Such environments might be functional in nature or could be the result of perturbation during sample preparation.

Additional evidence of the linkage between these features of the \( b \)-cytochrome \( \alpha \)-band observed between 560 nm and 565 nm is provided by the action of certain respiratory inhibitors known to interact with terminal oxidases. Figure 22b, curve 5 demonstrates that addition of
cyanide to a dithionite reduced washed membrane preparation abolished the entire red shoulder of the \( \alpha \)-band spectrum observed in absolute spectra, with a shift of the \( \alpha \)-band absorption maximum to 557 nm. Carbon monoxide, however, had a minimal effect upon the \( b \)-cytochrome \( \alpha \)-band region of membrane suspensions reduced with sodium dithionite suggesting that the cytochrome or cytochromes contributing to the cytochrome \( b_{560}^+ \) shoulder may not correspond to cytochrome \( \alpha \) itself, but rather to other components of the cytochrome \( \alpha \) complex (Fig. 22b; 2+3). Carbon monoxide is known to interact with both of the aerobic terminal oxidases of \( E. coli \) \([157, 216]\) and to perturb the Soret region of the absorbance spectrum as shown in Figure 17. The other respiratory inhibitors used in these experiments are also known to interact with the aerobic terminal oxidases through ligand interaction at the free sixth coordination site of the oxidases' haem groups which is reserved as the site of reaction with dioxygen \([87, 112, 157]\). Haems of other respiratory cytochromes have all six iron coordination sites occupied by linkage to amino acids of the apoprotein backbone as described in detail in the Introduction. Thus perturbation of reduced and/or oxidized cytochrome absorption spectra may be caused by direct interaction with a terminal oxidase, as is generally assumed to be the case with carbon monoxide \([216]\), or by an allosteric effect of such an interaction as may be the explanation for the observed influence of cyanide upon those components of the cytochrome \( \alpha \) complex with reduced minus oxidized \( \alpha \)-bands in the 560 nm to 565 nm spectral range.

A pertinent comparison is provided by the reaction of stationary-phase wild-type membrane suspensions to respiratory inhibitors acting upon the cytochrome \( d \) complex (Table VII). In these membranes the cytochrome \( \alpha \) complex was largely absent, as shown by the lack of material absorbing between 560 nm and 565 nm in the low temperature dithionite reduced absolute spectrum of Figure 22a; 5. Upon treating such a reduced sample with carbon monoxide, cytochrome \( d \) itself responded with a pronounced red shift of its \( \alpha \)-absorbance band from 626 nm to 633 nm, although the cytochrome \( b_{558} \) component of the complex showed no apparent change in absorption characteristics, its low temperature absorption maximum remaining at 559 nm (Figure 22a; 4+5). On the other hand the actions of azide and cyanide resulted in the partially resolved \( \alpha \)-band peaks of the type-\( b \) cytochromes moving together to form a sharp, overlapping peak with an absorption maximum between 557 nm and 558 nm, and although each of these inhibitors caused a blue shift of the cytochrome \( d \) \( \alpha \)-band cyanide virtually abolished the dithionite reduced \( \alpha \)-band absorbance spectrum of cytochrome \( d \) whereas azide had little effect on its intensity (Figure 22a; 5,6,7). At present it is unknown whether these inhibitors have more than one site at which they may act upon the cytochrome \( d \) complex although azide has been shown to have multiple effects upon respiration and oxidative phosphorylation, each with a characteristic \( k_i \) value \([103]\). High resolution \( \alpha \)-band
**Fig. 22:** High resolution α-band spectra: absolute reduced spectra of washed, resuspended membranes in the presence of respiratory inhibitors.

Cells of w⁺ strain GR17N and its cyd⁺ derivative GR19N were grown to stationary phase on M9K minimal medium containing DL-lactate; crude membranes were prepared from each culture and washed as described in Materials & Methods. Membrane samples were resuspended in 100 mM potassium phosphate buffer, pH 7.0 to a protein concentration of 2.9 mg mL⁻¹ (GR17N) or 5.2 mg mL⁻¹ (GR19N). Samples were reduced with a few crystals of Na₂S₂O₄ before addition of the respiratory inhibitor. The latter was added as a few crystals of KNO₃, NaNO₂, NaN₃ or NaCN or as CO bubbled gently through the reduced sample for 90 seconds. The CO-treated samples were then loaded into the cryogenic cuvette and frozen under a stream of CO. All sample preparation was carried out under dark conditions and absolute spectra were recorded at 77K as the average of three scans. Subsequent illumination of the CO-treated samples was performed by exposing the cryogenic cuvette to the light (and warmth) from a 150 W incandescent bulb at a distance of 5 cm for 180 s intervals before refreezing and rescanning. The glutathione treatment illustrated was by addition of a few crystals to a freshly prepared membrane suspension which was subsequently reduced with Na₂S₂O₄. Dithionite reduced controls ± NaCl crystals were used to ensure that dissolving and mixing inhibitors into the samples did not cause sample oxidation by introducing dioxygen.

**a.** Cytochrome content of membranes of strain GR17N, ΔA = 0.20:
1. dithionite reduced + nitrite,
2. dithionite reduced + nitrate,
3. dithionite reduced + carbon monoxide, illuminated for a total of 540 s,
4. dithionite reduced + carbon monoxide, unilluminated,
5. dithionite reduced,
6. dithionite reduced + azide,
7. dithionite reduced + cyanide,
8. endogenous + glutathione, then dithionite reduced,
9. endogenous, (oxidized, oxygenated).

**b.** Cytochrome content of membranes of strain GR19N, ΔA = 0.20:
1. dithionite reduced + carbon monoxide, illuminated for a total of 180 s,
2. dithionite reduced + carbon monoxide, unilluminated,
3. dithionite reduced,
4. dithionite reduced + azide,
5. dithionite reduced + cyanide,
6. endogenous, (oxidized, oxygenated).
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<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>cytochrome b $\lambda^\gamma$ (nm)</th>
<th>cytochrome b $\lambda^\alpha$ (nm)</th>
<th>cytochrome d $\lambda^\alpha$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR17N</td>
<td>dithionite reduced</td>
<td>426</td>
<td>556.5 (558)</td>
<td>628</td>
</tr>
<tr>
<td></td>
<td>dithionite + nitrite</td>
<td>426</td>
<td>556.0 (558)</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>dithionite + nitrate</td>
<td>426</td>
<td>556.0 (558)</td>
<td>(635+642)</td>
</tr>
<tr>
<td></td>
<td>dithionite + carbon monoxide</td>
<td>425</td>
<td>556.0 (557)</td>
<td>636</td>
</tr>
<tr>
<td></td>
<td>dithionite + CO + hv$^a$</td>
<td>424</td>
<td>556.0 (558)</td>
<td>635</td>
</tr>
<tr>
<td></td>
<td>dithionite + azide</td>
<td>424</td>
<td>556.5 (560)</td>
<td>622</td>
</tr>
<tr>
<td></td>
<td>glutathione, then reduced</td>
<td>426</td>
<td>555.0 (559)</td>
<td>(636+642)</td>
</tr>
</tbody>
</table>

| GR19N           | dithionite reduced         | 426                                 | 555.0 (560$^+$)                     | —                                   |
|                 | dithionite + carbon monoxide | 425                              | 555.0 (560$^+$)                     | —                                   |
|                 | dithionite + CO + hv$^b$   | 425                                 | 555.0 (560$^+$)                     | —                                   |
|                 | dithionite + azide         | 424                                 | 556.0 (565)                         | —                                   |
|                 | dithionite + cyanide       | 425                                 | 557.0 —                             | —                                   |

$^a$ Illumination for 540 seconds as described in Figure 22.
$^b$ Illumination for 180 seconds as described in Figure 22.

Table VII: Wavelengths of absorption maxima of cytochrome b components in membranes exposed to terminal oxidase inhibitors.

Low temperature absorption maxima of reduced minus oxidized spectra of b-cytochromes in membranes from cells grown to stationary phase on D-glucose. Wavelengths are expressed in nanometres, values in parentheses indicate absorption peak 'shoulders'.
studies may prove a fruitful source of information regarding functions of specific terminal oxidase components in addition to the generally accepted ambient temperature spectrophotometric analyses of the overall Soret-band absorption characteristics of each complex.

Detailed descriptions of the effects of respiratory inhibitors upon the terminal oxidases of relevance to this study are provided below: sections II.C.i and II.C.ii describe membrane studies of cytochromes $d$ and $o$ respectively and sections III.C.v+vii relate to investigations of solubilized and purified cytochrome $o$ preparations.

(c) Use of membranes prepared from $cyd^-$ strains of *E. coli*

A number of benefits of using $cyd^-$ strains have been described and illustrated above. These strains, unable to express the components of the cytochrome $d$ complex under the conditions of growth promoted resolution of the overlapping $\alpha$-bands of 77 K reduced minus oxidized difference spectra because they lack a cytochrome $b_{558}$ (Fig. 18, 22). In addition spectral analysis following interaction of cytochromes with carbon monoxide or other respiratory inhibitors was simplified for there was no interference with the absorption spectra of cytochrome $o$ in the Soret region by cytochrome $d$ (Fig. 17, 22). Moreover, as detailed above, these strains provided a stable model of cell membranes containing a cytochrome complement usually observed under early exponential phase growth conditions and they enabled large quantities of cells and membranes to be prepared in this state. Not only were the large quantities of cells required for the physical studies of cytochromes described in this study difficult to prepare as coordinated, adequately aerated early exponential phase cultures, but delays inherent in the cooling and harvesting procedures inevitably resulted in some anaerobiosis of the samples and consequent initiation of induction of the cytochrome $d$ respiratory pathway by wild-type cells. Consequently the $cydA$ strains provided by the laboratory of R. B. Gennis during the course of this study and $cyd^-$ derivatives generated from these have been used throughout these investigations both independently and in comparison with their wild-type parental strains.

The investigation of mechanisms controlling the expression of the components of the cytochrome $d$ terminal oxidase will be discussed in relation to other cytochrome $d$ results, in section II.C.i.f.
(d) **Analysis of mutant strains with an altered type-6 cytochrome complement**

Additional attempts to improve resolution and characterization of type-6 cytochrome α-band characteristics utilised membranes from a series of putative cyb mutants which had been generated as described under Materials & Methods (Fig. 23). Each strain illustrated contained an aberrant complement of type-6 cytochromes. It is unclear from the reduced minus oxidized difference spectra alone whether these mutants contained one or more altered cytochromes or if the spectral modifications were caused by altered proportions of cytochromes normally present in such membranes. Table VIII lists the spectral properties of a number of such mutants which represent classes of strains which contained similar cytochrome profiles: precise wavelengths were determined with the aid of fourth order derivative analyses.

A selection of representative mutant strains were subjected to further analysis. Besides the potentiometric and kinetic studies which are described in subsequent sections, spectrophotometric analyses suggested that certain of the selected mutants, e.g. KW107, may have been deficient in a cytochrome component with an α-band absorption maximum at 556.0 nm and others, e.g. most of the ‘KW400’ series mutants, appeared to be lacking a component absorbing at 554.5 nm. These mutants were selected on the basis of their inability to utilize the cytochrome o respiratory pathway as described under Materials & Methods. Certain of them are described in sections II.A.ii.e and II.C.ii.b+c with respect to cytochrome interactions with carbon monoxide. The spectra portrayed in Figure 23 indicate the difficulties encountered with interpretation of the mutants’ α-bands, for the blue shoulder observed at 554 nm in membrane suspensions of KW107 could have been caused by the over-production of a component absorbing light at this specific wavelength or by the lack of material absorbing strongly at 556 nm in control samples.

Nevertheless, high resolution spectral analyses of the putative cyb" mutant preparations reinforced the earlier categorization and tentative spectral identification of type-6 cytochrome components of the aerobic respiratory pathways on the basis of their absorption maxima, for specific components were consistently absent from the fourth order derivative analyses of redox α-band spectra of membrane suspensions from many of these strains (Table VIII). Indications that some of the cytochrome $b_{560}^+$ features could be found in specific mutant strains without the appearance of other $b_{560}^+$ features was not taken as evidence that each of these spectral characteristics was caused by a separate component. An equally feasible result of an induced mutation would be the prevention of the formation of one or more specific molecular environments affecting distinct forms or states of a single cytochrome responsible for generating all of these related features. These strains provide a
**Fig. 23:** High resolution redox spectroscopy of membrane preparations from putative cyb<sup>-</sup> mutants.

Cells of several mutant strains were grown to mid exponential phase on **CYD** minimal medium containing succinate; crude membranes were prepared from each culture and washed as described in **Materials & Methods**. Membrane samples were resuspended in 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0 to a protein concentration of 10 mg mL<sup>-1</sup>. The cyd± notation refers to phenotypic character under these culture conditions.

### a. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.

<table>
<thead>
<tr>
<th>Strain</th>
<th>(cyd&lt;sup&gt;+&lt;/sup&gt;)</th>
<th>ΔA</th>
<th>Spectrum Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLJ01</td>
<td>ΔA = 0.150</td>
<td>1'</td>
<td>fourth order finite difference spectrum.</td>
</tr>
<tr>
<td>KW102</td>
<td>ΔA = 0.150</td>
<td>2'</td>
<td>fourth order finite difference spectrum.</td>
</tr>
<tr>
<td>KW105</td>
<td>ΔA = 0.120</td>
<td>3'</td>
<td>fourth order finite difference spectrum.</td>
</tr>
<tr>
<td>KW107</td>
<td>ΔA = 0.120</td>
<td>4'</td>
<td>fourth order finite difference spectrum.</td>
</tr>
<tr>
<td>KW183</td>
<td>ΔA = 0.125</td>
<td>5'</td>
<td>fourth order finite difference spectrum.</td>
</tr>
</tbody>
</table>

### b. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.

<table>
<thead>
<tr>
<th>Strain</th>
<th>(cyd&lt;sup&gt;+&lt;/sup&gt;)</th>
<th>ΔA</th>
<th>Spectrum Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLJ04</td>
<td>ΔA = 0.250</td>
<td>1'</td>
<td>fourth order finite difference spectrum.</td>
</tr>
<tr>
<td>KW401</td>
<td>ΔA = 0.012</td>
<td>2'</td>
<td>fourth order finite difference spectrum.</td>
</tr>
<tr>
<td>KW407</td>
<td>ΔA = 0.001</td>
<td>3'</td>
<td>fourth order finite difference spectrum.</td>
</tr>
<tr>
<td>KW417</td>
<td>ΔA = 0.125</td>
<td>4'</td>
<td>fourth order finite difference spectrum.</td>
</tr>
<tr>
<td>KW420</td>
<td>ΔA = 0.250</td>
<td>5'</td>
<td>fourth order finite difference spectrum.</td>
</tr>
<tr>
<td>Strain</td>
<td>C/E source</td>
<td>cyt. d</td>
<td>cytochrome ( b ) ( \alpha )-band absorption maxima</td>
</tr>
<tr>
<td>----------</td>
<td>------------</td>
<td>--------</td>
<td>----------------------------------------------------</td>
</tr>
<tr>
<td>GR17N</td>
<td>D-glucose</td>
<td>++</td>
<td>556.0 557.5 559.0 — — —</td>
</tr>
<tr>
<td>PLJ01</td>
<td>D-glucose</td>
<td>—</td>
<td>554.5 556.0 — — 560.5 562.0 565.0</td>
</tr>
<tr>
<td>KW102</td>
<td>D-glucose</td>
<td>++</td>
<td>556.0 557.5 559.0 trace — —</td>
</tr>
<tr>
<td>KW105</td>
<td>D-glucose</td>
<td>++</td>
<td>556.0 557.5 559.0 trace — —</td>
</tr>
<tr>
<td>KW107</td>
<td>D-glucose</td>
<td>+ trace</td>
<td>557.5 trace — 562.0 565.0</td>
</tr>
<tr>
<td>KW110</td>
<td>D-glucose</td>
<td>++</td>
<td>556.0 559.0 trace — —</td>
</tr>
<tr>
<td>KW131</td>
<td>D-glucose</td>
<td>++</td>
<td>556.0 557.5 trace — 562.0</td>
</tr>
<tr>
<td>KW144</td>
<td>D-glucose</td>
<td>++</td>
<td>556.0 559.0 560.5 trace —</td>
</tr>
<tr>
<td>KW183</td>
<td>D-glucose</td>
<td>—</td>
<td>554.5 556.0 557.5 — 562.0 565.0</td>
</tr>
<tr>
<td>KW201</td>
<td>D-glucose</td>
<td>++</td>
<td>556.0 — 559.0 — 562.0 565.0</td>
</tr>
<tr>
<td>KW203</td>
<td>D-glucose</td>
<td>++ trace</td>
<td>556.0 — 560.5 562.0 565.0</td>
</tr>
<tr>
<td>KW205</td>
<td>D-glucose</td>
<td>++ trace</td>
<td>556.0 trace 560.5 562.0 565.0</td>
</tr>
<tr>
<td>KW401</td>
<td>D-glucose</td>
<td>—</td>
<td>554.5 556.0 557.5 — 560.5 562.0 565.0</td>
</tr>
<tr>
<td>KW407</td>
<td>D-glucose</td>
<td>++</td>
<td>554.5 trace — 559.0 — trace</td>
</tr>
<tr>
<td>KW417</td>
<td>D-glucose</td>
<td>—</td>
<td>554.5 557.5 trace 562.0 565.0</td>
</tr>
<tr>
<td>KW420</td>
<td>D-glucose</td>
<td>++</td>
<td>554.5 556.0 trace 560.5 565.0</td>
</tr>
<tr>
<td>KW424</td>
<td>D-glucose</td>
<td>++</td>
<td>554.5 trace 560.5 565.0</td>
</tr>
<tr>
<td>KW425</td>
<td>D-glucose</td>
<td>++</td>
<td>554.5 557.5 — 560.5 565.0</td>
</tr>
<tr>
<td>PLJ01</td>
<td>succinate</td>
<td>—</td>
<td>554.5 556.0 — 560.5 562.0 565.0</td>
</tr>
<tr>
<td>KW102</td>
<td>succinate</td>
<td>—</td>
<td>556.0 557.5 — 560.5 trace 565.0</td>
</tr>
<tr>
<td>KW105</td>
<td>succinate</td>
<td>—</td>
<td>556.0 557.5 — 560.5 562.0 565.0</td>
</tr>
<tr>
<td>KW107</td>
<td>succinate</td>
<td>— trace</td>
<td>557.5 trace trace 565.0</td>
</tr>
<tr>
<td>KW183</td>
<td>succinate</td>
<td>— trace</td>
<td>556.0 — 560.5 562.0 565.0</td>
</tr>
<tr>
<td>KW401</td>
<td>succinate</td>
<td>—</td>
<td>554.5 556.0 — trace 560.5 565.0</td>
</tr>
<tr>
<td>KW407</td>
<td>succinate</td>
<td>—</td>
<td>554.5 557.5 — trace 565.0</td>
</tr>
<tr>
<td>KW417</td>
<td>succinate</td>
<td>—</td>
<td>554.5 557.5 trace — 562.0 565.0</td>
</tr>
<tr>
<td>KW420</td>
<td>succinate</td>
<td>—</td>
<td>554.5 556.0 trace 560.5 562.0 565.0</td>
</tr>
</tbody>
</table>

**Table VIII:** Wavelengths of absorption maxima of cytochrome \( b \) components in membranes from classes of putative cyb\(^{-}\) mutants.

Low temperature absorption maxima of \( b \)-cytochrome \( \alpha \)-absorption bands from reduced minus oxidized spectra of membranes from cells grown to stationary phase on D-glucose or succinate. Wavelength values are expressed in nanometres and derived from fourth order derivative analyses of the original partially resolved high resolution \( \alpha \)-band spectra.
useful resource for future investigations of these phenomena.

The case of KW107 was particularly intriguing for the interpretation that it lacked cytochrome $b_{556}$ was supported by its failure to utilize the respiratory chain terminating in cytochrome $o$ while retaining the ability to express and use that terminating in cytochrome $d$. Yet at the time this mutant was isolated the order of electron transfer between the cytochrome components of the former pathway was still being disputed by the laboratories of Anraku, Gennis and Poole as described in detail in the Introduction [95, 117, 128, 180]. Consequently this strain was subjected to further analyses and its respiratory cytochromes were solubilized and fractionated by liquid chromatography (section III, A+B). Investigations into the identity of 'cytochrome $b_{556}$' and the relationship of this fraction to cellular dehydrogenase activities are also described in section III.B.

(ii) **Potentiometric Titrations**

(a) **Membrane cytochrome complement of cells grown on specific carbon-energy sources**

Potentiometric analyses of cells' respiratory response to growth on a variety of specific carbon-energy sources was detected by redox difference spectrophotometry of membrane suspensions in the absorption range of the type- $b$ cytochromes' $\alpha$-band in the presence of a platinum electrode as described under Materials & Methods. It was impractical to use the more sensitive Soret region due to spectral interference from the multiple amphipathic electrophilic mediators that were required to bring the cytochromes present in the sample membranes into equilibrium with the electrode. However, by using the $\alpha$-band region of the absorption spectrum to determine the proportion of cytochrome reduced at selected potentials additional information was gathered at specified wavelengths which were representative of certain partially resolved $\alpha$-bands even at the temperature of 305 K at which the titrations were performed. Thus a comparison could be made between reduction potentials of those cytochromes displaying absorption maxima from 562 nm to 565 nm and those cytochromes absorbing between 558 nm and 560 nm at the titration temperature.

Titrations of resuspended, washed membranes from cells grown aerobically on minimal medium showed distinct profiles which were dependent upon the strain of cells being used, upon the carbon-energy source available and upon the phase of growth at which the cells were harvested [66, 69, 117]. Therefore the basis of comparison for this study was a series of cyd$^*$ cell lines derived from wild-type strain GR17N grown on a minimal, defined medium (CYD) in the presence of the
Fig. 24: Differences in potentiometric titrations of membrane preparations from cells grown to stationary phase on either D-glucose or on DL-lactate.

Wild-type strain GR17N was grown aerobically to stationary phase on CYD minimal medium supplemented with either

a. D-glucose (•—•—•), or
b. DL-lactate (O--O--O).

Membranes were harvested, washed and resuspended in degassed 100 mM potassium phosphate buffer, pH 7.0 at a protein concentration of  (a) 25.9 mg mL\(^{-1}\), (b) 14.6 mg mL\(^{-1}\).
non-fermentable carbon-energy source DL-lactate. These strains have been used by the laboratory of R. B. Gennis for numerous investigations of aerobic respiratory pathways in *Escherichia coli* and the published results include several potentiometric titrations [8, 62, 117]. DL-lactate has been used as a carbon-energy source by the majority of laboratories studying aerobic respiratory pathways of *E. coli* [60, 100, 102, 130] although the current investigations demonstrated that there were disadvantages associated with its use when preparing membrane suspensions for electrochemical studies (v.i.). Figure 24 shows potentiometric titration profiles for type-6 cytochromes of membranes from wild-type GR17N cells grown to stationary phase on either DL-lactate or on D-glucose. The redox difference spectra and CO-binding spectra in Figure 25 indicate that under these growth conditions the respiratory pathway terminating in cytochrome *d* predominated and that relatively little cytochrome *o* was present, especially in the glucose-grown cells (see also Fig. 16 to 20). Nevertheless the two titration profiles deviate substantially at both low and high potentials as did the spectral determinations of the cytochrome *b* complement of each set of membranes (Fig. 24, 25). Although evidence will be presented in subsequent sections suggesting that certain low-potential characteristics of each profile were probably related to differences in dehydrogenase complement caused by adaptation to the carbon-energy source provided, the high-potential, endogenous response of freshly resuspended membranes from cells grown on DL-lactate displayed an anomalous quantity of reduced cytochrome: approximately 25% of the total. Furthermore there was a rapid rate of spontaneous reduction of these 'lactate-grown' samples which stabilised at a potential of approximately +25 mV. In order to obtain a complete reduction profile the reduced samples were routinely reoxidized with K$_3$Fe(CN)$_6$ as illustrated in Figure 26b.

The two panels of Figure 26 show that detectable differences in type-*b* cytochrome complement between wild-type strain GR17N and its cyd<sup>+</sup> derivative GR19N were limited to high potential cytochromes when both strains were grown to stationary phase under aerobic conditions. This result may be surprising in light of the distinct features of the low-temperature redox difference spectra provided by similarly prepared membrane samples from these strains which indicated that under such conditions the predominant respiratory chain produced by the parent strain was that of the cytochrome *d* complex while the cyd<sup>-</sup> derivative relied on that terminating in cytochrome *o* (Fig. 25). It is suggested that only a limited number of the type-*b* cytochromes resolvable by high resolution difference spectroscopy were distinguishable by potentiometric titration of resuspended membranes as implemented in this study. This restriction may have been a result of a limited number of those spectrally resolvable cytochromes being major contributors to the α-band absorption spectrum of the membranes, or the major contributors to the spectrum may have had mid-point
Fig. 25: Ambient temperature redox difference spectra and carbon monoxide binding spectra of wild-type cells grown on either D-glucose or DL-lactate.

Cells of $w^+$ strain GR17N were grown to stationary phase on CYD minimal medium supplemented with carbon-energy sources of either D-glucose or DL-lactate; crude membranes were prepared from each culture and washed as described in Materials & Methods. Membrane samples were resuspended in 100 mM potassium phosphate buffer, pH 7.0 to protein concentrations of 21.7 mg mL$^{-1}$ (DL-lactate), 21.0 mg mL$^{-1}$ (D-glucose).

**a.** Dithionite reduced plus carbon monoxide minus dithionite reduced difference spectra obtained at 295 K.
1. Strain GR17N grown on DL-lactate to stationary phase, $\Delta A = 0.10$.
2. Strain GR17N grown on D-glucose to stationary phase, $\Delta A = 0.03$.

**b.** Dithionite reduced minus peroxide oxidized difference spectra obtained at 295 K.
At wavelengths greater than 500 nm, $\Delta A = 0.10$; below 500 nm, $\Delta A = 0.33$.
1. Strain GR17N grown on DL-lactate to stationary phase.
2. Strain GR17N grown on D-glucose to stationary phase.
**Fig. 26:** Potentiometric differences between membrane preparations from $w^+$ and $cyd^-$ cells grown to stationary phase.

Washed membranes were prepared from *E. coli* strains GR17N ($w^+$) and GR19N ($cyd^-$) after aerobic growth to stationary phase in CYD minimal medium in the presence of:

- **a.** D-glucose : (O--O--O), GR17N ; (+-+-+), GR19N ; or
- **b.** DL-lactate : (O--O--O), GR17N ; (+-+-+), GR19N.

The washed membranes were resuspended in degassed 100 mM potassium phosphate buffer, pH 7.0 at protein concentrations of:

- **a.** GR17N, 25.9 mg mL$^{-1}$, GR19N, 33.4 mg mL$^{-1}$ ; $\Delta A = 0.050$,
- **b.** GR17N, 14.6 mg mL$^{-1}$, GR19N, 22.8 mg mL$^{-1}$ ; $\Delta A = 0.040$.

Arrows refer to potentials at which samples were removed and rapidly frozen for high resolution spectrophotometric analysis (see Fig. 28).
### Results & Discussion

**Graph a.**

- Reduced b cytochrome $(\Delta A + X_{\text{red}})$
- GR19N baseline
- GR17N baseline
- $E_h$ (mV vs NHE)

**Graph b.**

- Reduced b cytochrome $(\Delta A + X_{\text{red}})$
- GR19N baseline
- GR17N baseline
- $E_h$ (mV vs NHE)
potentials that were grouped such that under the electrochemically equilibrated conditions of the titration procedure brought about by the influence of the mediators they acted as pools of $b$-cytochrome and appeared as a smaller number of electrochemically distinguishable entities.

Note that part of the spectral evidence for the greater number of type-$b$ cytochromes being present in the membrane comes from fourth order derivative analyses which are known to be qualitative in nature and extremely sensitive so that minor components, undetected by other analytical methods, may have been disproportionately emphasised (section I.i.d) \[22, 90\].

Thus it is proposed that the differences in the low potential type-$b$ cytochrome complement of the cells grown on glucose or lactate were caused by distinct cytochromes associated with the dehydrogenases induced under each set of growth conditions (Figure 26a vs. 26b). Similarities between the two strains’ mid-potential cytochromes (-25 mV to +125 mV) suggest that the two aerobic respiratory chains may share $b$-cytochromes with common characteristics, one candidate being cytochrome $b_{556}$ which was found to be ubiquitous under aerobic conditions (Figure 25 a+b) \[4, 87, 180, 186\]. When the two strains were grown on the same substrate, whether glucose or lactate, differences between their type-$b$ cytochromes were restricted to the high potential range (+125 mV and above), implying that this was the region in which differences between the two terminal oxidases were being observed (Figure 26 a,b).

(b) Electrochemical characteristics of the aerobic respiratory type-$b$ cytochromes

The $cyd^-$ strains were essential for investigating electrochemical characteristics of the high-aeration respiratory pathway terminating in cytochrome $o$ : harvesting cells in early exponential phase by the procedures available would have provided insufficient material for analysis by potentiometric titration. The type-$b$ cytochromes detected by this procedure and associated with cytochrome $o$ respiration are shown to possess mid-point potentials of approximately +220 mV by comparison of the curves in both panels of Figure 26. Table IX displays $E_h$ values for $b$-cytochromes determined from titrations of membrane suspensions derived from several strains, these values being within the range published by other laboratories \[157, 216\].

The high potential type-$b$ cytochrome present in membranes of GR17N wild-type cells grown to stationary phase on either substrate may have been the cytochrome $b_{558}$ of the cytochrome $d$ complex : it was not revealed by titrations of $cyd^-$ membranes, it demonstrated an absorption maximum of approximately 559 nm at 305 K and the investigations of R. B. Gennis have shown it to be particularly sensitive to environmental manipulation \[119\]. In these and related experiments
### Growth on DL-lactate:

<table>
<thead>
<tr>
<th>GR17N (+thiamine; n=3)</th>
<th>GR19N (+thiamine; n=4)</th>
<th>GR19N (-thiamine; n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E_h )</td>
<td>( E_h )</td>
<td>( E_h )</td>
</tr>
<tr>
<td>+363 (± 6)</td>
<td>+202 (±22)</td>
<td>+379 (± 34)</td>
</tr>
<tr>
<td>+211 (±13)</td>
<td>+69 (±17)</td>
<td>+201 (± 40)</td>
</tr>
<tr>
<td>+ 90 (±20)</td>
<td>- 27 (±31)</td>
<td>70 (± 31)</td>
</tr>
<tr>
<td>- 38 (±32)</td>
<td>-173 (±11)</td>
<td>- 59 (± 18)</td>
</tr>
<tr>
<td>-232 (±26)</td>
<td>214 (+46)</td>
<td>-214 (± 14)</td>
</tr>
</tbody>
</table>

### Growth on D-glucose:

<table>
<thead>
<tr>
<th>GR17N (+thiamine)</th>
<th>GR19N (+thiamine)</th>
<th>PLJ01 (+thiamine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E_h )</td>
<td>( E_h )</td>
<td>( E_h )</td>
</tr>
<tr>
<td>+262</td>
<td>+214</td>
<td>+275</td>
</tr>
<tr>
<td>+129</td>
<td>+101</td>
<td>+103</td>
</tr>
<tr>
<td>+ 30</td>
<td>+ 37</td>
<td>+ 24</td>
</tr>
<tr>
<td>- 118</td>
<td>-109</td>
<td>- 71</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PLJ04 (+thiamine)</th>
<th>PLJ07 (+thiamine)</th>
<th>KW107 (+thiamine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E_h )</td>
<td>( E_h )</td>
<td>( E_h )</td>
</tr>
<tr>
<td>+263</td>
<td>+232</td>
<td>+223</td>
</tr>
<tr>
<td>+178</td>
<td>+162</td>
<td>+144</td>
</tr>
<tr>
<td>+ 76</td>
<td>+ 58</td>
<td>+ 33</td>
</tr>
<tr>
<td>- 8</td>
<td>- 82</td>
<td>- 224</td>
</tr>
</tbody>
</table>

**Table IX:** Cytochrome b complement of aerobically-grown cell membranes determined by potentiometric titration.

Mid-point potential values are provided as mV vs. NHE with the percentage contribution to total type-b cytochrome measured in the cytochromes during each titration. Cells were grown to stationary phase on CYD minimal medium supplemented with the carbon/energy sources indicated; values in parentheses are standard deviations. Four component analyses are taken as the basis for comparison, as these are the most generally applicable under the constraints described in Materials & Methods; three or five component analyses are shown where necessitated because a four component fit was inadequate.
the complex was titrated in membranes and also after solubilization and purification in different detergent solutions, showing the mid-point potential of cytochrome $b_{558}$ to vary from +61 mV in octylglucoside solution to +180 mV in membrane suspensions [8, 105, 117, 119]. Although both of these values are much lower than those determined in the current study, the cytochrome $d$ complex exhibits radically different behaviour in an oxygenated or partially oxygenated state, so sample preparation procedures may be influential (v.i.). Alternatively, the high potential titration feature may be related to the $\beta$-band of cytochrome $b_{595}$ which absorbs at 562 nm in reduced minus oxidized difference spectra at ambient temperatures and possesses a mid-point potential of +113 mV [105, 117]. Again, although this is substantially less than the value of +235 mV depicted in this titration, Fig. 27, it has not yet been determined whether cytochrome $b_{595}$ interacts directly with molecular oxygen: an oxygenated form may exhibit distinct properties. Analysis of the properties of the cytochrome $d$ complex in greater detail (v.i.) suggested that although free oxygen had been rigorously excluded from these membrane titrations it is possible that this terminal oxidase, which has a particularly low $k_m$ for dioxygen [4, 112, 157], retained bound oxygen and behaved as an oxygenated complex (section II.B.ii) [80, 105, 157].

Multiple wild-type strains were available which generated different patterns of type-$b$ cytochromes in their $\alpha$-band redox difference spectra, indicating that they contained varying quantities of similar cytochromes when grown under comparable conditions (Fig. 21). The incomplete resolution of type-$b$ cytochrome $\alpha$-bands achieved by low temperature redox difference spectrophotometry prevented accurate quantitative estimation of the individual $b$-cytochromes present. When coupled with fourth order derivatization the techniques provided accurate spectral identification of those type-$b$ cytochromes but remained solely qualitative. The potentiometric titration data characterized a limited number of discernable type-$b$ cytochromes both quantitatively as their percentage contribution to the total cytochrome $b$ $\alpha$-band absorbance and qualitatively by electrochemical identification in terms of mid-point potential. Consequently unambiguous correlation between cytochromes detected in each of these two analytical methods was impossible, even when the aerobic respiratory chains were investigated independently by the selection of genetic and growth conditions described above.

Attempts to achieve such correlations were made by means of several procedures. Investigations were conducted into observed perturbations of the electrochemical response of certain type-$b$ cytochromes by manipulating conditions of cell growth or of the titration itself. Samples were removed from titrations poised at known potentials for low temperature spectrophotometric analysis. Strains containing putative cytochrome $b$ mutations in addition to the $cyd^+$ characteristics
Fig. 27: Modification of the titration characteristics of specific membrane cytochromes by cell growth without thiamine and by treatment of membrane preparations with ferricyanide.

Washed membranes were prepared from cyd⁺ strain GR19N after aerobic growth to stationary phase in CYD minimal medium supplemented with DL-lactate. The washed membranes were resuspended in degassed 100 mM potassium phosphate buffer, pH 7.0.

a. Cells grown in the presence (+--++) or absence (O-O-O) of thiamine.HCl, which was added to a concentration of 10 μg mL⁻¹ under standard conditions.

b. Successive reductive titrations of a membrane suspension, the sample being reoxidized with minimal quantities of titration buffer saturated with K₃Fe(CN)₆ between each titration:

(+-++++) initial reduction profile,
(-O--O-) secondary titration following reoxidation,
(+a-a+) tertiary titration following second reoxidation.
were generated and analysed in order to obtain additional simplification of the cytochromes expressed. The following sections describe these studies.

(c) Perturbation of the electrochemical response of certain type-b cytochromes

Perturbation of the electrochemical response of certain type- \( \text{-b} \) cytochromes as determined by potentiometric titration could be achieved by several means. Apart from adding ligands that would bind to the terminal oxidase, a technique discussed in detail below (sections II.B.i+ii, III.B.iv.f & III.C.iv.a), the growth of cells without a thiamine supplement in the medium and the addition of ferricyanide to reduced titration samples both dramatically affected the \( \text{b-cytochrome} \) titration profile of membrane suspensions. The effects of the latter two procedures are illustrated by Figure 27 in which panel 'a' depicts representative titrations of cells grown under equivalent conditions other than the presence or absence of a thiamine supplement to a final concentration of 10 \( \mu \text{g mL}^{-1} \) and panel 'b' shows the effect of successive reoxidation of a reduced membrane suspension with ferricyanide ion on subsequent reductive titrations.

All strains of \( \text{Escherichia coli} \) used in these investigations were K-12 derivatives and as such are categorised as thiamine auxotrophs. Nevertheless these cells will grow, albeit slowly, on minimal media in the absence of added thiamine indicating that the requirement is not absolute and suggesting that the \( \text{thi-1} \) lesion itself is 'leaky'. In both procaryotes and eucaryotes thiamine pyrophosphate is the cofactor for decarboxylases acting on \( \alpha \)-keto acids, transketolases and other reactions involving transfer of groups derived from a ketone [36]. The pyruvate decarboxylase complex is of central metabolic importance to an actively respiring bacterium growing aerobically in a defined, minimal medium with either glucose or lactate as sole carbon/energy source, as are pyruvate oxidase and the \( \alpha \)-ketoglutarate dehydrogenase complex, two other enzymes that require thiamine pyrophosphate \{64\}.

An inadequate supply of the cofactor required for activity of these enzymes would disrupt intracellular metabolic pools and the dynamic equilibria between reduced and oxidized forms of the pyridine nucleotides and between the adenosine phosphates (phosphorylation potential '\( \Delta G_p \)' or energy charge '\( ec \)') \{5, 64\}. A feasible consequence of such a situation might be an adaptation of the electron transport pathways involving an alteration of cytochrome components. However, whereas quantitative or qualitative changes in the expression of dehydrogenases might be expected in these circumstances, revealed by a low potential modification of type- \( \text{-b} \) cytochromes, a more general
response was observed. As indicated in Figure 27a the entire cytochrome-\textit{b} profile of membrane suspensions from cells grown in the absence of thiamine is shifted to higher potential in comparison with controls. This general displacement of some 100 mV is difficult to explain without postulating the complete replacement of respiratory pathways. An alternative interpretation of the data is that the effect of growth without thiamine caused a respiratory adaptation in which the cells failed to express the low potential type-\textit{b} cytochromes. Since the data are illustrated as the proportion of total cytochrome reduced the latter situation would cause the high potential cytochromes to be emphasised in the titration profile which would consequently appear to undergo a gross shift to higher potential. The concentrations of type-\textit{b} cytochrome per milligram of membrane protein were not comparable between cells grown with and without thiamine, and so a direct test of this phenomenon cannot be made until there are accurate means of identifying which cytochromes are being reduced at each stage of a potentiometric titration. Nevertheless a metabolic cause for the loss of dehydrogenase expression is relatively simple to postulate if thiamine is absent, since lactate utilization \textit{via} pyruvate would be slower as would the passage of metabolites through the early steps of the tricarboxylic acid cycle resulting in lower concentrations of a number of potential dehydrogenase substrates. Thus the means by which thiamine influences respiration and cytochrome expression merit further investigation.

Further evidence of a major change in respiratory complement was obtained by membrane solubilization with Triton detergents and anion-exchange chromatographic fractionation of the extracts. In cells grown without thiamine these extracts contained significant quantities of a cytochrome with reduced \textit{minus} oxidized absorption maxima of 423.0 nm, 524.0 nm and 553.5 nm, indicative of a \textit{c}-type cytochrome, the original membranes having a pronounced \textit{\alpha}-band absorption shoulder at approximately 550.0 nm. (data not shown). Fourth order derivative analysis indicated that the \textit{\alpha}-band peak comprised two components, one of 552.0 nm and the other of 554.5 nm which may be indicative of two cytochromes or may be a feature of a respiratory cytochrome \textit{c} \textit{(c.f. section I.i.b; Fig 5)}. The quantity of this material was difficult to estimate because of spectral overlap and incomplete chromatographic fractionation, but this cytochrome was resistant to both urea and cholate stripping of the membrane and total Soret absorbance of the cytochrome \textit{c} fractions from the extract was approximately one quarter of that of the pooled cytochrome \textit{o} complex, with which it partially overlapped (data not shown). The appearance of this novel cytochrome under such conditions of nutritional stress is curious because of the lack of \textit{c}-type cytochrome in \textit{E. coli} K-12 cells grown aerobically with an adequate thiamine supply, and it may be related to the perturbation observed in the potentiometric membrane titration (Fig. 27).

A second form of electrochemical perturbation of cytochromes was discovered during the
course of potentiometric titrations of membranes from lactate-grown cells. The differential effect of ferricyanide was subsequently exploited to distinguish certain type-b cytochromes within membrane suspensions and solubilized preparations. As described above, freshly resuspended membranes prepared from cells grown on DL-lactate tend to auto-reduce, possibly as a result of endogenous substrates being present even after extensive washing of the membrane preparation with aqueous buffer in repeated cycles of homogenization and centrifugation.

In order to obtain data points at the high potential range of the titration profile it was necessary to reoxidize the sample. When oxygenating reagents were used for this purpose, such as small aliquots of concentrated solutions of \((\text{NH}_4)_2\text{S}_2\text{O}_8\) or \(\text{H}_2\text{O}_2\), an extended period was required before the sample re-equilibrated with the electrode as indicated by inconsistent data and considerable hysteresis on comparing reducing and oxidizing curves. Following full reoxidation with these reagents and adequate equilibration times of 45 min to 60 min subsequent reductive titrations closely followed the original pattern (data not shown). However, if oxidation of the reduced sample was carried out with \(\text{K}_3\text{Fe(CN)}_6\) a displacement of part of the titration curve was observed while most of it remained constant. The modification indicated that a cytochrome generally comprising about 20% of the total and with a potential of approximately +25 mV was undergoing a dramatic shift in its electrochemical properties such that after the ferricyanide treatment it behaved with a new mid-point potential of -175 mV. The clarity with which this effect could be observed depended upon the source of the membrane sample: the proportions of individual cytochromes and the resolution of components in any specific section of a titration profile was dependent upon the bacterial strain and the growth conditions used. In a few cases the original cytochrome was detectable from the initial reductive titration profile whereas in the majority of samples it appeared as part of a larger, unresolved portion of the total cytochrome having a mid-point potential of approximately +45 mV. The appearance of the displaced component was clearly resolved in either situation after the ferricyanide treatment since there were no cytochromes with mid-point potentials interfering in the -200 mV region of the titrations.

No significant alterations in the complement of type-b cytochromes were apparent from the \(\alpha\)-bands of the redox difference spectra performed at 305 K as part of the potentiometric procedure nor from spectrophotometric analysis of poised potential samples taken during the course of the titrations (section II.A.ii.d). This was an additional indication that neither spectrophotometric nor potentiometric analytical methods were capable of resolving all the type-b cytochromes present in a typical membrane sample from aerobically-grown cells.

This phenomenon complicated the collection of high-potential titration data from membrane
samples derived from cells grown on DL-lactate. These samples also auto-reduced and were difficult to maintain in the oxidized state required for the spectroscopic reference cell during the extended titration procedure. Therefore disodium succinate was routinely used as a non-fermentable carbon-energy source for not only did it eliminate these procedural difficulties it also provided higher yields of respiratory cytochrome.

The selective perturbation of cytochrome mid-point potentials by ferricyanide suggested the possibility of its use as a technique for resolving and identifying certain type-6 cytochromes. However, the inability to correlate spectrophotometric and potentiometric data, even when incorporating the available cyd' strains and variations of growth conditions into the experiments, indicated that this procedure would have to be used in conjunction with additional methods of separating the complex mixtures of cytochromes present in membrane preparations.

(d) Poised potential high resolution redox difference spectrophotometry

In spite of the limited resolution provided by the redox difference spectra collected at 305 K as part of the potentiometric titration procedure it was apparent that in membranes from cyd' strains and in those from wild-type cells grown to exponential phase the cytochromes being reduced at the highest potentials displayed a biphasic α-band. It could not be determined whether one or multiple cytochromes caused these features, but a broad absorption peak at 565 nm was accompanied by a slightly smaller broad absorption peak at 558-560 nm. The lack of spectral resolution at these temperatures and the small absorption change observed in the α-band region due to the reduction of these components prevented more detailed analysis and suggested that potentiometric poising coupled with high-resolution spectrophotometric analyses would be fruitful.

As described in section I.i.e the procedure of high resolution spectrophotometry of samples poised at specific potentials offered an opportunity to simplify the number of cytochromes contributing the α-band redox difference spectrum of a membrane sample. By taking samples for high resolution spectrophotometry which had been poised at a range of potentials throughout the titration of a membrane preparation increasing proportions of the constituent cytochromes would be electrochemically reduced. High resolution spectrophotometry could then be employed at 77 K to indicate the combined spectral characteristics of those cytochromes reduced at each 'poised' potential. It was anticipated that subtraction of selected digitised spectra would yield high-resolution spectral data for those cytochromes undergoing reduction between each pair of 'poised' potentials. It should be
remembered that low temperature spectrophotometric data from samples poised at cited potentials cannot be taken as representing the state of reduction of similar samples maintained at ambient temperature at the same potential. The poising technique only provides a valid direct comparison between similarly treated samples although it appears to be indicative of the sample components' relative sensitivity to reduction if low temperature analyses are performed on a series of samples poised at a progression of potentials (section I.ii.c).

Figure 28 portrays low temperature spectra of samples removed and poised at several potentials during the wild-type and cyd' strain membrane titrations illustrated in Figure 26. These high resolution spectra show the $\alpha$- and $\beta$-bands of the type-$b$ cytochromes and the $\alpha$-band region of cytochrome $d$. When each of the two cell strains had been grown to stationary phase the low potential spectra of the wild-type, GR17N, showed that it contained very significant quantities of cytochrome $d$ by the combined peak and trough between 600 nm and 700 nm, but there was no detectable $b$-cytochrome $\alpha$-band shoulder between 560 nm and 565 nm which would have been attributable to the cytochrome $o$ complex. The cyd' strain GR19N displays minimal cytochrome $d$ but a substantial red shoulder on the $b$-cytochrome $\alpha$-band, confirming that these two preparations are representative models for investigating the two alternative aerobic terminal oxidases. (The small amount of cytochrome $d$ complex expressed by GR19N when grown to stationary phase upon DL-lactate has been commented upon elsewhere by Johnson & Bragg [89]. This phenomenon was not observed when these cells were grown on succinate, nor when the derivative strain PLJ01 was grown on either of these non-fermentable carbon sources.) However, the presence of other cytochromes, especially those of low potential, in membranes containing the cytochrome $o$ terminal oxidase which have been derived from cyd' cells grown to stationary phase may not be representative of cytochromes normally associated with the cytochrome $o$ complex. Under the batch conditions of this study, stationary phase cyd' cells had necessarily been grown under abnormal conditions, for in wild-type strains the cytochrome $o$ respiratory chain would have been replaced by that of cytochrome $d$ as soon as the dissolved oxygen concentration fell below a critical level, as described in the Introduction. Thus the cytochrome $o$ complex may not be functioning effectively in cyd' cells approaching stationary phase and components of other respiratory chains may be expressed as the cells adapt to the carbon and energy sources resulting from sub-optimal utilization of aerobic respiratory metabolism of the nutrients in the medium supplied.

Fourth order derivative analyses of the poised potential spectra are shown in Figure 28b and their peak positions identify the $b$-cytochromes reduced at each potential. The red $\alpha$-band shoulder associated with cytochrome $o$ that was present in the cyd' cell membranes at +98.5 mV was
Fig. 28: Poised potential high resolution difference spectra of $w^+$ and $cyd^-$ cells from the titrations illustrated in Fig. 26.

High resolution reduced minus oxidized difference spectra of poised potential samples removed during the titrations illustrated in Figure 26 and measured at 77 K.

Strains GR17N ($w^+$) and GR19N ($cyd^-$) had been grown aerobically to stationary phase in CYD minimal medium in the presence of D-glucose. Washed membranes were prepared and resuspended in degassed 100 mM potassium phosphate buffer, pH 7.0 at protein concentrations of 25.9 mg mL$^{-1}$ (GR17N) and 33.4 mg mL$^{-1}$ (GR17N) for the titrations. Potential values indicate the potential at which the sample was removed from the titration vessel.

**a.** GR17N membranes, $\Delta A = 0.020$,

1. $-337.5$ mV vs. NHE, versus reference reoxidized with H$_2$O$_2$.
2. $-337.5$ mV vs. NHE,
3. $-111.5$ mV vs. NHE,
4. $-26.0$ mV vs. NHE,
5. $+185.0$ mV vs. NHE,

2'. fourth order finite difference spectrum.
3'. fourth order finite difference spectrum.
4'. fourth order finite difference spectrum.
5'. fourth order finite difference spectrum.

**b.** GR19N membranes, $\Delta A = 0.020$,

1. $-348.5$ mV vs. NHE,
2. $-90.5$ mV vs. NHE,
3. $-25.5$ mV vs. NHE,
4. $+98.0$ mV vs. NHE,
5. $+264.5$ mV vs. NHE.

1'. fourth order finite difference spectrum.
2'. fourth order finite difference spectrum.
3'. fourth order finite difference spectrum.
accompanied by a distinct, albeit smaller, peak at 556 nm reflecting the observations obtained from the 305 K spectra. This peak increased in size as the potential was lowered until it became the dominant feature of the spectrum. Because of the overlap between these two absorption maxima and the change in size of the one peaking at lower wavelength it was not possible to determine whether the higher wavelength peak was also increasing in amplitude as the potential was decreased. While any increase in the size of the red shoulder was significantly less than that of the 556 nm peak an accurate determination of relative amplitude would have required curve deconvolution analyses of the spectra — a technique that has been used by other laboratories for determination of component contributions to ambient temperature spectra but one that was not available for the current investigation (203, 204). The fourth order derivative analyses indicated that those cytochromes undergoing reduction at potentials below +100 mV had redox absorption maxima of 556 nm at 77 K. The cytochromes that were reduced after adjustment of the potential to +100 mV, which included those associated with the cytochrome $a$ complex, had low temperature absorption maxima at 555.0 nm, 557.5 nm, 562.0 nm and 565.0 nm. The two higher wavelength peaks are components of the red shoulder, there being insufficient absorption by these poised potential samples to determine whether or not a third component of the red shoulder was present, as observed in fully reduced minus fully oxidized membrane suspensions. At lower potentials the fourth order derivative spectra exhibited decreased resolution of these components, which may have been caused by an increase in concentration of a component absorbing at 564.5 nm or simply by the influence of the increasing quantities of electrochemically reduced cytochrome $b_{556}$. The latter explanation is favoured due to the results from high resolution fully reduced minus fully oxidized difference spectra of membrane preparations of this type (Fig. 19, 21).

The wild-type membranes were shown to have contained a simpler $b$-type cytochrome complement in addition to cytochrome $d$ (Fig. 28a). At +190.5 mV the cytochrome $d$ was fully reduced, suggesting that the large inflection in the potentiometric titration profile (Fig. 28) was caused by absorption by one or both of the $b$-cytochrome components of the cytochrome $d$ complex, cytochromes $b_{558}$ and $b_{595}$. The fourth order derivative spectra shown in Figure 28b indicate that there is an absorption peak at 559 nm, the wavelength associated with absorption by cytochrome $b_{558}$ although the original 77 K redox difference spectra suggested that between 50 % and 70 % of this cytochrome was not reduced at this high potential. However, because of the overlap of the two cytochrome $a$-bands the ‘total’ absorption at 558 nm at low potential would have incorporated a substantial contribution from cytochrome $b_{556}$, implying that the large, high potential inflection in the titration profile corresponded to the full reduction of cytochrome $b_{558}$ which itself is associated
with the terminal oxidase (Fig. 27, 28). Thus a precise estimation of percentage reduction of individual components could not be provided without deconvolution analysis since there was interference from the adjacent absorption maximum at 556 nm. Additionally the contribution to the combined peak from the $\beta$-band of cytochrome $b_{595}$ could not be gauged accurately: the broad, low amplitude absorption peak caused by the $\alpha$-band of cytochrome $b_{595}$ was too small for accurate quantitation \cite{118}. Nevertheless there was little change in amplitude of either the small $\alpha$-band attributed to the latter cytochrome's high-spin haem or the small shoulder at approximately 550 nm which has been suggested to be caused by its $\beta$-band \cite{118} indicating that a substantial portion, if not all of the cytochrome $b_{595}$ was reduced at this high potential, as was cytochrome $d$ itself.

As the potential was lowered there was a gradual shift in the absorbance maximum of the lower wavelength component, changing from 554.0 nm at +190.5 mV to 555.5 nm at +532.0 mV. It is uncertain whether this change was significant, although examination of the titration profile in Figure 26 indicates that several electrochemically distinct type-$b$ cytochromes were reduced over this potential range. In spite of this, there are few alterations visible in the poised potential redox difference spectra (Fig. 28b). The $b$-cytochrome $\alpha$-band increased in height by a factor of 2.5 between +190.0 mV and -20.5 mV. Cytochrome $b_{558}$ was responsible for a significant portion of this change as described above, but an increasing contribution was due to one or more type-$b$ cytochromes absorbing between 555 nm and 556 nm. Moreover, although there was no apparent alteration of the cytochrome $d$ $\alpha$-band at potentials above -20.5 mV at each of the lower potentials one observed a progressive decrease in amplitude of both the 626 nm 'reduced' peak and of the 648 nm 'oxygenated' trough and a shift of the former from 626 nm to 635 nm. The cause of this absorbance shift is unknown, although the sensitivity of the reduced peak of cytochrome $d$ to perturbation by the addition of certain ligands was demonstrated in Figure 22: the decrease in amplitude of the adjacent peak and trough may simply be a consequence of greater overlap between these two spectral features as the peak shifts to higher wavelength.

The results illustrated in Figure 28 indicate that although the poised potential technique for obtaining high resolution redox difference spectra at distinct potentials provided some additional information about the components of the two terminal oxidase complexes it was of little assistance in resolving the complexities of the other $b$-type cytochromes with lower mid-point potentials.
Analysis of mutant strains with an altered type-\(b\) cytochrome complement

Generation of mutants was intended to simplify the cytochrome-\(b\) content of relevant cell membranes in order that the remaining cytochromes might be resolved more thoroughly upon subsequent spectrophotometric or potentiometric investigation. The advantages of this approach were evident from the utility of the \(cyd'\) strains used throughout these studies which removed interference by cytochromes associated with the cytochrome \(d\) terminal oxidase. However, further simplification of the membranes' cytochrome content was required if the type-\(b\) cytochromes of the central and early segments of the electron transport chains were to be analysed, as has been shown in previous sections by the inability of the applied methodologies to accomplish adequate resolution of these components.

In addition it was anticipated that if \(b\)-cytochrome mutants could be prepared they might also serve as keys with which to correlate the information provided by each of these analytical techniques. Two types of mutants were sought: those with defective expression of constituents of the cytochrome \(o\) complex which would be used in an analogous manner to the \(cyd'\) strains that were already available (\(cyo'\) strains), and mutants which expressed abnormal type-\(b\) respiratory cytochromes or that failed to express one or more of these cytochromes, useful in studies of the order of electron flow between respiratory components (\(cyb'\) strains).

Selection procedures were developed to promote the isolation of mutants defective in the activity of the respiratory chain terminating in cytochrome \(o\) yet spectroscopic techniques were unable to make positive identification of any strains failing to express the cytochrome \(o\) complex (see, however, section II.C.ii.b). However, certain mutant strains were observed to generate abnormal 'reduced plus carbon monoxide minus reduced' spectra with minimal features in the Soret region of cytochrome \(o\) itself. Following nitrosoguanidine mutagenesis enrichment and selection procedures for potential \(cyo'\) mutants were performed as described under Materials & Methods. Strains KW420, KW424 and KW425 were each distinguished by the lack of a clear absorbance trough at 430 nm in CO-binding spectra indicating the possibility that cytochrome \(o\) was absent from their membranes (Fig. 29). Interpretation of these reduced plus carbon monoxide minus reduced difference spectra was complicated by the overlapping absorbance shifts resulting from CO binding to the cytochrome \(d\) complex, which was induced under the growth conditions provided. These strains were maintained and subcultured under anaerobic conditions in order to minimize reversion, but as found with the \(cyo',cyd'\) strains when competing respiratory chains were eliminated the reversion rate was sufficient to induce enough cytochrome \(o\) revertants to dominate the culture. This would even occur in cultures maintained under strict anaerobic conditions until the final aerobic
batch culturing of the cells for analysis of the aerobic oxidases, respiring revertants rapidly outgrowing the fermentative mutant cultures (v.i.). Thus maintaining and using pure cultures of strains carrying point mutations in the cytochrome \( \alpha \) terminal oxidase was not possible unless an alternative respiratory chain was able to be induced under the relevant growth conditions: in the case of this study the alternative chain was that terminating in cytochrome \( d \), effectively negating attempts to simplify the cytochrome content of the respiratory apparatus for spectrophotometric or other analyses.

The absence of cytochrome \( \alpha \) was unable to be confirmed from the CO-binding spectra of membrane preparations from the putative \( \text{cyo}^-,\text{cyd}^+ \) strains KW420, KW424 and KW425 because of the overlap between the Soret bands of the two aerobic terminal oxidase complexes, coupled with that from their CO derivatives (Fig. 29). Nevertheless, these analyses showed that if present, any cytochrome \( \alpha \) that existed in these membranes was in greatly reduced quantities when compared to preparations from the parental strain PLJ04. A 560 nm CO-binding spectral trough was still visible in these spectra, as was the red shoulder on the redox \( \alpha \)-band between 560 nm and 565 nm (Fig. 29). It is therefore possible that these spectral features are only related to cytochrome \( \alpha \) indirectly, and may be caused by another cytochrome component of the oxidase.

Since considerable progress was being reported by the laboratory of R. B. Gennis in locating the chromosomal position of the \( \text{cyo} \) operon in addition to the generation of \( \text{cyo}^- \) strains as a preparatory step in cloning the gene products responsible for this oxidase activity, attempts at creating and characterizing additional \( \text{cyo}^- \) mutants in the current study were abandoned. R. B. Gennis generously provided \( \text{cyo}^-,\text{cyd}^- \) strain RG167 which had been produced from strains with single site polar mutations in each of these two operons but under aerobic conditions the selection pressure for respirationally competent back mutations was sufficiently great to prevent a pure culture from being maintained. Subculturing plus spectroscopy of carbon monoxide derivatives showed that even single revertant strains were unstable, generating a series of respiratory phenotypes in which features of the membranes’ CO-binding spectra could be predicted from certain characteristics of each strain’s colony morphology (data not shown).

The \( b \)-cytochrome mutants generated and isolated in this study were classified into groups on the basis of their parentage and any cytochrome abnormalities detected by low temperature redox difference spectrophotometry coupled with fourth order derivative analysis. Spectral characteristics of the major groups of putative \( \text{cyb}^- \) strains are listed in Table VIII. Strain KW107 is a representative example of those mutants which appeared to have, from low temperature spectrophotometry of membrane suspensions, a minimal cytochrome \( b_{556} \) content (Fig. 23). Electrochemical properties
Fig. 29: Carbon monoxide binding spectra from putative cyb\textsuperscript{−} and potential cyo\textsuperscript{−} mutant isolates.

Ambient temperature reduced plus carbon monoxide minus reduced difference spectra of washed membranes of strains grown aerobically to stationary phase on CYD minimal medium supplemented with D-glucose. Membranes were resuspended at the stated protein concentrations in 100 mM potassium phosphate buffer, pH 7.0. Procedures for preparation and gassing of samples are described under Materials & Methods.

1. Parental strain PLJ04, 2.5 mg mL\textsuperscript{−1}, \( \Delta A = 0.050 \).
2. Derivative strain KW420, 1.0 mg mL\textsuperscript{−1}, \( \Delta A = 0.005 \).
3. Derivative strain KW424, 1.0 mg mL\textsuperscript{−1}, \( \Delta A = 0.005 \).
4. Derivative strain KW425, 1.0 mg mL\textsuperscript{−1}, \( \Delta A = 0.005 \).
of KW107 are listed in Table IX and illustrated in Figure 30 in comparison to titration profiles of membranes from GR17N & GR19N grown under the same conditions. It is apparent that membranes of cyd' strain KW107 contained virtually no cytochromes with mid-point potentials lower than 0 mV when it was grown on minimal medium supplemented with glucose. This strain is a derivative of PLJ01 and consequently expresses cytochrome d components when grown on glucose, but not when grown on succinate or DL-lactate. It grows relatively slowly under aerobic conditions which would be expected to delay the oxygen-dependent onset of expression of the cytochrome d complex: a phenomenon demonstrated by the spectra in Figure 31a which show the mutant to have levels of both terminal oxidases that are intermediate between the exclusive reliance on either the cytochrome d complex or on the cytochrome o complex displayed respectively by GR17N and GR19N when each of the three strains is grown to stationary phase. (Cytochromes associated with the cytochrome o complex absorb in the 560 nm to 565 nm range of these redox difference spectra and cytochrome d α-band absorption is visible between 615 nm and 695 nm). If the high potential cytochromes are those associated with the terminal oxidase complexes, the identity and spectroscopic properties of those cytochromes of KW107 shown to be reduced over the range of 0 mV to +100 mV by Figure 31b are pertinent to the elucidation of organization of the aerobic respiratory chains, especially if these cells do indeed lack the cytochrome b555. These phenomena are addressed below, as is the difficulty of identifying ‘cytochrome b556’ unambiguously.

Solubilization and fractionation of membrane cytochromes from strain KW107 resulted in essentially ‘normal’ elution profiles from standard anion exchange chromatographic separations, including significant quantities of cytochrome with redox α-band absorption maxima of 556 nm at 77 K suggesting that the cytochromes b556 solubilized from cell membranes by the standard procedure were not related to the ‘central respiratory cytochrome b556’ of the cytochrome o electron transport chain (sections III.A.i+ii).

(iii) Reduction Kinetics

(a) Overview of technique

Utilization of dual wavelength spectrophotometry for investigating the reduction kinetics of cytochromes enabled small changes of absorbance to be detected in the cytochromes’ Soret or α-band regions even when the sample generated a high degree of incident beam light scattering, such as that
Fig. 30: Potentiometric titration of mutant and parental strains with different complements of type-\(b\) cytochrome.

Washed membranes were prepared from parental strain GR17N (\(w^+\)) and derivative strains GR19N (\(cyd^+\)) and KW107 (cytochrome \(d\) complex under the control of growth substrate and possible cytochrome \(b\) modifications) after each had been grown aerobically to stationary phase in CYD minimal medium in the presence of D-glucose. The washed membranes were resuspended in degassed 100 mM potassium phosphate buffer, pH 7.0 at the following protein concentrations:

- GR17N, 25.9 mg mL\(^{-1}\), (---);
- GR19N, 33.4 mg mL\(^{-1}\), (-----);
- KW107, 15.7 mg mL\(^{-1}\), (■ ■ ■).

Data points for the GR17N and GR19N curves are provided in Figure 26 but have been omitted here for clarity.
**Fig. 31:** High resolution redox difference spectra of mutant strains with different complements of cytochrome.

Washed membranes were prepared and resuspended for potentiometric titration from strains GR17N, GR19N and KW107 grown aerobically to stationary phase in CYD minimal medium in the presence of D-glucose (Fig. 30). Spectrophotometry was performed at 77 K following dilution of the samples to final concentrations of 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0, at the protein concentrations indicated:

**a.** Dithionite reduced minus peroxide oxidized redox difference spectra.
1. GR17N, 13.0 mg mL\(^{-1}\);
2. KW107, 7.9 mg mL\(^{-1}\);
3. GR19N, 16.7 mg mL\(^{-1}\).

**b.** Fourth order finite difference spectra calculated from the corresponding redox difference spectra in panel ‘a’.
caused by membrane suspensions [90]. In practice the \( \alpha \)-band absorbance change was most often used to indicate the proportion of cytochrome in the reduced state to circumvent interference from the coloured mediator phenazine methosulphate which was frequently used as an intermediary for the electronic equilibration between respiratory components and the experimental electron donor.

Addition of a moderate excess of substrate to the single cuvette containing the resuspended sample results in an absorbance change indicative of the steady state level of reduction of the cytochromes present. Electrons flowing into the respiratory chains contained within the sample membranes pass through the various components and are transferred to dioxygen by the terminal oxidase or oxidases until all the oxygen dissolved in the sample has been consumed. When the suspension becomes anaerobic the loss of electrons from the terminal oxidases will cease while electron flow into the respiratory chains continues until all components susceptible to reduction have been reduced. Several complications are immediately apparent. Without addition of an electrochemical mediator only certain biological substrates are capable of transferring electrons into the respiratory chains: this may be used as the basis of assays for specific dehydrogenase activities. Notable exceptions are quinol analogues, many of which are soluble in the membrane lipid phase and interact with cytochrome quinol binding sites; other exceptions are strong redox reagents such as \( \text{Na}_2\text{S}_2\text{O}_4 \) and \( \text{K}_3\text{Fe(CN)}_6 \). The sample membranes are impermeable to some of these reagents (e.g. \( \text{(NH}_4\text{)}_2\text{S}_2\text{O}_8 \)) which may thus be used for topological studies. The relative rates of electron transfer into and out of each component of the respiratory chains present will affect the size and duration of the steady states observed. If the respiratory systems in the resuspended membranes are intact transfer of electrons into electron transport chains from a biological substrate through a dehydrogenase will lead to reduction of only those cytochromes functionally linked to the dehydrogenase. This topic of functional linkage between cytochromes required clarification at the time of these studies, since it had been proposed that natural quinol analogues in the membranes of \textit{Escherichia coli} (ubiquinol-8, menaquinol-8) would create an equilibrium between all respiratory components at the potential generated by the biological reductant supplied, should an appropriate dehydrogenase be present. If the \( b \)-cytochrome components of the respiratory chains could be distinguished one might determine whether this was the case or whether restricted populations of cytochromes were preferentially reduced by specific substrates, while others of equivalent potential remained isolated: were the multiple respiratory chains of \textit{E. coli} functionally or structurally distinct or did electronic equilibration occur between them? Sequential addition of reductants and oxidants could be used to determine the amounts of cytochrome sensitive to each of these reagents after specific pretreatment, and respiratory inhibitors were available to provide blocks in respiratory
pathways enabling functional groups of cytochromes to be determined.

As implemented the method provided absorbance data at a specific wavelength relative to that at an isosbestic point. Consequently the variation in quantity of reduced cytochrome was determined over time as a proportion of the total absorbance change due to fully reduced cytochrome at the selected wavelength pair, although the identity of those cytochromes being reduced at any specific stage of the experiment was unknown. Steady state poising experiments were therefore linked with the procedure, as described under Materials & Methods, in order to obtain high resolution redox difference spectra from stages in the redox manipulation of cytochromes. These redox states were attained in the cryogenic sample holder at predetermined intervals after reagent addition.

(b) Membrane cytochrome analysis

When using an analytical procedure with so many variables meaningful interpretation of the results requires initial simplification of the system in order that the individual components may be recognised and distinguished. To this end cyd' strains were used for dual wavelength studies of the effect on the total cytochrome content of membranes from cells grown on different carbon/energy sources when succinate is added to the suspension (Fig. 32). Following an initial steady state of cytochrome reduction there was a rapid and a subsequent slow phase of dynamic cytochrome reduction, neither of which exhibited a first order rate. The changes in the rate of cytochrome reduction in each of these traces confirmed the results from other analyses that there were multiple respiratory type-6 cytochromes present in cells grown under conditions causing them to channel electrons from a single substrate, chosen from several carbon/energy sources tested, to the cytochrome o terminal oxidase. With all four membrane preparations resuspended at an equivalent protein concentration the total amounts of type-6 cytochrome in these variously-grown cells are seen to be different, the concentrations achieved, expressed as nmol (mg membrane protein)$^{-1}$, being approximately 0.17 (glucose), 0.20 (succinate), 0.20 (DL-lactate) and 0.30 (L-proline) [49,97].

The absorbance change attained at the initial steady state is indicative of the proportion of total cytochrome reduced, which itself is dependent upon the relative rates of electron influx and efflux through individual respiratory cytochromes as the substrate is oxidized and electrons transferred to dioxygen. Since the reductant used to create each trace in Figure 32 was disodium succinate the steady state absorbance reflects the dynamic reduction level of b-cytochromes associated with succinate oxidase activity. This level of steady state reduction varies considerably between the various
Fig. 32: Reduction kinetics of membrane cytochromes from cyd⁻ cells grown aerobically to stationary phase on various carbon/energy sources.

Washed membranes were prepared from cells of cyd⁻ strain GR19N grown aerobically to stationary phase on CYD minimal medium supplemented with the carbon/energy sources indicated below. Dual wavelength analyses were carried out upon membranes resuspended in 100 mM potassium phosphate buffer, pH 7.0 at 4.0 mg mL⁻¹, as described under Materials & Methods, sections 1+n.

Substrates were added to the final concentrations shown as indicated: S, disodium succinate (5.0 mM); N, NADH (0.8 mM); D, sodium dithionite (A slight excess, freshly saturated in buffer solution to provide complete reduction of sample). Maximal succinate reduction was obtained over the time course indicated with membranes from cells grown on DL-lactate or L-proline: it required considerably longer than 30 min in the other cases and the level of reduction achieved is indicated by the pointer $S_m$.

1. DL-lactate (sodium),
2. succinate (disodium),
3. L-proline,
4. D-glucose.
preparations: 49.4% (glucose), 40.0% (succinate), 44.7% (DL-lactate) and 3.0% (L-proline) indicating that the rate of electron flow through respiratory cytochromes from this substrate is markedly different in the proline grown cells. Among parameters affecting the steady state reduction level are the succinate dehydrogenase activity (supplying electrons to the respiratory chain), susceptibility of respiratory cytochromes to reduction by succinate (relative activities of succinate dehydrogenase and ‘succinate oxidase’), and the ability of other respiratory cytochromes to withdraw electrons from those components of the succinate oxidase chain (‘ancillary oxidase activities’, secondary cytochrome pools). The duration of the initial, aerobic steady state demonstrates how rapidly oxygen dissolved in the preparation is consumed and thus measures the efficiency of electron transfer through the available pathways from succinate to dioxygen. Oxidase activities expressed by these preparations were, in nmol min\(^{-1}\) (mg protein\(^{-1}\)), 0.022 (glucose), 0.063 (succinate), 0.137 (DL-lactate) and 0.032 (L-proline). The total amount of type-\(b\) cytochrome that was reduced by succinate was 80.2% (glucose), 93.6% (succinate), 92.6% (DL-lactate) and 73.2% (L-proline), reflecting the results of potentiometric titrations which demonstrated that cells grown on glucose or proline had greater quantities of low potential cytochromes than those grown on lactate (Table IX, Fig. 33), the mid-point potential for the succinate/fumarate redox couple being +31 mV \(\text{[145]}\). These succinate oxidase activities were competitively inhibited by malonate and oxaloacetate (data not shown).

Potentiometric titrations illustrate the inherent electrochemical properties of all the type-\(b\) cytochromes present in a sample by progressively reducing each cytochrome with all components kept in electronic equilibrium by the added mediators. In contrast the absorbance changes observed in dual wavelength kinetic studies are the result of dynamic processes observed in progress and may be caused by partial reduction of several cytochromes simultaneously. Thus even when comparative data was available for the action of certain reductants upon membranes derived from cells grown under different nutritional conditions it was still not possible to identify the redox status of specific cytochrome species. Further simplification of the cytochrome complement of sample membranes was one response to this dilemma, using the putative cyb\(^{\text{c}}\) strains that had been generated in order to identify kinetic abnormalities which could be related to lesions affecting cytochromes and identified from spectral and potentiometric studies, as described elsewhere and discussed in the following section \(\text{[214]}\).
Fig. 33: Potentiometric titrations of membrane preparations from cyd<sup>−</sup> cells grown to stationary phase on L-proline, D-glucose or DL-lactate.

Washed membranes were prepared from cells of cyd<sup>−</sup> strain GR19N grown aerobically to stationary phase on CYD minimal medium supplemented with L-proline, D-glucose or DL-lactate as indicated below. Error bars show the standard deviation of mid-point potential and percentage contribution to total type-b cytochrome, in ‘n’ sample preparations, each cytochrome b being resolved by theoretical fits of the data by curves with the stated number of components.

a. D-glucose supplement,  n = 2,  three component fit.

b. DL-lactate supplement,  n = 3,  four component fit.

c. L-proline supplement,  n = 3,  four component fit.

d. L-proline +Mo/Se supplements,  n = 3,  four component fit.

e. L-proline ±Mo/Se supplements,  n = 1,  five component fit, (see Materials & Methods):
   solid bars,  + Mo/Se supplement,
   clear bars,  - Mo/Se supplement.
{B) Functional Pools of Respiratory Cytochrome

(i) Respiratory Cytochromes of Cells Grown Aerobically on L-Proline

(a) Overview of Investigative Procedure

The combined experimental approach to the interpretation of $b$-cytochrome reduction in dual wavelength kinetic experiments required concentration upon the kinetic responses of membranes from a standard source, coupled with the analytical techniques described above, plus that of steady state poising for high resolution redox difference spectrophotometry. In the course of these investigations using a variety of reductants, oxidants and respiratory inhibitors, the addition of electrochemical mediators such as PMS to the reaction mixture enabled electrons to be distributed more rapidly to the multiple cytochromes present. This brought into question whether certain cytochromes are sequestered in distinct pools within the membrane with substantially slower electron transfer between cytochromes in different pools and, if so, how the cytochrome pools remain distinct in the presence of the high concentrations of natural quinols found in these membranes \cite{115,214}.

The aerobic growth conditions provided for the cyd$^{-}$ strains which were used for this part of the investigation incorporated the defined medium MMP using L-proline as carbon/energy source plus organic and inorganic supplements as described under Materials & Methods. The cyd$^{-}$ character of the bacterial strains ensured that the cytochrome $o$ complex was the only terminal oxidase present, but the supplemented medium, which included selenium and molybdate, permitted the synthesis of several medium to low potential respiratory cytochromes $b$, as illustrated in Figure 33. The mechanisms and restrictions upon electron transfer between these cytochromes and their organization into functional respiratory chains when present in the same membrane required elucidation. Although shortly before this study the laboratories of both Gennis and Kaback had constructed functional oxidase models with proteoliposomes containing only three constituent proteins — a dehydrogenase, a quinol and a terminal oxidase — the current data had shown that the assembly of respiratory chains in a natural membrane was far more complex \cite{104,126,128}. 
(b) Titration of Membranes from L-Proline Grown Cells

Potentiometric titration measurements of membrane samples from \textit{cyd}^+ strain GR19N had indicated that this technique was capable of resolving a minimum of four cytochrome components clearly \textit{(e.g.} Fig. 30, 33). When Se and Mo supplements had been added to the growth medium the proportion of cytochrome with mid-point potential of -30 mV was increased at the expense of a cytochrome with potential of about +250 mV and a lowering of the contribution of another cytochrome with $E_{h} = +50$ mV \textit{(Fig. 33).} Selenium and molybdenum are known to act as cofactor constituents in certain respiratory components such as formate dehydrogenase and nitrate reductase and to affect their expression \textit{(18, 66, 87).} Five components were resolved in several titrations, apparently the result of a marginal divergence of two components of the major contributor such that they became distinguishable by the analytical software \textit{(see Materials \\& Methods re limitations of the technique).} For comparison with Figure 33, Figure 30 shows data for the same strain grown on the minimal CYD medium with carbon/energy source of either glucose or DL-lactate.

(c) Redox Difference Spectrophotometry of Membranes from L-Proline Grown Cells

Reduced \textit{minus} oxidized difference spectrophotometry at 77 K revealed that in addition to those cytochromes absorbing in the region of the \textit{\alpha}-band from 560 nm to 565 nm and associated with cytochrome \textit{o} a broad absorption peak was present in the \textit{\alpha}-band at 556 nm \textit{(Fig. 34).} Fourth order derivatives indicated that multiple cytochromes generated the latter peak \textit{(Fig. 34,b,1'),} undamped analyses resolving three components with absorption maxima at 554 nm to 555 nm, 555 nm to 556 nm and 556 nm to 557 nm \textit{(data not shown).} The minor variations observed in these components’ maximal wavelengths was attributed to the superimposition of their absorption peaks. The presence of cytochrome \textit{o} was confirmed by reduced plus carbon monoxide \textit{minus} reduced spectroscopy \textit{(data not shown).}

(d) Kinetics of Cytochrome Reduction in Membranes from L-Proline Grown Cells

These kinetic data have been fully described and presented elsewhere as part of a collaborative investigation \textit{(214).} They are reviewed briefly in the following paragraphs.

Dual wavelength kinetic studies showed that with the addition of any one of a variety of
Fig. 34: High resolution redox difference spectra of membrane preparations from cyd<sup>−</sup> cells grown to stationary phase on L-proline.

Washed membranes were prepared from cyd<sup>−</sup> strain GR19N after aerobic growth to stationary phase in CYD minimal medium in the presence of L-proline. The washed membranes were resuspended and spectrophotometry was performed at 77 K at final concentrations of 100 mM potassium phosphate buffer, 40 mM Tris-HCl buffer, 1.0 mM MgCl, 1.0 M sucrose, pH 7.0, at a protein concentration of 2.0 mg mL<sup>−1</sup>.

a. Reduced minus oxidized difference spectra, ∆A = 0.01.
   1. Dithionite reduced minus peroxide oxidized difference spectra.
   2. Duraquinol reduced minus peroxide oxidized difference spectra.
   3. Spectrum #1 minus spectrum #2.

b. Fourth order finite difference spectra calculated from the corresponding redox difference spectra in panel ‘a’.
biological substrates two general features could be observed in the progress of overall cytochrome reduction: these were a rapid initial phase of reduction followed by a much slower secondary phase as in Figure 32. Neither of these two phases was found to be homogeneous upon inspection of the dual wavelength traces, discontinuities being found in the curves depicting the absorbance changes resulting from net cytochrome reduction in each phase.

The rapidity with which the 'fast phase' cytochromes were reduced precluded more detailed kinetic analysis of this phase of reduction, although by using duroquinol as reductant, with its relatively high redox potential and consequently lower reaction rate the non-uniform nature of the fast phase of reduction was more clearly demonstrated.

Low temperature spectrophotometric data collected from samples poised at the end of the 'fast phase' of reduction displayed a complex spectrum which included the features of the $\alpha$-band spectrum between 560 nm and 565 nm associated with the cytochrome $\alpha$ complex as well as a broad peak at 554 nm to 557 nm. The latter peak was shown to comprise at least two components by minor variations in its profile following reduction with different substrates and by fourth order derivative analyses. Assays of specific dehydrogenase activities in these membrane preparations, combined with spectral and potentiometric data suggested the identity of certain cytochromes that might be contributing to the lower wavelength $\alpha$-band of the fast phase components. At the time these experiments were completed both formate dehydrogenase and succinate dehydrogenase were thought to incorporate a cytochrome $b$, the properties of each being: formate dehydrogenase, $\lambda_{max} = 555 \text{ nm}$, $E_m = -100 \text{ mV}$ (66, 68) and succinate dehydrogenase, $\lambda_{max} = 556 \text{ nm}$, $E_m = -45 \text{ mV}$ in detergent solubilized form (102, 144). Identification of cytochromes associated with formate and succinate dehydrogenase activities progressed with contributions from several laboratories during the course of these studies. These topics are dealt with in detail in sections III.A.1.b, III.A.v.

The observation that the initial steady state level of reduction is generally below 15% of the total cytochrome reduced during the rapid reduction phase suggests that the oxidation step of the terminal oxidase by dioxygen was not the rate limiting step in the reduction of the respiratory chain by the substrates tested. Thus the cytochrome $\alpha$ complex would be transferring electrons to dissolved dioxygen more rapidly than the substrates could provide them to the dehydrogenases. Addition to the membranes of formate, the substrate having the lowest redox potential of those used, illicited greater reduction of cytochromes during the initial aerobic steady state than was observed with other substrates. Transition of the sample to anaerobic conditions was then marked by the rapid reduction of the 'fast phase' cytochromes. Because of the probable inclusion of type-$b$ cytochromes associated with formate dehydrogenase and succinate dehydrogenase in the fast phase group, and their...
α-band absorption maxima approximating 556 nm, the ‘respiratory cytochrome $b_{556}$’ which has been linked with one or both aerobic respiratory chains by many workers could not be recognised [4, 87, 95, 112, 117, 118].

Reduction of samples by duroquinol was found to reduce approximately half of the total membrane cytochrome and spectrophotometric results showed that the cytochrome reduced by the duroquinol ‘fast phase’ largely comprised those of the cytochrome $o$ complex. The aerobic steady state of reduction achieved with duroquinol was about 25% of the total cytochrome reducible with this high potential quinol analogue suggesting that it was donating electrons more directly to the cytochrome $o$ complex than the other, lower potential substrates and that it was incapable of reducing any other cytochromes, which appear to have potentials less than that of cytochrome $o$ [87, 112, 157].

If PMS, an electrochemical mediator, was added to the samples before the substrate a substantial increase in the reduction rate of the ‘slow phase’ cytochromes resulted, although the final level of reduction achieved was similar in the presence or absence of PMS. Slow phase reduction initiated by substrates in the presence of PMS was insensitive to cyanide inhibition except for that brought about by ascorbate. The latter reductant presumably donated electrons to the slow phase cytochromes sufficiently slowly that inhibition of cytochrome $o$ by cyanide brought about a rapid reduction of intermediate cytochromes. The other reductants appeared capable of reducing these intermediate cytochromes more rapidly than they could be reoxidized by cytochrome $o$ with the result that cyanide inhibition of the terminal oxidase had no apparent effect on cytochrome reduction in the presence or absence of PMS. Steady state poised spectrophotometry of the slow phase cytochromes at 77 K, obtained by subtracting a spectrum from a sample poised during the preceding fast phase, produced a redox difference spectrum with a broad α-band peaking at 556.5 nm. Although all of this slow phase cytochrome was oxidizable by ferricyanide ions only about half of this cytochrome pool was susceptible to oxidation by fumarate.

The suggested interpretation of these results is that biological substrates rapidly reduce a pool of cytochromes comprising significant proportions of the cytochromes closely associated with the appropriate dehydrogenases and those closely associated with the terminal oxidase cytochrome $o$. The reaction of dissolved dioxygen with the cytochrome $o$ is faster than the rate of reduction of the terminal oxidase resulting in the formation of a steady state in which only the cytochromes of the dehydrogenases are partially reduced while oxygen is available. The rate limiting step in the reactions occurring during this steady state is therefore between electron transfer from the dehydrogenase cytochrome and to the subsequent cytochrome in the pathway, suggesting that it may involve the
quinone components of the respiratory chain. The two procaryotic quinones ubiquinone-8 and menaquinone-8 are capable of reduction by the dehydrogenases, or directly through the action of PMS if this mediator is present. When the oxygen supply in the assay cuvette is exhausted the ‘fast phase’ reactions proceed: reoxidation of cytochrome $o$ ceases and the oxidase complex is rapidly reduced, as are the other components of the ‘direct’ electron transport pathway from the dehydrogenases. Reduction of the remaining cytochromes then occurs at a distinctly slower rate: menaquinone-8 is capable of transferring electrons to cytochromes associated with fumarate reductase activity and another component of this ‘slow phase’ cytochrome pool, reoxidizable by ferricyanide, appears to be reduced by either of the two quinones.

It thus appears that the majority of type- $b$ cytochrome species existing in the cell membrane are, indeed, capable of reduction or oxidation by the excess quantities of quinones that are present [95, 115]. However, cytochromes associated closely with fumarate reductase activity are not reduced by ubiquinol-8, but are reduced by menaquinol-8 [31]. Moreover, of those cytochromes capable of reduction by either quinol some are reduced at a far greater rate, leading to the observation of the fast and slow reduction phases, indicating a functional separation of the respiratory cytochromes of these membranes [214]. The moderately paced reduction of the cytochrome $o$ complex by exogenously added duroquinol supports the interpretation that whereas the natural complement of membrane quinols provides a means by which electrons may be distributed between cytochromes — and possibly other respiratory chain components — this distributary effect is not only far slower than the rates of reduction of certain cytochromes by other mechanisms but is also dependent upon the electrochemical potential of the reductant for determination of which cytochromes are susceptible to reduction.

Significantly the ‘fast phase’ cytochromes include those of the cytochrome $o$ complex, irrespective of the substrate used to reduce the sample. Since the respiratory chain linking dehydrogenases with cytochrome $o$ is known to contain ubiquinone-8 as a component (the participation of menaquinone-8 has been disputed [87, 95, 197]) this quinone at least is shown to interact preferentially with these groups of cytochromes [127, 197].

A number of experiments exploiting the distinct properties of the two terminal oxidases of the aerobic respiratory chains were able to be carried out on resuspended membranes without interference from other cytochromes. This work is described in sections II.C.i+ii below.
Apart from these two 'special cases', it was apparent that because of the complexities resulting from the multiple respiratory chains present in these membrane vesicles the foregoing identification of separate pools of cytochrome within the cell membrane was as far as the available technologies would be able to take the investigation and characterization of cytochromes in that environment. Consequently further studies concentrated upon the fractionation and characterization of individual cytochromes following detergent solubilization: they are presented in section III.
(C) **Aerobic Terminal Oxidases**

(i) **Membrane Studies of Cytochrome d**

(a) **Spectroscopic Properties of Cytochrome d Studied in Wild-type Cell Membranes**

The cytochrome d complex contains two cytochromes with α-bands well separated from any others detected by redox difference spectrophotometry of aerobically grown cell membranes. That of cytochrome b595 is of minimal analytical utility because of the small extinction coefficient of this cytochrome which contains high-spin haem iron. Although it has not been isolated from the other components of the cytochrome d complex the reduced minus oxidized difference spectrum proposed for it is similar to that of cytochrome c peroxidase, with a relatively large β-band overlapping the absorption wavelengths of the majority of type-b cytochromes which contain low spin haem iron (118). The complicated α-band absorption pattern of cytochrome d itself exhibits distinct features caused by both reduced and oxidized states of the molecule.

Figures 16 + 18 show spectra from membranes containing the cytochrome d terminal oxidase. The former illustrates absolute reduced and absolute oxidized spectra collected at ambient temperature plus the redox difference spectrum derived from them. Figure 18 displays high resolution reduced minus oxidized difference spectra of membrane preparations collected at 77 K: a comparison is provided showing spectral features in the presence and absence of cytochrome d. R. B. Gennis has described many of the properties of the cytochrome d complex and its constituent cytochromes in work published during the course of the current investigations {63, 105, 118, 119, 135, 145, 205}. The reduced and oxidized forms of the cytochrome exhibit broad α-band absorption maxima at 628 nm and 650 nm respectively. Thus in reduced minus oxidized difference spectra a combined peak plus trough are observed, these two features overlapping to some extent: interestingly, as mentioned earlier, no peaks are observed in the cytochrome d α-band region upon subjecting the difference spectrum to fourth order derivatization. It was observed that redox difference spectra of samples measured against a peroxide oxidized reference displayed an additional minor trough at approximately 680 nm, appearing as a broad extension of the oxidized trough and visible in Figure 18. Samples measured against a ferricyanide oxidized reference did not always produce this extra
detail, the result being dependent upon the strain of cells from which the membranes had been prepared and also on the preparation method. The 680 nm shoulder was lost if the reference had been fully reduced with dithionite or NADH before adding ferricyanide as oxidant, suggesting that the endogenous potential of the sample and its effect upon the redox state of cytochrome $d$ within the resuspended membranes might be responsible for the variations observed.

Digitization of the output signal from the Perkin-Elmer 356 spectrophotometer through an interface to independent processors circumvented absorbance anomalies during spectral scanning caused by automatic filter changes at 620 nm and 690 nm, for it enabled baseline subtractions and accurate spectral analysis of the complex cytochrome $d$ $\alpha$-band region could be performed on stored spectra. Figure 35 illustrates the $\alpha$- and $\beta$-band regions of reduced minus oxidized difference spectra of membranes containing the cytochrome $d$ complex, obtained with hydrogen peroxide or ferricyanide oxidized references and measured at both 295 K and 77 K. At 77 K the wavelengths of the absorption maximum and minimum were 626 nm and 648 nm respectively. At 295 K both values were increased by 4 nm. The overlap between a peak and trough of similar amplitude accounts for the minor variation in values for the wavelengths of maximum and minimum absorption of reduced minus oxidized difference spectra that are observed and which are also found in the literature (87, 157). Different absolute concentrations of the chromophore would only provide constant wavelength values if the two overlapping features were mutual mirror images: the possibility of more than two forms of the cytochrome being present complicates the situation further (161, 167, 168, 169).

The difference between the spectra resulting from these two methods of oxidizing the reference are also shown in Figure 35. Each secondary difference spectrum demonstrates that although the most noticeable feature of the original reduced minus oxidized difference spectra is the trough at 680 nm observed with an H$_2$O$_2$ oxidized reference and not with a ferricyanide oxidized reference, another major feature is the increased absorbance at 644 nm occurring with the ferricyanide oxidized reference at 77 K (646 nm at 295 K). Absolute spectra of endogenous preparations and those treated with either H$_2$O$_2$ or (NH$_4$)$_2$S$_2$O$_8$ displayed the high wavelength trough whereas samples which had been reduced and then reoxidized with [Fe(CN)$_6$]$^{3-}$ did not (data not shown). It was proposed that the 680 nm shoulder was associated with an oxygenated form of the cytochrome $d$ terminal oxidase rather than an oxidized form, and this supposition has subsequently been confirmed by recent work from the Gennis laboratory (105, 118). After purifying the complex, this research group also derived cytochrome $d$ extinction coefficients of 7.4 mM$^{-1}$ cm$^{-1}$ for the wavelength pair (628 - 607) nm (118). These wavelengths relate the absorption maximum of the reduced form
Fig. 35: Membranes containing the cytochrome $d$ complex: redox difference spectra after treating the reference material with either oxygenating and/or oxidizing agents.

Washed membranes were prepared from cells of $w^+$ strain ML308-225 grown aerobically to stationary phase on complex medium. Dithionite reduced minus oxidized difference spectrophotometry was performed at the temperatures indicated after dilution of the samples to final concentrations of 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0, at a protein concentration of 10 mg mL$^{-1}$. The reference materials were treated with the reagent specified below.

**a.** Temperature 295 K, $\Delta A = 0.05,$
1. reference treatment = $[\text{Fe(CN)}_6]^{3-},$
2. reference treatment = $\text{H}_2\text{O}_2,$
3. curve #2 minus curve #1.

**b.** Temperature 77 K, $\Delta A = 0.05,$
1. reference treatment = $[\text{Fe(CN)}_6]^{3-},$
2. reference treatment = $\text{H}_2\text{O}_2,$
3. curve #2 minus curve #1.
to that at an isosbestic point, thereby allowing estimation from absolute or difference spectra and avoiding the requirement of knowing whether or not a sample was binding dioxygen. The nature of the 77 K reduced minus oxidized absorbance peaks at 626 nm and 630 nm is described below in the light of data from poised potential high resolution spectrophotometry (section II.C.i.e).

The Soret region of the redox difference spectrum of the cytochrome \( d \) complex displays a peak overlapping that of the type-\( b \) cytochromes (Figure 16). Although the purified form of the two subunit cytochrome \( d \) complex contains cytochrome \( b_{595} \) attempts at isolation of the three cytochrome components from the intact complex have yielded only cytochromes \( b_{558} \) and \( d \) \[62, 105\]. Spectral deconvolution studies of the constituents of the complex have been reported yet reconstructed spectra of the Soret region of the individual components have not been published \[105, 118\]. Thus the Soret shoulder cannot be attributed unambiguously to either cytochrome \( b_{595} \) or to cytochrome \( d \) at present, although the correct assignation of this spectral feature is of relevance to interpretation of ligand binding data and the understanding of component function (sections II.C.i,b+c).

(b) **Spectroscopic Investigation of Cytochrome \( d \) Ligand Binding**

Perturbation of the cytochrome \( d \) visible absorption spectrum by dioxygen reflects the physiological action of the terminal oxidase but several alternative ligands also cause alterations in its spectra as shown in Figure 22. Because of the width and proximity of the absorbance maxima of the reduced and oxidized forms of the terminal oxidase the spectral shifts are more clearly seen in absolute spectra than in redox difference spectra.

High resolution 'absolute reduced' spectra of membrane preparations containing cytochrome \( d \) which have been collected in the presence of a series of respiratory inhibitors with structural similarities to dioxygen suggest that the interactions between the terminal oxidase and the ligands tested may be complex since a variety of cytochrome \( d \) \( \alpha \)-band responses to the ligands are observed (Fig. 22a). For reference curve 9 shows the oxygenated form observed in 'endogenous' membranes and having absorption maxima at 648 nm and 680 nm; curve 5 illustrates the dithionite reduced form with a single peak at 628 nm.

There is a red shift of the reduced cytochrome \( d \) \( \alpha \)-band to 638 nm upon treatment with carbon monoxide, the absorption at this higher wavelength being somewhat less than the original absorbance at 626 nm. The modified absorption maximum of the cytochrome \( d \) spectrum is
maintained after extensive illumination at low temperature but the absorbance values at both 556 nm and 638 nm are restored to the values of the original maxima (Fig. 22a; 3,4). The reactions of the cytochrome d complex with carbon monoxide are described in greater detail below (section II.C.i.c).

Nitrite and nitrate ions both produce a greater red shift in the reduced absorption maximum than CO, although the peak width extends dramatically, and close inspection of curves 1 and 2 indicates that twin peaks may be present in each case, at 638 nm and at 643 nm. It is possible that two alternative bound states are permitted with each of these latter ligands, or the result may be caused by partial turnover of the ligand binding reaction. These results support the conclusions of Poole’s laboratory in suggesting that several oxides of nitrogen are able to react with cytochrome d and shift the α-band absorption spectrum of the reduced form to longer wavelengths [85]. However, earlier workers had noted several concentration-dependent inhibitory effects of silver ions upon respiration in similar membrane preparations to which AgNO₃ had been added, including spectral alterations of cytochrome d, at that time termed cytochrome a₂ [20]. While the lower concentration effects may have been caused by the silver cations themselves the apparent decrease of absorbance of the cytochrome d reduced minus oxidized α-band absorption that was observed at the higher concentrations tested can be explained by the interaction of cytochrome d with the nitrate anion which would cause decreased absorbance in the difference spectrum because of greater overlap between the reduced peak and oxidized trough [20, 85]. This may explain the ‘discrepancy’ stated to exist between reports from the two laboratories [87].

Although an explanation of the preceding results do not require the postulation of an intermediate form of cytochrome d lacking absorbance between the wavelengths of 610 nm and 670 nm, other evidence for such a species, relating to the transition between the reduced and oxidized or oxygenated states, has been presented [20, 168, 169]. The interpretation of this data has been controversial, however, with the identity of the ‘invisible’ form as either the oxygenated or the oxidized state of the cytochrome being disputed [20, 161, 167, 168, 169]. In the current study a decrease in the α-band absorption of cytochrome d was the major spectroscopic effect when potassium cyanide reacted with the reduced form of this cytochrome although reaction of reduced cytochrome d with either cyanide or azide caused a slight blue shift to 621 nm in the α-band of the absolute reduced absorption spectrum (Fig. 20a; 6,7). Thus in the presence of cyanide there may be an analogue of an intermediate functional form of cytochrome d generated which possesses minimal α-band absorptive properties [168, 169]. Reaction of the cytochrome d complex with either cyanide or azide also caused an apparent decrease in the absorbance of the cytochrome b₅₅₈.
α-band absorption raising the possibility that interaction with the terminal oxidase might be indirect and mediated through the type-b cytochrome. Nevertheless, because of structural similarities between dioxygen and these ions, and their known ability to act as ligands with other terminal oxidases it is probable that the reverse is true: that they react with cytochrome $d$, subunit II of the cytochrome $d$ complex, in a distinctly different manner to that of the previously discussed ligands, and that there is an allostERIC effect upon subunit I of the complex which comprises cytochrome $b_{558}$ \[62\]. Owing to the substantial overlap of the type-$b$ cytochrome α-bands no attempt was made to quantify these spectral effects upon cytochrome $b_{558}$. Other studies have indicated that cyanide will react slowly with the oxygenated or oxidized form of cytochrome $d$, there being some dispute over the spectral characteristics of these two forms \[161,168,169\].

The reduced form of glutathione was known to inactivate certain cytochromes. It reacts with the cytochrome $b^{\text{AT}}$ component of the nitrate reductase of *Escherichia coli* in which treatment with 1.0 mM GSH not only prevents autoxidation but also largely abolishes the absorbance changes normally observed upon reduction of a ferricyanide oxidized sample with dithionite \[28\]. Thus redox difference spectra performed in buffers containing GSH display minimal absorption changes, suggesting that the cytochrome $b^{\text{AT}}$ is no longer capable of participating in electron transfer reactions (data not shown). Presumably one or more critical disulphide linkages is reduced by the GSH treatment resulting in a conformational change within cytochrome $b^{\text{AT}}$ which prevents the haem iron changing its oxidation state. Mammalian mitochondrial cytochrome $c$ provides another of several examples of cytochromes undergoing glutathione modification and it has been suggested that the protohaem transfer protein donating haem to cytochrome $b_5$ in rat liver is a glutathione S-transferase \[50,184,190\].

Thus the effects of reduced glutathione upon cytochrome $d$ that are illustrated in Figure 22a,8 need not be the result of this cytochrome’s reactivity with dioxygen: as a terminal oxidase the cytochrome $d$ in *E. coli* membrane preparations may interact with glutathione because of greater exposure of susceptible haem than occurs in many other cytochromes or it may undergo conformational change after reduction of specific disulphide bonds within the apoprotein. It is of interest that the alteration of the absolute spectrum to generate a broad peak resulting from reduction of cytochrome $d$ in the presence of GSH is not observed if glutathione is added to the reduced form of the cytochrome. Additionally the broad peak shown in curve 8 appears to comprise two components with maxima at approximately 640 nm and at 645 nm, extremely similar to the effect on the reduced terminal oxidase by nitrate or nitrite. This may indicate that reduced glutathione interacts with the haem iron to form a ligand adduct rather than effecting reduction of a conformationally
critical disulphide link.

(e) Photochemical Degradation of the Cytochrome $d$—Carbon Monoxide Complex

Further analysis of the interaction between carbon monoxide and cytochrome $d$ in membrane preparations entailed high resolution spectrophotometry at 77K and an investigation of the photodissociation reaction between the ligand and the cytochrome complex which incorporated trapping of intermediates at several temperatures from 77 K to 273 K. The resulting shifts in size and position of absorbance peaks and consequent overlapping of absorption maxima and minima created complicated spectral patterns: especially in the $\alpha$-absorption region of the cytochrome $d$ spectrum which is multiphasic even in the standard redox difference spectrum. Attempts to simplify the results by means of scanning dual wavelength spectrophotometry, yielding absolute 'reduced plus carbon monoxide' spectra versus an isosbestic reference wavelength of 575 nm are shown in Figure 36. The quantitative changes to the cytochrome $b_{558}$ and cytochrome $d$ '626 nm' absorption peaks caused by photodissociation of the carbon monoxide complex observed at ambient temperature (Figure 22) were shown to occur during illumination at liquid nitrogen temperatures, a dewar with an unsilvered window being used for this purpose (Fig. 36). The absorption increase was also observed after illumination with the sample poised at higher temperatures (198 K, 243 K, 261 K and 273 K) (data not shown). As was expected from the 295 K results (Figure 22) no significant alterations were observed in the wavelengths of absorption maxima at the low temperatures (Fig. 36). Thus two distinct phases of photodissociation are indicated. One is responsible for the restoration of full absorption of the $b$- and $d$-type cytochromes, which is able to take place at temperatures as low as 77 K, and a second appears to be required for the return of the cytochrome $d$ 'reduced' absorption peak to its pre-complexed position, 626 nm. This second step was not observed in these experiments and it may require either the complete removal of CO from the sample or the addition of dioxygen followed by rereduction [116, 163]. Background absorption and the signal quality of these single scan difference spectra indicated that comprehensive comparison and interpretation of such results would require the digitization, averaging and mathematical treatment of the analogue output from the spectrophotometer available at this time, as suggested by comparable experiments performed by R. K. Poole [163]. When this capability had been established the advances in analysing the cytochrome $d$ complex that were being published by the laboratory of R.B. Gennis had dictated a further change in the orientation of this investigation [76, 101, 104, 163].
**Fig. 36:** Scanning dual wavelength spectra of membranes containing the cytochrome $d$ complex following carbon monoxide treatment and low temperature photodissociation.

Spectra of the carbon monoxide complex of membrane cytochromes before and after exposure to visible illumination at 77 K and 273 K. Single samples were held at the temperatures indicated and scanned from 730 nm to 380 nm with the spectrophotometer in dual wavelength mode using 575 nm as the reference wavelength.

Washed membranes were prepared from wild-type strain GR17N grown aerobically to stationary phase on CYD minimal medium supplemented with DL-lactate. The membranes were resuspended at a protein concentration of 14.4 mg mL$^{-1}$ in 100 mM potassium phosphate buffer, pH 7.0, reduced with dithionite and gassed with carbon monoxide as described under Materials & Methods. All procedures were carried out in total darkness, including sample transfer and storage under liquid nitrogen during re-equilibration of the cryogenic apparatus at different temperatures. Sample illumination was achieved at the stated temperatures with a 150 W incandescent source at a distance of 5 cm directed through the unsilvered window of the sample dewar for 600 seconds, all detectable spectral changes being complete within this period.

**a.** Soret region, $\Delta A = 0.20$,

1. Baseline,

2a. 77 K : post illumination.  
3a. 273 K : post illumination.

2b. 77 K : pre-illumination.  
3b. 273 K : pre-illumination.

**b.** $\alpha$-band region, $\Delta A = 0.20$,

1. Baseline,

2a. 77 K : post illumination.  
3a. 273 K : post illumination.

2b. 77 K : pre-illumination.  
3b. 273 K : pre-illumination.
(d) **Potentiometric Titration of Components of the Cytochrome d Complex**

It is possible to perform simultaneous potentiometric titrations of each of the three types of cytochrome present in the cytochrome d complex because of their well-separated absorption bands in the 550 nm to 700 nm wavelength range. Of the standard mix of electrochemical mediators used in these titrations only 2,6-dichlorophenolindophenol ($E_m = +224$ mV) need be omitted in order to prevent spectroscopic interference between 600 nm and 700 nm. Data for potentiometric titrations of cytochromes b$_{595}$ and d in resuspended membrane preparations are shown in Figures 37+38. The titration curves for both cytochromes display pronounced differences between oxidative titrations performed with ferricyanide ion and those in which H$_2$O$_2$ or oxygen-saturated water was used for oxidation. The lack of hysteresis between oxidative titrations and subsequent reductive titrations in these experiments indicated that the removal of DCPIP from the titration buffer had no deleterious effect upon sample equilibration with the platinum combination electrode and that the different responses observed were effects of the oxidants. Absorption measurements of the cytochrome b$_{595}$ $\alpha$-band peak at ambient temperature ($\lambda_{max}=595$ nm) are reduced in accuracy by its small size, while those of the cytochrome d peak ($\lambda_{max}=626$ nm) and trough ($\lambda_{max}=650$ nm) are complicated by their partial overlap: the redox behaviour of the latter is more clearly observed at 662 nm (Fig. 38c).

The endogenous potential of these resuspended membrane preparations was approximately +250 mV. Reduction to +75 mV with NADH resulted in little change in the absorbance features of cytochrome d but the absorbance of the 595 nm peak approximately doubled (Fig. 37). Subsequent oxidation with ferricyanide restored the endogenous value of cytochrome b$_{595}$ absorbance whereas treatment with H$_2$O$_2$ or dissolved dioxygen completely abolished this absorption peak (Fig. 37). The response of the cytochrome d redox absorption peak and trough were different during the titrations as illustrated in Figure 38. NADH reduction of cytochrome d from its initial, preparatory state resulted in little absorbance change. Oxidation of the reduced cytochrome with ferricyanide caused a decrease in absorption of the redox absorbance peak at 626 nm, with a mid-point potential of +262 ($\pm5$) mV. The effect of ferricyanide oxidation on the redox absorbance trough, observed at 626 nm, was minimal, in agreement with the proposal that the oxidized form of cytochrome d has negligible absorbance and that the reference cell contained the oxygenated form which was absorbing at 626 nm to form the redox trough. Oxidation with H$_2$O$_2$ totally abolished the absorbance of both features of the cytochrome d $\alpha$-band as would be expected if both sample and reference were oxygenated. As the reference had been oxidized with ferricyanide this result supported the suggestion
**Fig. 37:** Potentiometric titrations of the cytochrome $b_{595}$ component of the cytochrome $d$ complex in resuspended membrane preparations.

Washed membranes were prepared from cells of wild-type strain HfrH grown aerobically to stationary phase on CYD minimal medium with glucose as carbon/energy source. These membranes were resuspended at a protein concentration of 15.0 mg mL$^{-1}$ in 100 mM potassium phosphate buffer, pH 7.0 to which were added all the standard electrochemical mediators except dichlorophenolindophenol, which would have interfered with the spectroscopic estimation of the cytochrome $d$ $\alpha$-bands. Contents of the reference cuvette were oxidized with ferricyanide.

**a.** Cytochrome $b_{595}$: proportion of reduced cytochrome estimated from $\Delta A$ at $\lambda_{\text{max}}^{\alpha}$. The small size of the cytochrome $b_{595}$ $\alpha$-band limited the accuracy with which measurements of absorption resulting from this component could be made.

- [ ] oxidation by ferricyanide and subsequent NADH reduction.
- [ ] oxidation by hydrogen peroxide and subsequent NADH reduction.

**b.** Cytochrome $d$: proportion of reduced cytochrome estimated from the $\alpha$-band peak-to-trough amplitude, $\Delta A$ (626 nm - 650 nm).

- [ ] oxidation by ferricyanide and subsequent NADH reduction.
- [ ] oxidation by hydrogen peroxide and subsequent NADH reduction.
- [ ] oxidation by oxygen-saturated water.
Reduced cytochrome $b_{595}$ (estimated % total)

Reduced cytochrome $d$ (% total)
Fig. 38: Potentiometric titrations of the cytochrome $d$ component of the cytochrome $d$ complex in resuspended membrane preparations.

Washed membranes were prepared from cells of wild-type strain HfrH grown aerobically to stationary phase on CYD minimal medium with glucose as carbon/energy source. These membranes were resuspended at a protein concentration of 15.0 mg mL$^{-1}$ in 100 mM potassium phosphate buffer, pH 7.0 to which were added all the standard electrochemical mediators except dichlorophenolindophenol, which would have interfered with the spectroscopic estimation of the cytochrome $d$ $\alpha$-bands. Contents of the reference cuvette were oxidized with ferricyanide.

**a.** Cytochrome $d$:

- proportion of reduced cytochrome estimated from the $\alpha$-band peak; $\lambda = 626$ nm.
- *• - • - •* oxidation by ferricyanide and subsequent NADH reduction,
- o-o-o oxidation by $H_2O_2$ or oxygen-saturated water and subsequent NADH reduction.

**b.** Cytochrome $d$:

- proportion of reduced cytochrome estimated from the $\alpha$-band trough; $\lambda = 650$ nm.
- • - • - • oxidation by ferricyanide and subsequent NADH reduction,
- o-o-o oxidation by $H_2O_2$ or oxygen-saturated water and subsequent NADH reduction.

**c.** Cytochrome $d$:

- proportion of reduced cytochrome estimated from the $\alpha$-band trough; $\lambda = 662$ nm.
- • - • - • oxidation by ferricyanide and subsequent NADH reduction,
- o-o-o oxidation by $H_2O_2$ or oxygen-saturated water and subsequent NADH reduction.
that cytochrome d retains oxygen as a stable 'oxy' form and must be fully reduced before it is released. Reduction following treatment with H\textsubscript{2}O\textsubscript{2} produced an extremely sharp appearance of the redox absorption features centred at +206 (±8) mV. The disappearance of the dual peak and trough upon oxidation with either H\textsubscript{2}O\textsubscript{2} or dissolved oxygen and their reappearance upon subsequent reduction both occurred over an extremely narrow potential range indicating that these responses were not caused by straightforward electron transfer reactions. The behaviour of the cytochrome b\textsubscript{595} was similar to that of the reduced peak of cytochrome d although its mid-point potential for redox transitions resulting from treatment with ferricyanide, H\textsubscript{2}O\textsubscript{2} or O\textsubscript{2} was approximately +150 (±15) mV.

This behaviour indicated that a metastable form of the complex was probably being formed that would change suddenly to the alternate redox form even in the presence of the broad range of redox mediators used in the titration experiments. Knowing that cytochrome d has a particularly low \( K_m \) for dioxygen and that in forming a ligand to the iron oxygen would affect both spectroscopic and potentiometric properties of the haem the simplest explanation for the spectral observations during the titrations is that ferricyanide ions oxidize the cytochrome d complex and oxygen and peroxide both create a distinct, stable, oxygenated form of the terminal oxidase \{105, 157, 216\}. This is supported by the spectrophotometric studies described above and studies of the interaction between cytochrome d and cyanide ions which indicated that an alternate form of the complex existed, other than oxidized or reduced, with minimal absorption of light in the haem-d \( \alpha \)-band region \{167, 168, 169\}.

That there was no spectrophotometric alteration of the 'oxidized trough' at 626 nm on treatment with reductant or ferricyanide suggests that oxidized cytochrome d may require oxygenation before being able to undergo the transition between fully oxidized and fully reduced states. As the spectrophotometric measurements of these titrations were performed using a reference preparation oxidized with ferricyanide, an explanation is required as to the lack of equivalence between sample and reference after treatment of the former with the same oxidant. The reference preparation would have had access in the reference cuvette to limited amounts of both oxygen and the endogenous substrates common to such membrane preparations. Consequently a certain amount of turnover of the various states of the terminal oxidase would have been possible in the presence of the ferricyanide added as oxidant. The sample preparation would have been unable to undergo any turnover since oxygen was removed by flushing the titration vessel with inert gas. As a result of its high mid-point potential cytochrome d would thus be trapped in partially reduced form. It is therefore suggested that there is not only an intermediate form of cytochrome d between the reduced and oxidized states, but that turnover of the terminal oxidase reaction is required for interconversion of these two states and that the
intermediate is associated with the oxygenated form of the oxidase complex. Comparison of Figures 38a and 38c shows that although this explanation fits the behaviour of the 650 nm 'oxidized' trough the 626 nm 'reduced' peak indicates that transition between the reduced state of the cytochrome and some form of the 'invisible' intermediate may be possible even in the absence of oxygen, for the reduced peak is lessened upon treatment of the sample with ferricyanide without a corresponding increase in the oxidized trough.

In more recent reports Poole has supported the existence of a third, 'invisible' form of cytochrome $d$ (although the assignation of spectral characteristics to the oxidized and oxygenated states has been disputed) \{157, 161\}. By comparative titrations of membrane preparations from $cyd^+$ and $cyd^-$ strains Gennis has determined the following $E_m$ values for the components of the cytochrome $d$ complex: cytochrome $d$, $E_m$ = +260 mV; cytochrome $b_{595}$, $E_m$ = +150 mV; cytochrome $b_{558}$, $E_m$ = +180 mV. It should be noted that the cytochrome $b_{558}$ values were obtained from analyses that assumed the presence of fewer type-$b$ cytochrome components than the current study indicates to have been warranted \{62\}. Moreover, cytochrome $d$ analysis was conducted by measuring the peak height at 628 nm which would yield 'partial' redox behaviour as illustrated in Figure 38a \{117\}. Gennis has also suggested the presence of a distinct 'oxy' or oxygenated state as an explanation for the lack of equilibration in potentiometric titrations of membranes containing the cytochrome $d$ complex that was noted by the laboratory of Hendler \{80, 105, 161\}. Rather than postulating complex electron transfer mechanisms to explain the previously observed behaviour in terms of direct transitions between reduced and oxidized states of the terminal oxidase Gennis and coworkers obtained straightforward single electron redox titration data for each of the components of the cytochrome $d$ complex after solubilizing and isolating it from cell membranes \{63, 105\}. They ensured that all traces of dioxygen had been removed from their sample and apparatus, incorporating a prolonged incubation of the sample at low potentials before initiating the titration procedure. In this way they anticipated that all dioxygen liganded to the complex would be removed and a uniform population of reduced sample molecules could then be titrated, instead of a mixed sample in which oxidized molecules had been reduced but oxygenated molecules displayed radically distinct redox behaviour. It is possible that in these experiments the mechanism of interconversion between redox states of the terminal oxidase was altered by detergent action for the mid-point potentials of the components of the cytochrome $d$ complex were found to be markedly dependent upon the solubilizing detergent as well as upon the pH of the titration buffer \{63, 105, 117, 119\}.

In a report published as the writing of this thesis was being completed, R. B. Gennis and coworkers have consolidated earlier findings and presented additional spectroscopic evidence to support
a proposal that the purified cytochrome \( d \) complex may exist in any of several stable states at room temperature including oxidized, oxygenated, peroxy and 'peroxy intermediate' forms \[116].

(e) High Resolution Redox Difference Spectrophotometry at Poised Potentials

Samples removed at specified potentials and poised at low temperature during the course of the titration could be used for high resolution, multiple scan difference spectrophotometry versus an oxidized reference at 77 K. Figure 28 indicates that at the highest potential tested the cytochrome \( d \) was largely reduced (85 % maximal peak to trough amplitude) whereas the type-\( b \) cytochrome \( \alpha \)-band is only partially reduced (34 % maximal peak height at 556 nm, 42 % maximal peak height at 558 nm). As described above (sections I.ii.e and II.C.i.d), the redox state of the cytochromes cannot be equated with that at a specific potential at ambient temperature but the sequence of spectra taken over a range of potentials confirms that the cytochrome \( d \) has a higher potential than the majority of type-\( b \) cytochromes and that the cytochrome \( b_{558} \) associated with the cytochrome \( d \) complex also has a higher potential than the bulk of the type-\( b \) cytochrome in the sample. Cytochrome \( b_{595} \) appears to be fully reduced in all of these spectra, although the small size of its \( \alpha \)-band absorption prevents accurate estimation.

As the potential of the sample is lowered to a nominal value of about -100 mV the cytochrome \( d \) \( \alpha \)-band spectrum alters profoundly. The ‘reduced’ peak appears to shift towards the red from 626 nm. This causes greater overlap between the reduced peak and oxidized trough, which while undergoing very little change in its wavelength of minimal absorption, is decreased in amplitude in consequence (Fig. 28). Reduction of cytochromes in membrane suspensions by the addition of dithionite prior to standard low temperature reduced minus oxidized difference spectrophotometry lowers the potential of the sample below that at which the wavelength of the cytochrome \( d \) reduced peak was seen to alter from the ‘typical’ 626 nm in these poised potential experiments. Consequently the observations may be the result of a structural change in cytochrome \( d \) resulting from electron transfer to a sensitive part of the molecule susceptible to interaction with the electrochemical mediators present in the titration buffer. Cytochrome \( b_{558} \) is now known to bear the functional quinol binding site of the complex yet the spectrum of this component of the oxidase did not appear to change \[115, 219\]. Alternatively the observation may be a consequence of tightly bound oxygen loss experienced during prolonged incubation at low potentials. Thus rapid freezing of a sample to 77 K soon after reduction with dithionite may not completely dissociate the oxygen bound to cytochrome \( d \). Thus the small red shift of the reduced minus oxidized peak observed at
low potentials in the poised potential redox difference spectra would be the result of total dissociation from oxygen \( \lambda_{\text{max}} = 629 \text{ nm at 77 K} \); Fig. 28) whereas the peak in typical redox difference spectra is caused by a complex retaining tightly bound oxygen \( \lambda_{\text{max}} = 626 \text{ nm at 77 K} \); Fig. 35). At ambient temperatures the spectral features are broader and any shift less pronounced so that it would be effectively hidden in reduced minus oxidized spectra even though treatment with dithionite would result in the slow removal of the tightly bound oxygen (Fig. 35). Thus there is a correlation with the results of oxidizing and oxygenating the contents of the reference cuvette as described above. Both sets of experiments show a similar red shift in the cytochrome \( d \) redox \( \alpha \)-band peak, yet in the poised potential spectra the sample is modified while the reference is held constant and the situation is reversed in those experiments testing the spectral effects of \([\text{Fe(CN)}_6]^3-\) and \( \text{H}_2\text{O}_2 \) on the reference material. An oxygenated absorption peak with a smaller extinction coefficient than that of the oxidized cytochrome, in addition to the formation of the red shoulder at 680 nm would account for the apparent shift of the 'reduced' peak in the redox difference spectra. However, demonstration of such a quantitative absorption difference was equivocal, possibly because of the broad nature of the absolute cytochrome \( d \) spectra following treatment with either ferricyanide or hydrogen peroxide.

If the proposal is correct, the loss of tightly bound oxygen from low potential samples in the poised potential experiments and the resulting conversion of those samples to the fully reduced state would cause a red shift in the sample spectrum, as is observed. Thus lowering the potential in those experiments would produce a transition from 'reduced plus oxygenated minus oxidized' to 'reduced minus oxidized' spectra. This may be compared to the variable reference experiments which illustrate the difference between 'reduced plus oxygenated minus oxidized' and 'reduced plus oxygenated minus oxidized plus oxygenated' spectra. Hence the similar result obtained from dissimilar operations.

(f) Control of Expression of the Cytochrome \( d \) Complex by Growth Substrate

Studies of the control mechanisms governing expression of the cytochrome \( d \) complex had been initiated in the host laboratory after it was determined that GR19N, the original cyd \( A1 \) strain which had been produced from GR17N by nitrosoguanidine mutagenesis, was a double mutant \( \{89, 60\} \). GR19N is unable to synthesize the complex under any growth conditions while the parent strain GR17N is capable of expressing it during growth on any poorly aerated medium \( \{60\} \). In strain PLJ01, a single-site revertant from GR19N, expression of the cytochrome \( d \) complex was
influenced by the availability of certain carbon-energy sources during growth on minimal media: induction of the complex was observed when cells were grown with glucose or glycerol, but not when the carbon-energy source was succinate, lactate, pyruvate, alanine or proline [89]. Isolation and characterization of strain PLJ01 was originally based upon this ability to synthesize functional cytochrome \( d \) complex only when grown on specific carbon/energy sources [89].

Other parameters governing the induction of the components of the respiratory chain terminating in the cytochrome \( d \) complex have been extensively investigated and well documented in the literature. A major factor is the availability of limiting concentrations of dioxygen, although other favourable conditions include growth into late exponential or stationary phase (often resulting in lowered oxygen tensions due to high cell population densities), certain anaerobic growth conditions, mutants in certain operons inducing anaerobic respiratory components, enzymes related to quinol metabolism or production of cyclic AMP or catabolite-gene activator protein and growth under sulphate-limiting conditions or in the presence of cyanide [87, 157, 172].

Cells of PLJ01 containing the F8 episome were capable of producing wild-type quantities of cytochrome \( d \) under all growth conditions tested. Therefore induction of the \( cyd \) operon located on this episome was not repressed by any host cell mechanisms for the control of \( cyd \) expression under those growth conditions in which PLJ01 normally failed to synthesize the cytochrome \( d \) complex [89]. The amount of cytochrome \( d \) produced in any culture is dependent on several criteria, particularly the degree of oxygenation of the medium, the rate of consumption of dissolved dioxygen by the culture and the duration of oxygen-limited growth. Consequently a factor of two is well within the limits of experimental accuracy for such estimations and further analyses of control in the presence of the single copy episome were not attempted.

Growth rates of the wild-type progenitor GR17N were similar to those of PLJ01 during the early phases of growth although the former maintained their exponential character for a longer period than similar cultures of PLJ01. At higher cell densities the latter cultures developed a linear rate of culture growth which was thought to be dependent upon the rate of solution of oxygen into the medium through the liquid surface since more vigorous agitation decreased their late-growth generation time (Fig. 39a). These results indicate that the dissimilarity in cytochrome \( d \) induction between the strains was not caused by an inability of PLJ01 to grow sufficiently rapidly to exhaust the supply of dioxygen in the culture; they were confirmed by culturing GR19N under equivalent conditions. In the present study it had been intended to carry out competitive growth experiments in the presence of dual substrates and to use the anticipated biphasic growth patterns (diauxie) to investigate whether there is an overall repressive mechanism of control or whether induction is a
Fig. 39: Aerobic growth curves of strain PLJ01: effects of increased aeration and dual carbon/energy sources.

Growth of cultures in CYD minimal medium was estimated from optical density measurements of appropriately diluted samples at a wavelength of 600 nm in a Perkin-Elmer model 124 double beam spectrophotometer. Cultures were of 750 mL volume in 2 L Erlenmeyer flasks and were aerated by rotation at the stated speeds as described under Materials & Methods.

a. DL-lactate supplemented medium, temperature = 37°C:
   1. + - + +  strain GR17N (ω+ phenotype), rotation speed = 300 rpm;
   2. o - o - o  strain PLJ01 (cdt+ phenotype), rotation speed = 300 rpm;
   3. • - • •  strain PLJ01 (cdt+ phenotype), rotation speed = 50 rpm.

b. i. Variably supplemented medium, temperature = 30°C, rotation speed = 300 rpm:
   1. o - o - o  strain PLJ01 (cdt+ phenotype), glucose plus succinate, each at 10 mM;
   2. • - • •  strain PLJ01 (cdt+ phenotype), glucose plus succinate, each at 5 mM;
   3. + - + +  strain PLJ01 (cdt+ phenotype), glucose at 5 mM.

ii. Variably supplemented medium, temperature = 30°C, rotation speed = 300 rpm:
   1. o - o - o  strain PLJ01 (cdt+ phenotype), glycerol plus succinate, each at 10 mM;
   2. • - • •  strain PLJ01 (cdt+ phenotype), glycerol plus succinate, each at 5 mM;
   3. + - + +  strain PLJ01 (cdt+ phenotype), glycerol at 10 mM.
specific response to certain molecular stimuli. Figure 39b shows that in the later stages of growth of PLJ01 in CYD minimal medium, during which the induction of the cytochrome \(d\) complex occurs under favourable conditions (56, 89, 172), the separate diauxie growth phases could not be distinguished clearly. This also suggests that under these conditions the carbon/energy source does not limit the growth rate. Therefore carbon/energy sources were supplied in the medium in pairs and cytochrome \(d\) was assayed by redox difference spectrophotometry. Table X shows that the overall control of expression of the cytochrome \(d\) complex is inductive and not repressive since substrates with which expression was not observed when used alone (e.g. lactate, succinate) did not prevent induction when used in combination with substrates permitting expression on their own (e.g. glucose, glycerol).

As noted in the earlier study the rationalization of the observed pattern of control by growth substrate is not simple (89). It does not appear to be advantageous for the organism to prevent expression of a potentially useful respiratory pathway in the presence of non-fermentable substrates and to induce it when fermentable carbon/energy sources are available and respiration is not obligatory. The phenomenon is probably linked to the complicated interactions of the multiple regulatory mechanisms alluded to above; one which may be particularly relevant being the possibility of a novel method of indirect control over synthesis of the cytochrome \(d\) complex by cyclic AMP or the catabolite-gene activator protein (34). Thus glucose (although not glycerol) might cause derepression of the genes coding for the terminal oxidase whereas the other ‘negative’ substrates would not have this ability.

Another interpretation of the data provides a functionally related explanation and is based upon the metabolic relationships of the various carbon/energy sources analysed. Aerobically grown *E. coli* catabolizes glucose primarily by glycolysis, the Embden-Meyerhof-Parnas pathway (64). Of the substrates tested with strain PLJ01 growing to stationary phase on minimal media, ‘glycolytic’ precursors of the reactions catalysed by 3-phosphoglyceraldehyde dehydrogenase and triose isomerase were found to be associated with the expression of the cytochrome \(d\) complex whereas products of pyruvate kinase activity associated with the tricarboxylic acid cycle did not enable the complex to be expressed. This observation suggested a possible correlation between the regulation of the expression of the cytochrome \(d\) complex and the well documented ‘Pasteur Effect’ in which the introduction of oxygen into an actively growing anaerobic culture causes a dramatic inhibition of both glycolysis and lactate production with a simultaneous induction of respiratory activity (36, 64). The high affinity for dioxygen of cytochrome \(d\) and its expression under low oxygen tensions make it an appropriate terminal oxidase for such conditions. Although
<table>
<thead>
<tr>
<th>C/E Source</th>
<th>Mole ratio cytochromes b : d</th>
<th>C/E Source</th>
<th>Mole ratio cytochromes b : d</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUCOSE (40 mM)</td>
<td>1.7 ± 0.1 ; n=4</td>
<td>LACTATE (40 mM)</td>
<td>15.3 ± 2.4</td>
</tr>
<tr>
<td>GLYCEROL (40 mM)</td>
<td>5.8 ± 1.6 ; n=5</td>
<td>PYRUVATE (40 mM)</td>
<td>23.9 ± 6.2 ; n=6</td>
</tr>
<tr>
<td>GLYCEROL (40 mM)+PYRUVATE (40 mM)</td>
<td>5.1 ± 0.7 ; n=5</td>
<td>GLYCEROL (20 mM) +PYRUVATE (20 mM)</td>
<td>3.8 ± 0.9 ; n=5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mole ratio cytochromes b : d</th>
</tr>
</thead>
<tbody>
<tr>
<td>LACTOSE (5 mM): 2.1</td>
</tr>
<tr>
<td>PYRUVATE (20 mM) + LACTOSE (5 mM): 4.6</td>
</tr>
<tr>
<td>GLYCEROL (20 mM) + LACTOSE (5 mM): 3.6</td>
</tr>
<tr>
<td>PYRUVATE (20 mM) + IPTG (5 mM): 25.1</td>
</tr>
<tr>
<td>GLYCEROL (20 mM) + IPTG (5 mM): 4.6</td>
</tr>
</tbody>
</table>

**Table X:** Production of cytochrome d by cells of PLJ01 grown on specific carbon/energy sources.

Cells of strain PLJ01 were grown under low aeration to stationary phase on the indicated carbon/energy sources as described in Materials & Methods. The mole ratio for cytochromes b : d was derived from reduced minus oxidized difference spectroscopy in conjunction with the extinction coefficients for α-absorption of these membrane constituents provided in references {104,118,135}. 
phosphofructokinase is known to be a major control step in the regulation of glycolytic catabolism, acting as a metabolic cross-over point under allosteric control by both ATP and citrate, it cannot be the major regulatory factor in the control of cytochrome d production by growth substrate for glycerol causes the same response as glucose \(36, 64\). The genes coding for phosphofructokinase and triose isomerase are known to lie in adjacent positions on the \textit{E. coli} genome, located at 87.7 min and 87.8 min respectively \(155\). Several substrates for glycolytic steps following the phosphofructokinase reaction were used in attempts to determine whether a precise step could be determined to affect cytochrome d expression and whether it might coincide with such known control points in the glycolytic sequence as 3-phosphoglyceraldehyde dehydrogenase, triose isomerase or pyruvate kinase.

These enzymes were at the extremes of the segment of the glycolytic pathway delimited by substrates associated with the induction of \textit{cyd} (glycerol) and those unable to cause induction (pyruvate). Pyruvate kinase and 3-phosphoglyceraldehyde dehydrogenase are subject to allosteric control by respiratory products, pyruvate kinase undergoing inhibition by ATP (amongst other effector control mechanisms) and 3-phosphoglyceraldehyde dehydrogenase being regulated by its use of the cofactor NAD\(^+\) as both substrate and allosteric activator (its four binding sites displaying negative cooperativity). Thus both of these activities are sensitive to the energy charge of the cell \(5\). The set of reactions between these two enzymes is also used by the other major routes of bacterial carbohydrate catabolism, the Entner-Doudoroff pathway and the hexose monophosphate shunt/heterolactic pathway, the latter being used in addition to glycolysis by rapidly growing \textit{E. coli} cells \(36\). Tests of cytochrome d induction by growth substrate using metabolic intermediates occurring in this catabolic path were obstructed by their instability (phosphoenolpyruvate, 1,2-diphosphoglycerate), their insolubility (D(-)-3-phosphoglycerate), or their cost — also related to stability — even as racemic mixtures (DL-3-phosphoglyceraldehyde, 2-phosphoglycerate). Additionally it is unclear in which form these phosphorylated triose intermediates would have been transported into the cell. Although 2,3-diphospho-D-glycerate was tested as a carbon/energy source for PLJ01 it resulted in such a slow growth rate \(\mu = 4\) hours \(\) that the oxygen content of the cultures would not drop below concentrations that would themselves inhibit the expression of the cytochrome d complex (data not shown). Similarly, attempts to use L-serine as growth substrate in order to form D(-)-3-phosphoglycerate \textit{via} phosphoserine resulted in generation times exceeding three hours.

Glyoxylate might be used as a carbon/energy source since \textit{E. coli} uses it in the dicarboxylic acid cycle to generate phosphoenolpyruvate, pyruvate and acetyl-CoA as well as using it for
regeneration of the phosphoenolpyruvate and acetyl-CoA by means of the glycerate pathway in which 3-phosphoglycerate is formed \[64\]. Although no substrate for 3-phosphoglyceraldehyde dehydrogenase would be present, the formation of unknown quantities of both substrate and product of pyruvate kinase would prevent unambiguous interpretation of any observed effect upon the induction of the cytochrome \(d\) complex. Studies of the inhibitory effects of arsenate or iodoacetate upon 3-phosphoglyceraldehyde dehydrogenase during growth would slow oxygen consumption by the culture such that the concentration of dioxygen would exert its own regulatory effect on \(cyd\) expression \[36\].

Therefore it is suggested that these growth substrate experiments have been taken as far as is useful without the implementation of an oxystat and that further investigations be carried out from a kinetic approach to determine whether the critical enzyme activities, or their allosteric responses are altered in the various \(cyd\) strains. Methods exist for determining intracellular triose phosphate concentrations as well as \(NAD^+\)/NADH ratios and estimates of energy charge \[5, 36, 64, 155, 218\]. These factors may also be pertinent to the regulation of expression of the cytochrome \(d\) complex for induction of a functional respiratory system in the presence of minimal oxygen concentrations would radically affect the relative levels of both adenosine polyphosphates and pyridine nucleotides. Additionally, regulation of the cytochrome \(d\) complex is altered in strain PLJ01 and its derivatives without any measurable defect in fermentative growth (data not shown), suggesting that the lesion is restricted to an aberrant regulatory response of the cells' \(cyd\) operon to intracellular production or consumption of a cofactor by the relevant glycolytic enzymes rather than to a defect in these enzyme activities themselves.

Subsequent investigations by other laboratories have shown that regulation of expression of the cytochrome \(d\) complex requires gene products located on the genome at sites remote from the \(cydAB\) operon at locations greater than minute 16.5 on the chromosome \((cydC\) at 18.9 min is probably related to the biosynthesis of chlorin for haem \(d\)), that the control is transcriptional and that it is regulated by the \(fNR\) gene under anaerobic conditions — presumably for the purposes of scavenging oxygen \[54, 56, 57, 61, 62\].

The initial experiments into substrate control of \(cyd\) expression had used cotransduction techniques to transfer the structural defect \(cydA\) from GR19N into the \(nadA\) strain PA2-18, selecting for the loss of auxotrophy for nicotinic acid as a primary screen for transductants. Similarly the gene responsible for substrate control of cytochrome \(d\) expression was cotransduced with \(suc\) into strain W945T which is incapable of growing with succinate as sole carbon/energy source \[89\]. A single cotransductant was obtained in the latter experiments so an attempt was made to carry out large
scale cotransduction screening to locate the relative positions of the two cyd alleles more accurately. Strain KW202 was isolated as nadA\textsuperscript{−} following elimination of a nadA ::TnlO insertion from strain NK6033 by Bochner's technique \cite{13}. Cotransduction of the cyd substrate control characteristic and nadA\textsuperscript{+} from PLJ01 into KW202 was performed with primary screening for ability to grow without nicotinic acid. None of the first fifty transductants isolated carried the substrate control feature for governing cytochrome d expression, suggesting that the cotransduction rate was substantially less than 5\%, in which case further investment in the intensive screening procedures would not have yielded useful values (data not shown). It is suggested that as the rate of cotransduction of nadA and cydA was 40\% in the earlier work (2 of 5 strains tested) and the rate of cotransduction of nadA and substrate control of cyd was found to be less than 5\% in the current study, the genomic distance between them may be greater than 40 kb or one minute \cite{218}. However, the episome F8, which has been shown to extend only part of the distance from nadA (16.8 min) to sdh (16.6 min), restores normal control over cyd expression in PLJ01 indicating that the control site and the cyd gene are more closely situated \cite{10, 60, 89}. While there is still some uncertainty about the precise locations of some of these genetic markers one possible explanation is that the control lesion may be associated with the supE suppressor function (glutamine tRNA\textsubscript{2} amber suppressor) which is located at minute 15.5 and is active in strain PA2-18 (glnV\textsuperscript{−}), absent in NK6033 (glnV\textsuperscript{+}) and has not been reported for GR17N, GR19N or PLJ01 \cite{10}.

Because of the complexity of interpreting the data from these various methods of analysing the composition and activity of the cytochrome d complex and in view of information received from the laboratory of R. B. Gennis that work on the purification and also the cloning and over-expression of cyd operon products was far advanced, emphasis was transferred to investigations of the cytochrome o terminal oxidase complex \cite{57, 61, 62, 63, 105, 118, 135}.

(ii) Membrane Studies of Cytochrome o

(a) Spectroscopic Properties of Cytochrome o Studied in Cell Membranes

The cytochrome o complex has been associated with type-b cytochrome α-band absorption shoulders observed in redox difference spectra of resuspended membrane preparations at 560 nm to 565 nm as illustrated previously in section II.A.i.b.1+3. The analysis of the component peak maxima (section II.A.i.b.2) contributing to this fused absorption feature have also been described.
above. The association of specific cytochromes with the cytochrome o complex does not imply that these may be identified with the cytochrome o terminal oxidase itself, just as the cytochrome d complex has been shown to comprise several individual cytochromes \cite{62, 98, 108, 135}. The character of cytochrome o has been disputed for several years, having been variously identified as each of the following type-b cytochromes: \(b_{555} \) \cite{95, 202}, \(b_{556} \) \cite{164}, \(b_{557} \) \cite{174} and \(b_{555-562} \) \cite{117}. It is widely acknowledged that one or more type-b cytochromes with \(\alpha\)-band redox absorption is or are associated with cytochrome o, but the lack of alteration of this region of the absorption spectrum upon complex formation with carbon monoxide has generally been taken to indicate that this is not the terminal oxidase itself \cite{157}. Claims have been made that the cytochrome o complex both does \cite{74, 202} and does not \cite{159} contain high spin haem, which would possess a free coordination site as required for a terminal oxidase.

Although a high spin type-b haem might be expected to display redox absorption spectra similar to cytochrome c peroxidase or cytochrome \(b_{595} \) with a small, displaced \(\alpha\)-band and larger \(\beta\)-band no spectrophotometric evidence was obtained to support such a proposition \cite{118}. However, if a high-spin haem-b is present, it may exhibit a low amplitude, broad \(\alpha\)-band similar to that of mammalian catalase \cite{6} which would not be observed in the presence of the other b-cytochrome components, and its Soret band and pyridine haemochromogen spectra would also be indistinguishable from those of the accompanying cytochromes. No spectral alterations have been observed upon oxygen binding to cytochrome o situated in membrane preparations although spectral perturbations do occur when carbon monoxide reacts with the terminal oxidase \cite{157, 216}. Care must be taken in the preparation of samples and conduct of such experiments since partial denaturation of type-b cytochromes results in loss of full coordination by the haem iron and greater haem exposure, enabling it to react with CO to form a ligand adduct in a manner analogous to that of the terminal oxidase \cite{49, 157, 216}.

(b) Spectroscopic Investigation of Cytochrome o Ligand Binding

The absolute reduced and absolute oxidized absorption spectra of cyd\(^{-}\) cell membranes containing the cytochrome o complex are shown in Figure 22b,3+6. Nitrate, nitrite and glutathione had no appreciable spectral effect upon the \(\alpha\)-absorption band of reduced type-b cytochrome. Azide decreased the absorbance of the entire type-b cytochrome \(\alpha\)-band region and that of the \(\beta\)-band also. A possible explanation of this observation is that the cytochrome \(b_{555} \) component of the
cytochrome \( o \) complex displayed decreased absorption in the presence of azide which would also lessen the contribution of this peak to the overlapping regions of the cytochrome \( b_{560}^+ \) \( \alpha \)-bands. Support for this suggestion comes from the flattening of the red shoulder at about 560 nm into a plateau (Fig. 22b,4). Azide treatment of the isolated cytochrome \( o \) complex, solubilized in detergent, failed to show any spectral changes (data not shown). The effect of cyanide ions on the \( b \)-type cytochrome \( \alpha \)-band was to abolish the red shoulder and shift the absorption maximum of the resulting triangular peak from 556 nm to 557.5 nm. Interestingly the \( \beta \)-band of the \( b \)-cytochromes maintains its biphasic appearance after cyanide treatment. Although the preferential effect of azide and cyanide upon the cytochrome \( o \) terminal oxidase complex, rather than the cytochrome \( d \) complex, has been well documented this was attributed to the latter’s greater affinity for oxygen: the current data may indicate that these two ligands do not act upon cytochrome \( o \) in an equivalent manner \cite{4, 157, 216}.

Curves 1+2 of Figure 22b show that the effect of carbon monoxide on the reduced \( \alpha \)-band spectrum of the type-\( b \) cytochromes is minimal, although there is a slight decrease in the height of the absorption maximum relative to that of the red shoulder. The pre-treatment \( \alpha \)-band profile is restored after a single exposure to intense broad-spectrum visible light (Fig. 22b). (Significant changes were still occurring in parallel membrane preparations containing cytochrome \( d \) after exposure to three times as much light.)

(c) The cytochrome \( o \)--CO complex and low temperature photolysis

Reduced plus carbon monoxide \textit{minus} reduced difference spectra of \( cyd^- \) membranes bearing the cytochrome \( o \) complex, using dithionite as reductant, are illustrated in Figure 40, panels a+b. Very little \( \alpha \)-band absorbance was measured at ambient temperatures, in common with the results of previous investigators \cite{157}. This has suggested that the cytochrome of the terminal oxidase, known to contain type-\( b \) haem only, may undergo minimal \( \alpha \)-band perturbation during the formation of the CO-adduct or that it may exhibit a particularly small \( \alpha \)-band redox absorption, possibly due to the presence of a postulated high-spin haem iron resulting in a redox \( \alpha \)-absorption band of extremely low intensity and a ‘carbon monoxide binding spectrum’ analogous to the equivalent spectra of catalase (Fig. 6). (See Appendix I for a discussion of why the former suggestion is unlikely.) Nevertheless Y. Anraku and coworkers have used the redox \( \alpha \)-band absorption feature of the purified cytochrome \( o \) complex, coupled with a quantitative interpretation of a second order finite difference analysis of CO perturbations of this spectral region to provide evidence that the redox absorption of
Fig. 40: Broad range difference spectra of the carbon monoxide derivatives of cytochromes in membrane preparations from strains with cyd" phenotype: photodissociation studies at various temperatures.

Strain PLJ01 was grown to stationary phase on CYD minimal medium containing succinate as carbon/energy source. Washed membranes were prepared and resuspended at a protein concentration of 12.7 mg mL"1, treated with carbon monoxide as described under Materials & Methods and analysed in the cryogenic attachment of the PE-356 spectrophotometer operated in split beam mode. Gassing and all subsequent sample manipulations were performed in the dark unless stated otherwise. Sodium dithionite was used as reductant for the single scan spectra in panels a and b, NADH for those in panel c.

1. Temperature = 295 K. Soret band $\lambda_{\text{max}}= 417.5 \text{ nm}$, $\lambda_{\text{min}}= 430.0 \text{ nm}$.
2. Temperature = 77 K. Soret band $\lambda_{\text{max}}= 413.0 \text{ nm}$, $\lambda_{\text{min}}= 429.0 \text{ nm}$.
3. Temperature = 77 K. Soret band $\lambda_{\text{max}}= 416.5 \text{ nm} + 432.5 \text{ nm}$, $\lambda_{\text{min}}= 426.0 \text{ nm}$.
    Sample exposed to illumination by 150 W incandescent source at 10 cm for 10 min.
4. Temperature = 77 K. Soret band $\lambda_{\text{max}}= 411.0 \text{ nm} + 434.0 \text{ nm}$, $\lambda_{\text{min}}= 425.0 \text{ nm}$.
    Following illumination at 77 K the sample temperature was raised to 198 K for 45 min before recooling to 77 K and recording the spectrum.
5. Temperature = 77 K. Soret band $\lambda_{\text{max}}= 425.0 \text{ nm}$.
    Following illumination at 77 K the sample temperature was raised to 273 K for 45 min before recording the spectrum.

b. 6-9: reduced plus carbon monoxide minus reduced difference spectra. $\Delta \lambda = 0.01$ (1), 0.03 (2-5).

6. Temperature = 77 K. $\alpha$-band $\lambda_{\text{max}}= 564.5 \text{ nm}$, (+ blue shoulder).
7. Temperature = 77 K. $\alpha$-band $\lambda_{\text{max}}= 555.0 \text{ nm}, 561.0 \text{ nm}$ and 565.0 nm.
    Sample exposed to illumination by 150 W incandescent source at 10 cm for 10 min before recording spectrum '7'.
8. Temperature = 77 K. $\alpha$-band $\lambda_{\text{max}}= 561.0 \text{ nm}$ (+ blue shoulder), 565.0 nm.
    Following exposure to illumination by a 150 W incandescent source the sample was stored in the dark at 77 K for 45 min before recording spectrum '8'.
9. Temperature = 77 K. $\alpha$-band $\lambda_{\text{max}}= 555.5 \text{ nm}, 561.0 \text{ nm}, 565.0 \text{ nm}$.
    Following exposure to illumination by 150 W incandescent source the sample was stored in the dark at 77 K for 45 min and reilluminated before recording spectrum '9'.
Fig. 40: (continued)

10. Temperature = 77 K. Soret band $\lambda_{\text{max}} = 413.5$ nm.
11. Temperature = 77 K. Soret band $\lambda_{\text{max}} = 413.5$ nm (+ red shoulder).

The sample was illuminated at 273 K with a 150 W incandescent source before recording the spectrum at 77 K.

12. Temperature = 77 K. Soret band $\lambda_{\text{max}} = 418.0$ nm + 429.0 nm.

The sample was illuminated a second time with a 150 W incandescent source before recording the spectrum at 77 K.
cytochrome o itself is at 555 nm at 77 K \{97\}. Not only is the validity of such a quantitative second order analysis open to question but their interpretation relied upon the assumed maintenance of relative absorption patterns between two observed \(\alpha\)-band peaks at room temperature and at 77 K. Consequently a direct investigation of the low temperature spectra of the cytochrome o-CO complex was undertaken as part of the current study.

Upon cooling to 77 K the Soret absorbance maximum of reduced plus carbon monoxide minus reduced difference spectra shifts from about 418 nm to 414 nm and the minimum from 430 nm to 428 nm, the exact wavelength values being dependent upon the intensity of the absorption since the two features overlap (Fig. 40a). Earlier reports had demonstrated that minor differences in the shape of the 77 K \(\alpha\)-band trough were dependent upon the strain and growth conditions employed \{89\}. Exposure of samples to an incandescent light source resulted in decreased intensity of both peak and trough in the Soret region of room temperature spectra, suggesting that these twin features were originally caused by a shift of the ‘reduced’ cytochrome o absorbance, represented by the trough, to a lower wavelength, represented by the peak, upon reaction of the reduced cytochrome with carbon monoxide and that the CO-adduct is subject to photodissociation (data not shown) \{112, 216\}. The results of sample illumination and spectral analysis at low temperature were more complex although all spectra were measured at 77 K in order to prevent temperature effects causing spectral shifts and absorption changes (Fig. 40). At 77 K illumination resulted in decreased intensity of the ‘CO-bound’ peak with the formation of a ‘high wavelength’ Soret peak at 432.5 nm and consequent diminution of the overlapping Soret trough. This indicates the trapping of an intermediate in the mechanism of photolysis of the cytochrome o-carbon monoxide complex, this intermediate being stable at 77 K but not at ambient temperatures. When the temperature was raised still higher following illumination at liquid nitrogen temperatures subsequent measurement of the Soret maxima at 77 K demonstrated that both had decreased in intensity. The rate of this reaction was moderate at 198 K, apparently remaining incomplete after 45 min (Fig. 40a). Since the Soret trough was still a major spectral feature after these temperature shifts it is suggested that photolysis of the cytochrome o-CO complex proceeds by way of at least two intermediates. One of these is stable at 77 K and characterized by the ‘high wavelength’ Soret peak, \(\lambda_{\text{max}} = 434.0\) nm; this degrades at temperatures of 198 K and above to a second intermediate with Soret absorbance which is effectively masked in these spectra but which does not negate the ‘Soret deficiency’ of cytochrome o itself shown by the trough with \(\lambda_{\text{min}} = 425.0\) nm. At room temperature this second intermediate presumably degrades rapidly to cytochrome o.

Throughout these procedures spectral changes are also visible in the \(\alpha\)-band region at 77 K
Figure 40 curves a,1+2 show the increased detail visible at low temperature, the 77 K \(\alpha\)-band region being expanded in panel b, curve 1 to reveal the complex absorption pattern of the CO-bound sample with a broad peak at 565 nm and a blue shoulder centred at approximately 555.0 nm. On illumination at this temperature the intensities of absorbance are altered although there appears to be little alteration of the wavelengths of the component peaks. Most obvious is the increased absorption of the 'blue shoulder' which approximately doubles in size to form a distinct peak (Fig. 40a,2; b,7). The breadth of the 565.0 nm peak also decreases substantially with partial resolution of another peak at 561.0 nm which may be attributable to the increased intensity of the 555.0 nm wavelength component. Thus the \(\alpha\)-band absorbance at 555.0 nm may be a feature of the 'first photolytic intermediate' with Soret absorbance at 432.5 nm. Maintaining the sample in the dark at 77 K for 45 min caused a reversion of the 555.0 nm component to its previous intensity, although the resolution of the higher wavelength \(\alpha\)-band features was not lost. Reillumination of the dark-incubated sample restored the initial photo-treated \(\alpha\)-band absorption profile (Fig. 40b,7-9). It is possible that such a photolytic reaction, dark-reversible at liquid nitrogen temperatures, is caused by dissociation of carbon monoxide from the terminal oxidase without diffusion of the ligand from the binding site. These reduced samples were oxygen depleted and they are crystalline at 77 K which would decrease the probability of such diffusion. Raising the temperature might permit the ligand to leave the proximity of the haem iron and thus modify the cytochrome spectrum further in a manner analogous to that of the mammalian cytochrome oxidase, possibly through a combination of sufficient energy being made available for complete dissociation and the sample becoming vitreous (38). On raising the temperature of the samples to 198 K and to 273 K the major \(\alpha\)-band absorbance was observed to be at 555 nm with a red shoulder in the 565 nm region. Since the Soret region demonstrated a large trough signifying the absence of reduced cytochrome \(c\) in the sample cuvette this \(\alpha\)-absorbance must have been caused by one or more intermediates in the photolytic process.

Thus low temperature 'reduced plus carbon monoxide minus reduced' difference spectra and photodissociation techniques did not reveal the character of the \(\alpha\)-band absorption of cytochrome \(c\) and at ambient temperature, at which the fully dissociated material might be expected to form in solution, the \(\alpha\)-absorption region of these spectra provided insufficient resolution or intensity to be of use (Fig. 40a+b). Analysis of 'reduced plus carbon monoxide minus oxidized' difference spectra at 77 K provide the CO-bound spectrum of the terminal oxidase superimposed on the standard redox difference spectra of other cytochromes in the preparation. By using limiting quantities of NADH partial reduction of the sample may be achieved, as has been practised in the potentiometric
titration procedures described under Materials & Methods. Carbon monoxide binds only to reduced oxidases and by progressive addition of reductant and CO 'reduced plus CO minus oxidized' spectra may be obtained in which there is little interference from low potential cytochromes. Figure 40c illustrates such spectra in which intense Soret absorption is observed at about 414 nm, typical of the CO-bound form of cytochrome o, minimal absorption at 428 nm to 430 nm, typical of type-b cytochromes including cytochrome o, and a biphasic α-band profile with an absorption maximum at 565 nm and a pronounced red shoulder centred at 555 nm. This profile also suggests that the major effect of carbon monoxide on the α-band absorption properties of cytochrome o may be to increase the intensity of this absorption with little change in its wavelength (Fig. 22b,2+3; Fig. 40c,10). Relative to the size of the α-band the β-absorption band is much larger than in standard reduced minus oxidized difference spectra after carbon monoxide treatment (Fig. 40c,10). Illumination at temperatures below 273 K caused a spectral shift from the CO-bound 414 nm peak to an absorption peak at 430 nm showing that some 'free' reduced cytochrome o was formed in addition to the second dissociation intermediate at this temperature (Fig. 40a,3-5; c,11+12). This illumination also caused an alteration of the intensity of the α-band absorption features, causing a slight decrease of absorption at 565 nm and a marked increase at 555 nm. In addition a broad shoulder was observed in the 575 nm region of the spectrum.

Poole and Chance have performed related experiments in which dioxygen was readmitted to CO-treated broken cell preparations maintained in the dark at -25°C. These samples were then exposed to high intensity visible light flashes at temperatures from 218 K to 77 K [164, 165]. Although these cells contained both the cytochrome o and cytochrome d terminal oxidases, evidence was presented that cytochrome o itself forms CO and O₂ intermediates with similar spectral properties [165]. Investigation of low temperature reactions of cytochrome o in the presence of both dioxygen and carbon monoxide demonstrated a higher velocity and lower photolytic reversibility with the former than with CO and that a photolytic intermediate of the cytochrome o-O₂ adduct was stable below 175 K which may be similar to the CO-induced absorption peak at 432.5 nm in reduced plus carbon monoxide minus reduced difference spectra [164]. On the basis of such data it has been suggested that the spectrum of the O₂ adduct is similar to that of the CO-adduct in the Soret region but that it may resemble that of the oxidized enzyme in the α-band with minimal absorbance in this region — a situation analogous to that of oxygen binding to cytochrome d (section II.C.i.b+c) [116, 164, 169].

Problems of thermal equilibration within the sample, convection and the instability of the electrochemical potential under conditions of partial reduction and CO gassing or aeration precluded
the practice of thawing the sample to room temperature and refreezing in order to observe the reformation of cytochrome o itself following photolysis of the CO adduct.

(d) Potentiometric Titration of Components of the Cytochrome o Complex

The standard potentiometric titrations of type-\(b\) cytochromes in cell membranes indicate the reduction status of low-spin cytochrome \(b\) components in the cytochrome \(o\) complex. As the technique is carried out at 305 K and measures the redox difference spectrum \(\alpha\)-band absorption change in order to determine the proportion of cytochrome \(b\) reduced, other components — including high-spin haem that may be present — will make little contribution. Because they are involved in the final electron transfer reaction with oxygen, the cytochromes of the cytochrome \(o\) terminal oxidase are expected to possess relatively high mid-point potentials, as do those of cytochrome \(d\) described above.

Comparison of the membrane titration profiles for \(\text{cyd}^+\) and \(\text{cyd}^-\) strains demonstrates that there are significant differences in the extreme portion of the high potential region of the titration profile which are ascribed to the cytochrome \(d\) complex (Fig. 25). The component with the next highest potential has an \(E_m\) value of approximately +225 mV and is assumed to be associated with cytochrome \(o\) \((117)\). Spectrophotometric studies suggest that the cytochrome \(o\) complex contains a \(b_{555}\) component which would contribute fully to the observed \(\alpha\)-band absorbance change at the absorption maximum \((4, 87, 143)\). Additionally certain of the cytochrome \(o\) cytochromes are known to form a red shoulder on the total \(b\)-cytochrome \(\alpha\)-band (section II.A.i.b.1,3). As the sample is reduced the contribution of the latter as a percentage of the total \(b\)-cytochrome \(\alpha\)-band amplitude, measured at the \(\alpha\)-band absorption maximum, is inaccurately represented since the wavelength of the maximum absorbance progressively shifts toward the blue during the course of the titration \((v.i.)\).

Thus cytochrome \(o\) components provide a minimum of 15 % of the type-\(b\) cytochrome of the glucose grown \(\text{cyd}^+\) cells and 35 % and 15 % of that of \(\text{cyd}^+\) and \(w^+\) cells grown on DL-lactate under the conditions described in Figure 26. The amount of cytochrome \(o\) in wild-type cells was dependent upon the time of induction of cytochrome \(d\) before harvesting, since oxygen tensions low enough to induce cytochrome \(d\) prevent expression of cytochrome \(o\) and cytochrome \(d\) itself may repress expression of cytochrome \(o\) \((112, 106)\). Hence the minimal amount of high potential cytochrome attributed to the cytochrome \(o\) complex in membranes of the glucose grown \(w^+\) cells (less than 10 %) whereas under the slower growth conditions with lactate the oxygen tension remained
high for a longer period and significantly more cytochrome $o$ was detected in these cell membranes (35\%) (Fig. 26). It should be noted that there may be other components of cytochrome $o$ contributing to the titration profile besides that detected as being of highest potential in membranes of cyd$^-$ strains.

Attempts to correlate the components detected by potentiometric titration with those resolved in low temperature spectrophotometric analyses are illustrated in Figure 28. Even at the temperatures at which the titrations were performed, 305 K, and with the single scan operation of the Perkin-Elmer 356 instrument, a distinction could be drawn between the spectral characteristics of the type-$b$ cytochromes being reduced during the course of these experiments (Fig. 41). At high potentials the initial indication of a reduced cytochrome $b$ $\alpha$-band at 305 K was a very broad 'peak' which bore no resemblance to standard Gaussian or Laurenzian spectral profiles but which seemed to result from two fused peaks with maxima at approximately 560 nm and 563 nm. Of these two, which always appeared simultaneously to provide the broad $\alpha$-band feature, the 563 nm absorbance was slightly greater than that at 560 nm (Fig. 41). As the electrochemical potential of the sample was lowered the absorption of both component peaks increased, that at the lower wavelength increasing more rapidly so that at potentials below +125 mV it was the dominant feature of the $\alpha$-band spectrum. During the course of the titrations the wavelength of the absorption maxima changed from 560 nm plus 563 nm to 558 nm alone, the latter being equivalent to a wavelength of between 555 nm and 556 nm at 77 K. Many attempts were made to quantify this wavelength shift as a function of potential, but the lack of spectral resolution combined with the low signal:noise ratio obtained during the single scan spectrophotometric measurements of the opaque resuspended membrane samples produced excessive scatter in the data for them to be interpreted with confidence.

(e) **High Resolution Redox Difference Spectrophotometry at Poised Potentials**

An alternative to direct analysis of the 305 K spectra in which there was exaggerated overlap of the broad $\alpha$-bands was to remove poised potential samples during the course of the titrations and subject them to high resolution, multiple scan difference spectrophotometry *versus* an oxidized reference at liquid nitrogen temperatures. At high sample potentials the 77 K variably-reduced *minus* oxidized difference spectra of cyd$^-$ cell membranes displayed partially resolved twin peaks with absorbance maxima of approximately 556 nm and 563 nm (Fig. 28). Fourth order finite difference analysis revealed that each peak comprised at least two components: those at lower wavelength being of 555.0 nm and 557.5 nm and those at higher wavelength peaking at 562.0 nm and 565.0 nm.
**Fig. 41:** Real time redox difference spectra of high potential cytochromes measured during potentiometric titration of membrane preparations from strains with cyd⁻ phenotype.

Washed membranes were prepared from cyd⁻ strain GR19N after aerobic growth to stationary phase in CYD minimal medium in the presence of DL-glucose. The washed membranes were resuspended in degassed 100 mM potassium phosphate buffer, pH 7.0 at a protein concentration of 26.9 mg mL⁻¹. The sample was reduced slowly by endogenous substrates in the presence of redox mediators under an oxygen-free nitrogen atmosphere, single scan spectra being recorded from 585 nm to 535 nm at the electrochemical potentials indicated using ferricyanide oxidized reference material as described under Materials & Methods. ΔA = 0.02.

Sample potential measured as each of curves 1 to 19 was recorded:

1. +315.5 mV vs. NHE
2. +307.0 mV vs. NHE
3. +288.0 mV vs. NHE
4. +275.0 mV vs. NHE
5. +260.5 mV vs. NHE
6. +236.0 mV vs. NHE
7. +215.5 mV vs. NHE
8. +207.5 mV vs. NHE
9. +197.0 mV vs. NHE
10. +179.0 mV vs. NHE
11. +167.0 mV vs. NHE
12. +158.0 mV vs. NHE
13. +144.0 mV vs. NHE
14. +122.0 mV vs. NHE
15. +96.0 mV vs. NHE
16. +81.0 mV vs. NHE
17. +62.5 mV vs. NHE
18. +40.5 mV vs. NHE
19. +8.5 mV vs. NHE
As the potential of the sample was progressively lowered the difference spectra showed that the increase in the absorption of the two distinguishable peaks was emphasized in that at lower wavelength (Fig. 28). Fourth order derivatives of these spectra display broad peaks, suggesting incomplete resolution. The broadness of these peaks which have maxima at 556.0 nm and 564.0 nm is indicative of the presence of the additional components more clearly resolved at higher potentials. Figure 28 also demonstrates the insignificant quantity of cytochrome $b_{560}^{+-}$, and by inference cytochrome $o$, in stationary phase glucose grown $w^+$ cell membranes: these results are described above (section II.C.ii.d).

Thus the high potential type-$b$ cytochrome observed in the potentiometric titrations was partially attributable to those cytochromes $b$ with longer wavelength $\alpha$-band absorbance (560 nm to 565 nm) previously associated with the cytochrome $o$ complex from purely spectrophotometric studies (section II.C.ii.a). In addition this high potential cytochrome $b$ contained cytochromes with lower wavelength absorption maxima, in the 555 nm to 558 nm range. Because of the difficulty in assigning precise potential values to low temperature poised spectra (section I.ii.c) these data cannot be used to link all of these components with cytochrome $o$ unequivocally.

The application of a variety of methods both independently and in combination had proven inadequate to delineate the properties of the individual cytochromes of the aerobic respiratory chains of *Escherichia coli*. In order to proceed with the investigation of these cytochromes' individual properties and of how they might act in a coordinated manner within the cell membrane it became apparent that it would be necessary to fractionate them so that their properties could be determined independently.
III : **PURIFIED DETERGENT EXTRACTS:**

**{A} Cytochrome Solubilization & Fractionation**

It was apparent that in order to obtain precise biophysical data relating to the aerobic terminal oxidases they would need to be separated from other cytochromes present in the analytical samples. This was particularly necessary in the case of the cytochrome $a$ complex with its complement of type-$b$ cytochromes, the spectral characteristics of which were obscured by the multiple $b$-cytochrome components of those respiratory chains induced by growth under aerobic conditions. Thus the contribution of individual cytochromes to data from potentiometric and kinetic studies, as well as their complete resolution and identification in purely spectrophotometric analyses, remained unclear. The combination of these techniques plus the use of mutant strains and strictly controlled growth conditions had not proven adequate for unambiguous interpretation of the complex results. Moreover work in other laboratories was well advanced in developing cloned preparations of the cytochrome $d$ complex (section II.C.i.e above). Consequently cyd$^{-}$ strains were used to simplify the cytochrome content of cells grown aerobically under defined conditions in preparation for detergent solubilization of their membranes and subsequent fractionation of the respiratory cytochromes by liquid chromatography.

(i) **Membrane Solubilization**

(a) **Redox spectroscopy of extracted fractions**

Although a variety of ionic detergents were found to extract cytochromes efficiently from *E. coli* membrane suspensions the method of preference incorporated solubilization by non-ionic Triton series of detergents, these permitting the employment of a greater variety of fractionation techniques and being less likely to cause major perturbations of protein structure (77, 82, 179). These were found to be far more effective at solubilization than octylglucoside or the Brij detergent series (data
The extraction procedure has been described in full under Materials & Methods. The efficiency of cytochrome extraction was very dependent upon the strain of cells in use and on the growth phase at which they had been harvested, 15% to 35% of the type-β cytochromes being extracted from washed, resuspended membranes (data not shown). Triton X-114 was generally found to extract about 25% more cytochrome b than Triton X-100 when each was used in 5% (w/v) aqueous solution at 0°C as described. The differential solubilization technique comprising extraction with Triton X-114 and phase separation by raising the temperature of the extract above the detergent's cloud point (c. 20°C) was investigated as an initial purification step (152, 166). All visible chromophores in the extract precipitated in detergent micelles during this procedure and could not be redissolved. Triton X-114 was replaced with Triton X-100 during the liquid chromatographic fractionation procedure as described under Materials & Methods in order to simplify sample manipulation and spectrophotometry at ambient temperatures.

Figure 42 illustrates a typical extraction of wild-type cell membranes and Figure 43 one from cyd" cells' membranes. The detergent extract contained approximately 35% of the type-β cytochrome, determined from the reduced minus oxidized α-band absorption whereas only 25% of the cytochrome d was extracted. The lower efficiency of extraction of cytochrome d is reflected in the smaller proportion of cytochrome b_{558} to b_{556} in the separated extract (Fig. 42a,3), although inspection of the extraction mixture itself indicates that addition of the detergent to the membrane suspension may cause some disruption of the cytochrome d complex and decrease the relative absorption of cytochrome b_{558} (Fig. 42a,2; 42b,2). Electrochemical properties of cytochrome b_{558} in the solubilized and isolated cytochrome d complex have also been reported to be particularly detergent sensitive (105). Damped fourth order derivative spectra are shown for clarity, the major difference between the samples being the loss of detail in the β-band region of the β-cytochromes present in the extract, possibly indicative of conformational disruption (Fig. 42b,1-3).

The extraction of cytochromes from membranes of cyd" cells rarely exceeded 20%, most of the cyd" strains having been derived from GR17N. The reduced minus oxidized α-band spectra of the extracts differed substantially from those of the resuspended membranes from which they originated, and addition of detergent to the membranes affected the spectrum of the β-cytochromes associated with the terminal oxidase, as was seen to be the case with cytochrome b_{558} and the cytochrome d complex, described above (Fig. 43a,1+2). Fourth order finite difference analysis of the cytochromes b_{560}+ of the cytochrome o complex showed that upon addition of 'Triton X' detergents multiple components at 560.5 nm, 563.0 nm and 565.0 nm in the membrane suspensions merged into a single component with a broad fourth order derivative peak centred at 563.0 nm (Fig.
Fig. 42: High resolution α-band spectra of Triton X-114 detergent extracts from washed membranes of wild-type cells.

Cells of \( w^+ \) strain MR43L were grown to stationary phase on CYD minimal medium containing succinate. Washed membranes were prepared and extracted by the standard techniques. Membrane samples were resuspended in 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0; extracted samples were analysed in the presence of half-strength extraction buffer with 50 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0.

a. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.
   1. Washed membranes; protein concentration = 2.5 mg mL\(^{-1}\) \( \Delta A = 0.10 \),
   2. Washed membranes in detergent/extraction buffer; protein = 2.5 mg mL\(^{-1}\) \( \Delta A = 0.10 \),
   3. Extract from washed membranes, protein concentration = 0.76 mg mL\(^{-1}\) \( \Delta A = 0.04 \).

b. Fourth-order finite difference spectra calculated from corresponding curves ‘a’ by two successive second-order derivatizations.

c. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.
   1. Washed membranes; protein concentration = 2.5 mg mL\(^{-1}\) \( \Delta A = 0.20 \),
   2. Washed membranes in detergent/extraction buffer; protein = 2.5 mg mL\(^{-1}\) \( \Delta A = 0.20 \),
   3. Extract from washed membranes, protein concentration = 0.76 mg mL\(^{-1}\) \( \Delta A = 0.20 \).
Fig. 43: High resolution α-band spectra of Triton X-114 detergent extracts from washed membranes of cyd⁻ cells.

Cells of cyd⁻ strain PLJ01 were grown to stationary phase on CYD minimal medium containing succinate. Washed membranes were prepared and extracted by the standard techniques. Membrane samples and the non-extracted residue were resuspended in 1.0 M sucrose, 100 mM potassium phosphate buffer, pH 7.0; extracted samples were analysed in the presence of half-strength extraction buffer with 1.0 M sucrose, 50 mM potassium phosphate buffer, pH 7.0.

a. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.

1. Washed membranes; protein concentration = 2.50 mg mL⁻¹  
ΔA = 0.16,

2. Extract from washed membranes; protein concentration = 0.84 mg mL⁻¹  
ΔA = 0.04,

3. Non-extracted residue; protein concentration = 1.50 mg mL⁻¹  
ΔA = 0.16.

b. Fourth-order finite difference spectra calculated from corresponding curves 'a' by two successive second-order derivatizations.
Moreover, inspection of the residue following resuspension after removal of the extract showed a distinctive absorption profile comprising \( \alpha \)-band absorption maxima at 555.0 nm, 557.5 nm, and 563.0 nm (Fig. 43a,3; 43b,3). The nature of this material is discussed in greater detail below (section III.C): it was subsequently determined to comprise a large proportion of the cytochrome \( o \) complex found in the membranes.

The standard extraction procedure for purifying the cytochrome \( o \) complex incorporated both urea and cholate washes of the membranes before extraction of the terminal oxidases with Triton X-114. These reagents denatured or removed significant quantities of extrinsic proteins from the membranes but had little effect on the terminal oxidases suggesting that the latter were firmly anchored within the membrane \cite{4, 77, 157}. The observation that the Triton detergents affected the absorption spectrum of \( b \)-cytochromes within the complexes of both cytochromes \( o \) and \( d \) indicated that the molecular environment of the haem moiety of these components was susceptible to membrane disruption but that any denaturation occurring as the result of detergent addition was insufficient to cause loss of the prosthetic group which is not covalently bound.

(ii) **Cytochrome Fractionation by Liquid Chromatography**

(a) **Ion-exchange chromatography — elution profiles of solubilized cytochromes**

The initial liquid chromatographic step of the fractionation procedure served several functions. All cytochromes extracted from membranes of aerobically grown cells bound to the DEAE-BioGel.A ion-exchange matrix which allowed them to be concentrated from the large volume extraction and detergent dilution steps. Once bound to the column detergent exchange could be accomplished simply and an effective fractionation of the solubilized cytochromes could be obtained by implementing the gradient elution procedure described under **Materials & Methods**. Spectral analysis of the visibly coloured material that failed to bind to the anion exchange column indicated that it comprised iron-sulphur protein and/or flavoprotein: none of this material bound to the cation exchange matrix, CM-BioGel.A under equivalent conditions (data not shown).

Solubilized material binding to the top of the column at low ionic strength was dark brown in colour which separated into eluted bands varying from pale yellow through brick red to brown. The
**Fig. 44:** DEAE-BioGel.A elution profile of Triton membrane extracts from wild-type cells grown on glucose.

Cells of \( w^+ \) strain MR43L were grown to stationary phase on CYD minimal medium containing glucose. Washed membranes were prepared and extracted by the standard techniques. The DEAE-BioGel.A was eluted under standard conditions with a KCl gradient.

- **Absorbance profile:** \( \Delta A_{412} = 0.10 \).
- **Lowry protein assay profile:** \( \Delta A_{660} = 0.10 \); 0.90 mg mL\(^{-1}\) eluate.

(Figures illustrating cytochrome fractionation via DEAE-BioGel.A columns have standardized abscissas in order to facilitate comparison of elution profiles.)
intensity of each fraction’s absolute absorbance at 412 nm ($A_{412}$) was indicative of the presence of cytochrome, iron-sulphur protein or flavoprotein. Reduced minus oxidized difference spectrophotometry was employed to identify the chromophores within specific fractions. The effect of the cytochrome $d$ complex upon the efficacy of cytochrome fractionation is shown in Figure 44. The initial peak contained flavoprotein and three other distinct peaks were identified as described below. A major feature of the $A_{412}$ profile from extracts of wild-type cell membranes was the pronounced ‘background’ of absorbing material upon which the other peaks were superimposed (Fig. 44). This background absorption was caused by the Soret band of the cytochrome $d$ that was eluted by the KCl gradient over an extremely broad salt concentration range, imparting a grey-green colour to the column. As fractions from various parts of the gradient contained different proportions of cytochromes $b_{558}$ to $d$ it is suggested that the cytochrome $d$ complex progressively dissociated in the conditions under which the column was run, causing it to elute throughout a large part of the gradient (data not shown). It is of interest that the solubilization technique and all procedures prior to loading the ion-exchange column were performed at low ionic strength.

Comparison of the Lowry protein and $A_{412}$ elution profiles resulting from fractionation of a solubilized extract of membranes from cyd~ cells shows that a far more distinct separation of fractions containing cytochromes is obtained in the absence of the cytochrome $d$ complex (Fig. 45).

The conductivity of the eluted fractions, representative of the ionic strength of the elution buffer at any specific stage of the gradient, provided a precise method of recognizing the eluted 412 nm absorption peaks containing cytochrome, and data in those figures illustrating equivalent elution profiles from several experiments are aligned by the conductivity of their eluted fractions. The utility of this approach is seen clearly in Figure 46 in which extracts of cell membranes from a variety of sources are shown to produce $A_{412}$ elution peaks which group into distinct categories which were labelled from I to IV. Fractions within each of these groups of peaks contained a distinct cytochrome complement ($v.i.$).

Figure 46 also demonstrates that the relative peak heights differed significantly in $A_{412}$ elution profiles obtained from extracts of membranes from cyd~ cells grown under different conditions indicating that the quantities of component cytochromes also varied with these conditions. Peak II was observed in all extracts fractionated, including those of wild-type cells containing cytochrome $d$. Peak III was only observed on fractionation of membrane extracts from cells grown on L-proline. The heights of peaks I and IV (indicating the intensity of absorption at 412 nm) varied with respect to that of peak II and to each other. When cells were grown to stationary phase on minimal medium containing any one of the several carbon/energy sources tested in these studies
Fig. 45: DEAE-BioGel.A elution profile of Triton membrane extracts from cyd<sup>−</sup> cells grown on glucose.

Cells of cyd<sup>−</sup> strain PLJ01 were grown to stationary phase on CYD minimal medium containing glucose. Washed membranes were prepared and extracted by the standard techniques. The DEAE-BioGel.A was eluted under standard conditions with a KCl gradient.

a. Absorbance profile: $\Delta A_{412} = 0.25$.

b. Lowry protein assay profile: $\Delta A_{660} = 0.25$; 0.76 mg mL<sup>−1</sup> eluate.
Fig. 46: DEAE-BioGel.A elution profiles of Triton membrane extracts from cyd− cells grown on different carbon/energy sources.

Cells of cyd− strain GR19N were grown to stationary phase on CYD minimal medium supplemented with the carbon/energy sources indicated below. Washed membranes were prepared and extracted by the standard techniques. The DEAE-BioGel.A was eluted under standard conditions with a KCl gradient.

a. Lactate,  
b. Succinate,  
c. Proline,  
d. Glucose.
their solubilized membrane extracts all contained peaks II and IV while the amount of peak I material was observed to vary when compared to the others by either peak height or peak area. Thus peak I was larger than peak II when DL-lactate was the sole carbon/energy source, it was smaller than peak II (approximately half-height) when only succinate was provided, small but distinguishable when the cells were grown on L-proline alone and absent when the carbon/energy source was glucose (Fig. 46).

It has been shown above from spectrophotometric studies that the membrane complement of type-\(b\) cytochromes is altered by the carbon/energy source available to the cells (Fig. 20; section II.A.i.b.1). The dual wavelength kinetic analyses of cytochrome reduction in membrane suspensions from cells grown on different substrates showed that the proportion of the total cytochrome reduced by specific substrates varied between these preparations as did the steady state level of cytochrome reduction, suggesting an adaptation of the cytochrome content to the respiratory function appropriate to the cells' conditions of growth (Fig. 32; section II.A.ii.b). The extraction and fractionation procedure described under Materials & Methods provided a preliminary method of separating these cytochromes for further study.

(b) Spectral characteristics of fractionated cytochromes

Broad range high resolution reduced minus oxidized difference spectra of material from elution peaks I, II and IV show that cytochrome is present in each of them but that there is no iron-sulphur protein or flavoprotein in these fractions (Fig. 47). The \(b\)-cytochrome \(\alpha\)-band region is shown in greater detail in Figure 48a in which both peaks I and IV are seen to exhibit largely symmetrical absorption peaks with maxima of about 556 nm. Peak II displays a complicated \(\alpha\)-band with three distinguishable overlapping absorption peaks, that of higher wavelength being more prominent. Fourth order derivative analyses of the \(\alpha\)-bands provided the values of the absorption maxima listed in Table XI from which the absorption maxima of peak II are seen to correspond to those of the similarly shaped \(\alpha\)-band of the unextracted residue from \(\text{cyd}^-\) cell membranes (Fig. 43a,3; 43b,3; section III.A.i.a). The Soret bands of the three cytochrome fractions are also shown in Figure 47. Although that of peak I is unremarkable the cytochrome of peak II produces a single, symmetrical Soret band in spite of the complexity of its \(\alpha\)-absorbance. Conversely, the single, symmetrical \(\alpha\)-band of the peak IV cytochrome is associated with a complex absorbance pattern in the Soret region, which may comprise two components of about equal absorbance. It will be noted that in comparison with the Soret band absorption the relative height of the \(\alpha\)-band is approximately twice
Fig. 47: Low temperature broad-range visible redox spectra of ion-exchange chromatographic fractions enriched in cytochrome peaks I, II and IV.

DEAE-BioGel A fractionation of partially purified cytochromes extracted from washed membranes by standard techniques. Cells were of *cyd*<sup>c</sup> strain PLJ01 and grown on succinate.

a. Peak IV; protein concentration = 1.80 mg mL<sup>-1</sup> : ΔA = 0.09,
b. Peak I; protein concentration = 0.76 mg mL<sup>-1</sup> : ΔA = 0.16,
c. Peak II; protein concentration = 1.68 mg mL<sup>-1</sup> : ΔA = 0.04.
Fig. 48: High resolution α-band spectra of ion-exchange chromatographic fractions enriched in cytochrome peaks I, II and IV.

Washed membranes were prepared from cells of cyd strain PLJ01 grown on succinate and their cytochromes were extracted and fractionated by the standard techniques. Samples were resuspended in 1.0 M sucrose, 100 mM potassium phosphate buffer, pH 7.0.

a. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77K.

1. Peak II ; protein concentration = 1.68 mg mL⁻¹ : ΔA = 0.01,
2. Peak IV ; protein concentration = 1.80 mg mL⁻¹ : ΔA = 0.05,
3. Peak I ; protein concentration = 0.76 mg mL⁻¹ : ΔA = 0.16.

b. Fourth-order finite difference spectra calculated from corresponding curves `a` by four successive first-order derivatizations.
as great in the peak IV cytochrome as in that of peak I, suggesting that the former may comprise two cytochromes \( b_{556} \) with distinct Soret features.

\section*{B) Cytochrome Fractions From Ion-Exchange Chromatography}

(i) \textbf{Peak I: Cytochrome} \( b_{556} \text{ Associated with Growth on Succinate}

(a) \textbf{Control of expression by growth substrate}

A cytochrome termed ‘cytochrome \( b_{556} \)’ had been linked to the aerobic respiratory chains of \textit{E. coli} from spectrophotometric investigations of membranes and associated fourth order derivative analyses \( [95, 180, 186] \). Anraku and coworkers had provided evidence from steady state and respiratory inhibition studies that cytochrome \( b_{556} \) is situated between the dehydrogenases and the terminal oxidase complexes, electron flow being mediated to and from this cytochrome by the quinones ubiquinone-8 and menaquinone-8 \( [95] \). These investigators had also isolated cytochrome \( b_{556} \) from a membrane extract solubilized in an ionic detergent mixture of Sarkosyl+cholate by repetitive gel filtration steps and had characterized the cytochrome’s fundamental structural features \( [102] \). The first purifications of both the cytochrome \( o \) and the cytochrome \( d \) terminal oxidases were also accomplished in Anraku’s laboratory: in each case membranes were solubilized in non-ionic Triton X-100 before an initial fractionation of the detergent extract by liquid chromatography via an anion-exchange matrix \( [97, 98, 99] \). Both of these extraction plus fractionation procedures resulted in the separation of cytochrome \( b_{556} \) although inspection of the published data indicated an unexplained anomaly in that the salt concentrations at which this cytochrome eluted from the two columns were extremely different. The fractionation of the cytochrome \( o \) complex using DEAE-Sepharose showed cytochrome \( b_{556} \) eluting well before any other cytochromes whereas fractionation of the cytochrome \( d \) complex via DEAE-Sephacel resulted in cytochrome \( b_{556} \) eluting after the majority of cytochromes \( [97, 98, 99] \). The current study had shown by both spectrophotometric and potentiometric techniques that there were several \( b \)-type cytochromes present in such cell membranes in addition to those of the terminal oxidases and although Anraku’s two
Conductivity of Peak # eluted fraction (mS cm\(^{-1}\)) cytochrome \(\lambda^Y\)\(_{\text{max}}\) (nm) cytochrome \(\lambda^B\)\(_{\text{max}}\) (nm) cytochrome \(\lambda^A\)\(_{\text{max}}\) (nm)

<table>
<thead>
<tr>
<th>Peak</th>
<th>I</th>
<th>5</th>
<th>425</th>
<th>(526), 528</th>
<th>556.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak</td>
<td>II</td>
<td>18</td>
<td>430</td>
<td>(525), 528+537</td>
<td>554.5+557.0+563.5</td>
</tr>
<tr>
<td>Peak</td>
<td>III</td>
<td>24</td>
<td>428</td>
<td>(528), 530+534</td>
<td>556.0</td>
</tr>
<tr>
<td>Peak</td>
<td>IV</td>
<td>30</td>
<td>426 + 435</td>
<td>(526), 528</td>
<td>556.5</td>
</tr>
</tbody>
</table>

**Table XI:** Absorption maxima of cytochrome fractions eluted from initial ion-exchange separation of solubilized membrane extracts.

Absorption maxima of high resolution reduced *minus* oxidized difference spectra measured at 77 K of the four recognisable cytochrome peaks eluted from DEAE-BioGel.A with a KCl gradient during the standard preparatory procedure developed for separating solubilized respiratory cytochromes. Values in parentheses indicate absorption peak 'shoulders'.
preparatory procedures were not identical it was possible that the cytochromes $b_{556}$ separated in each case were distinct species with different functions (sections II.A.i.b, II.A.ii.a, III.A.ii.a-c).

Attempts to reproduce and standardize the purification procedures described by Anraku indicated that liquid chromatographic ion-exchange fractionation of Triton solubilized cytochromes through a matrix of DEAE-BioGelA provided superior resolution than the use of either DEAE-Sepharose or DEAE-Sephacel. The purification and fractionation procedures developed and refined in the current study as described under Methods & Materials showed that there were at least two populations of cytochrome $b_{556}$ present in cyd$^+$ cell membranes grown under the appropriate conditions. One of these eluted from the initial ion-exchange column at particularly low salt concentrations as peak I in the $A_{412}$ elution profile and the other, peak IV, eluted at higher salt concentrations than were required to elute all other cytochromes (Fig. 46; Table XI). Moreover the presence of elution peak I and its constituent cytochrome $b_{556}$ was dependent on the carbon/energy source upon which the cells were grown (Fig. 46). Cells used by the laboratories of both Anraku and Gennis for the purpose of purifying aerobic terminal oxidases were grown on defined media with sodium lactate as carbon/energy source: Figure 46 shows that this is an excellent medium for the production of both fractionated peaks of cytochrome $b_{556}$. High resolution redox spectrophotometry indicated that the cytochromes in the two populations were not equivalent implying that multiple cytochromes $b_{556}$ could be present in aerobically grown E. coli (section III.A.ii.b; Fig. 47+48). Dual wavelength kinetic studies detected no reduction of the cytochromes from either elution peak in the presence of biological reductants, indicating that in this solubilized form neither of these two fractionated cytochromes $b_{556}$ was functioning in respiratory dehydrogenase activities (data not shown).

The cytochrome $b_{556}$ eluting as peak I in the $A_{412}$ elution profile was initially observed from fractionation of detergent extracts from solubilized membranes of cells grown on minimal medium supplemented with succinate as sole carbon/energy source and grown with vigorous aeration to stationary phase. Spectrophotometric analyses of membranes from aerobically grown cells, including those grown on glucose, had shown that they all contained significant quantities of a cytochrome with a 77 K absorption maximum at 556 nm (section II.A.i.b; Fig.19-21, Table VI) which had been identified as the ‘central’ cytochrome $b_{556}$ of aerobic respiration by Anraku’s respiratory inhibition studies (95). The absence of an elution peak I when extracts from glucose grown cells were fractionated in the current investigation indicated that the cytochrome $b_{556}$ of peak I could not be the ‘central’ cytochrome $b_{556}$ of aerobic respiration. This had been implied by Anraku in his purifications of the terminal oxidases by the suggestion that there was only a single cytochrome $b_{556}$ in the cytoplasmic membrane of aerobically grown E. coli (95, 143). The most
carbon/energy sources is as part of a dehydrogenase complex — possibly, in the case of the peak I cytochrome $b_{556}$, as part of the succinate dehydrogenase or lactate dehydrogenase complexes [87].

(b) **Association with succinate dehydrogenase**

At this time the existence of a type-$b$ cytochrome as part of the succinate dehydrogenase complex of *E. coli* was subject to controversy. No evidence linking a cytochrome $b$ to this enzyme had been found, although most other succinate dehydrogenases isolated from eucaryotic and some bacterial sources had been shown to incorporate a cytochrome $b$ and the enzyme activity in *E. coli* was known to be particularly labile [75, 76, 87]. It had been suggested, based on the resemblance of the enzyme’s subunit structure in *E. coli* to that from other sources, that not only might one of these subunits be the apoprotein of a cytochrome $b$ component, but that a facility for losing the prosthetic haem could account for the lability of succinate dehydrogenase activity in preparations from this organism [75, 87, 170]. The fumarate reductase of *E. coli*, structurally and functionally related to succinate dehydrogenase, had not been shown to contain a cytochrome $b$ [31, 113].

As these investigations proceeded Anraku’s laboratory repeated the assertion that only one cytochrome $b_{556}$ existed in aerobically grown *E. coli*, assigned the gene *cybA* to this product and mapped it to the 16 min region of the chromosome on the basis of gene dosage effects of $F'$ factors [143]. In addition Anraku’s group reported that the deletion $\Delta(kdp-gltA)$ prevented cells of strain TK3D11 from expressing this ‘central’ aerobic respiratory cytochrome $b_{556}$ studied in their earlier inhibition experiments [95, 143]. Three further reports suggested a possible link between the peak I cytochrome $b_{556}$ observed in this study and dehydrogenase activities: a type-$b$ cytochrome was identified as one of the four subunits of succinate dehydrogenase; the *sdh* operon was defined and sequenced with four component genes, the *sdhC* gene sequence bearing homologies to other cytochrome $b$ sequences; the $\Delta(kdp-gltA)$ deletion of strain TK3D11 was shown to extend into the *sdhC* gene [32, 144, 215].

The cytochrome abnormalities of cells carrying the $\Delta(kdp-gltA)$ deletion were investigated by growing cells of strain TK3D11 under this study’s standard conditions of high aeration using CYD minimal medium supplemented with specific carbon/energy sources. Although these cells were incapable of growth on succinate, as would be expected of a mutant lacking a functional succinate dehydrogenase, an attempt was made to induce the *sdh* gene products by growing these cells, and those of control strain GR19N, on another carbon/energy source in the presence of succinate.
**Fig. 49:** High resolution $\alpha$-band spectra and fourth order derivative analyses of resuspended membranes from strains GR19N and TK3D11 grown on DL-lactate and/or succinate.

Washed membranes were prepared from strains GR19N ($cyd^+$) and TK3D11 ($sdh^-$) after aerobic growth to stationary phase in CYD minimal medium in the presence of DL-lactate and/or DL-lactate plus succinate. The washed membranes were resuspended and spectrophotometry was performed at 77 K at final concentrations of 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0, with sample protein concentrations as indicated:

**a.** Dithionite reduced minus peroxide oxidized redox difference spectra, $\Delta A = 0.05$.
1. GR19N, grown with DL-lactate, membranes at 5.0 (mg protein) mL$^{-1}$.
2. GR19N, grown with DL-lactate plus succinate, membranes at 5.0 (mg protein) mL$^{-1}$.
3. TK3D11, grown with DL-lactate, membranes at 5.0 (mg protein) mL$^{-1}$.
4. TK3D11, grown with DL-lactate plus succinate, membranes at 5.0 (mg protein) mL$^{-1}$.

**b.** Fourth order finite difference spectra calculated from the corresponding redox difference spectra in ‘a’.
Glucose had been reported to repress succinate dehydrogenase levels in *E. coli* whereas the reverse was true for cells grown on lactate so the carbon/energy sources provided were DL-lactate alone or DL-lactate plus succinate (87). The cells of strain TK3D11 grew sufficiently slowly under the standard high aeration conditions in either of their appropriately supplemented minimal media that concentrations of the cytochrome \( d \) complex were insignificant. Resuspended membranes of strain TK3D11 grown on either medium displayed a distinctly different reduced minus oxidized \( \alpha \)-band at 77 K from those of the cyd\(^{-}\) controls, although cytochrome \( b_{556} \) was clearly a major component (Fig. 49). As the Anraku publications provided spectra of solubilized, fractionated cytochromes only, and none of the original membranes it is suggested that one or more cytochrome \( b_{556} \) species is indeed present in the membranes, but that none were solubilized by that laboratory's extraction procedure (143).

In addition to being present in extracts of membranes from succinate grown cyd\(^{-}\) cells elution peak I was also observed on fractionation of equivalent extracts from cells grown on DL-lactate and L-proline (Fig. 46). The resolution of the fractionation technique developed in this study permitted the distinction between the elution of the sharp peak I obtained from extractions of succinate or proline grown preparations (\( \mu = 8.0 \text{ mS cm}^{-1} \)) and the broader peak I produced by fractionation of extracts from cells grown on DL-lactate (\( \mu = 5.6 \text{ mS cm}^{-1} \)). Growth of cyd\(^{-}\) cells on a combination of succinate and DL-lactate generated an elution profile in which the peak I material eluted over a wider conductivity range, from 5 to 8 mS cm\(^{-1}\) suggesting that a distinction might exist between the cytochromes eluting in this peak originating from lactate grown cells and from those grown on succinate or proline although no spectrophotometric difference was observed between the peak I cytochromes \( b_{556} \) obtained from lactate grown and from succinate grown cyd\(^{-}\) cells, both displaying \( \alpha \)-bands with a very slight high-wavelength asymmetry (Fig. 49, 50, 51).

Lactate and succinate oxidase activities of resuspended membrane preparations from the TK3D11 cells and GR19N controls are provided in Table XII, confirming that the former mutant, proposed to carry a *sdh* deletion, lacks succinate oxidase and that these preparations contain both D-lactate and L-lactate oxidase activities. The cytochrome content of TK3D11 membranes appeared to contain a cytochrome \( c \) with 77 K reduced minus oxidized Soret and \( \alpha \)-band absorption maxima of 416 nm and 550 nm respectively. Figure 50 shows that fractionation of cyd\(^{-}\) membrane extracts following cell growth on lactate or on lactate plus succinate resulted in peak I cytochromes being eluted over the ranges stated above whereas membrane extracts from TK3D11 displayed different profiles. When TK3D11 cells were grown on lactate plus succinate a peak I eluted at the earlier conductivity range typical of lactate grown cells, although this peak was found to contain some
**Fig. 50:** DEAE-Biogel-A elution profiles of Triton membrane extracts from strain TK3D11 which lacks succinate dehydrogenase activity.

Cells of strains GR19N (cyd*, sdh⁺) and TK3D11 (cyd⁺, kdp⁻, sdh⁻) were grown to late exponential phase under high aeration on CYD minimal medium supplemented with the carbon/energy sources indicated below. Potassium phosphates and biotin were added to the TK3D11 cultures to bring the final concentrations to 50.0 mM and 25.0 mg mL⁻¹ respectively. Washed membranes were prepared and extracted by the standard techniques. The DEAE-Biogel-A was eluted under standard conditions with a KCl gradient.

a. Membrane extract from strain GR19N grown on DL-lactate + succinate,
b. Membrane extract from strain GR19N grown on DL-lactate,
c. Membrane extract from strain GR19N grown on succinate,
d. Membrane extract from strain TK3D11 grown on DL-lactate,
e. Membrane extract from strain TK3D11 grown on DL-lactate + succinate.
Results & Discussion

Peak # I II IV

Absorbance at 412nm (arbitrary units)

Conductivity (mS cm⁻¹)

Elution Volume
**Fig. 51:** High resolution visible range redox difference spectra of partially purified 'Peak I' cytochrome.

Cells of cyd\(^{-}\) strains PLJ01 and GR19N were grown to stationary phase on the carbon-energy sources indicated and used for Peak I preparation from Triton extracted membranes by the standard methods. Samples were analysed in 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0. The lactate associated cytochrome elutes from DEAE.BioGel.A somewhat earlier than the Peak I cytochrome obtained after growth on succinate and is shown for comparative purposes.

**a.** Dithionite reduced minus peroxide oxidized difference spectra obtained at 77K.

1. PLJ01 grown on succinate: protein concentration = 110 mg mL\(^{-1}\); \(\Delta A = 0.03\).
2. GR19N grown on lactate: protein concentration = 120 mg mL\(^{-1}\); \(\Delta A = 0.006\).

**b.** Fourth-order finite difference spectra calculated from corresponding curves 'a' by four successive first-order derivatizations.

**c.** Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.

1. PLJ01 grown on succinate: protein concentration = 110 mg mL\(^{-1}\); \(\Delta A = 0.10\).
2. GR19N grown on lactate: protein concentration = 120 mg mL\(^{-1}\); \(\Delta A = 0.04\).
cytochrome c in addition to type-b cytochrome (Fig. 51). Cells of strain TK3D11 grew particularly poorly in the presence of lactate plus succinate and the resulting elution profile exhibited more scatter in the A₄₁₂ data than was usual. Nevertheless no absorption was found at conductivity values corresponding to the succinate associated peak I cytochrome b₅₅₆, in contrast to the findings from the cyd⁻ control, and the gradient elution position of the peak I that was observed corresponded to that of extracts from lactate grown cells: it comprised cytochromes of types b and c (Fig. 49, 50).

Another sdhC⁻ strain, GVK124, was obtained from R. B. Gennis and grown in the presence of lactate and also with lactate plus succinate using cyd⁻ strain PLJ01 as control. More rapid growth of strain GVK124 was observed on vigorously aerated CYD minimal medium supplemented with DL-lactate than had occurred with sdhC⁻ strain TK3D11 and fractionated detergent extracts from membranes of the former produced A₄₁₂ elution profiles displaying significant interference from components of the cytochrome d complex (data not shown; refer to Fig. 44). Derivative strain KW531 was constructed by generalized transduction from sdhC::kan strain GVK124 into cyd⁻ strain PLJ01 using bacteriophage Plₙ₁₅, selecting for kanamycin resistance and screening for inability to grow on succinate plus a lack of cytochrome d when grown to stationary phase on DL-lactate. Membranes from cells of KW531 grown in the presence of DL-lactate and also lactate plus succinate contained L-lactate and D-lactate oxidase activities but no succinate oxidase (Table XII). Extraction and fractionation of these membranes resulted in the lactate grown samples producing elution profiles that displayed a peak I containing cytochrome b₅₅₆ and eluting at 5-6 mS cm⁻¹, similar to that of the PLJ01 control. The membrane extract from lactate plus succinate grown PLJ01 produced the broad peak I of cytochrome b₅₅₆ eluting between 5 and 8 mS cm⁻¹ observed in earlier controls, but the equivalent extracts from KW531 contained no material eluting at 8 mS cm⁻¹ (data not shown).

(c) Synopsis of ion-exchange chromatographic data relating to peak I

Thus it is suggested that there are multiple cytochromes b₅₅₆ in the cytoplasmic membrane of E. coli, some of which are inducible by carbon/energy sources such as succinate and lactate. The 'central' aerobic respiratory cytochrome b₅₅₆ investigated in the inhibition and spectrophotometric studies of membrane samples by Anraku and coworkers is probably less easily solubilized than the substrate inducible cytochromes b₅₅₆ observed in these experiments and in the later experiments from Anraku's laboratory ultimately linking 'cytochrome b₅₅₆' to the sdhC gene (95, 143, 144).
Table XII: Lactate and succinate oxidase activities of resuspended membrane preparations from cells grown on different carbon/energy sources.

Cells were grown under high aeration on CYD minimal medium supplemented with the indicated carbon/energy sources at 0.4% (w/v). Rates are based upon depletion of dioxygen from membrane suspensions as indicated by measurement of total cytochrome reduction by dual wavelength studies at 560 nm minus 585 nm. The oxygen concentration at 25°C was assumed to be 258 μM (258 μ gram atom L⁻¹); activities are expressed as nmol O uptake min⁻¹ (mg protein)⁻¹ (126). Substrates, lithium salts of D- or L-lactate or disodium succinate were present at final concentrations of 50 mM.
are at least two cytochromes $b_{556}$ inducible by growth substrate that elute as components of peak I in the solubilization and fractionation technique described in this study: one of these elutes in from the DEAE-BioGel.A column as the salt gradient generates a conductivity of $7.5$ to $8.5\, \text{mS cm}^{-1}$ ($n > 20$), has been associated with the presence of succinate dehydrogenase activity in cells and is absent from cells incapable of expressing the succinate dehydrogenase cytochrome $b$, the $sdhC$ gene product. The other peak I cytochrome $b_{556}$ elutes in the salt gradient at a slightly lower conductivity value of $5.0$ to $6.0\, \text{mS cm}^{-1}$ ($n = 4$) and is associated with growth in the presence of DL-lactate. Since both L-lactate and D-lactate oxidase activities are present in the cell membranes cytochromes of this fraction may function in association with the related dehydrogenase activities.

A recent collaborative publication describes the expression and purification of an active preparation of cloned succinate dehydrogenase using Lubrol PX as the solubilizing detergent and confirming the identity of the $sdhC$ gene product as a cytochrome $b_{556}$ [101].

(d) **Biophysical parameters of 'succinate grown' Peak I cytochrome $b_{556}$**

The succinate inducible cytochrome $b_{556}$ from elution peak I was subjected to exclusion chromatography through a calibrated Sephacryl S-200 column which provided a Stokes' radius value for this protein of $52\,500 \pm 1\,500$ (data not shown). This is close to the estimates of the combined $M_r$ of the three smallest subunits of the *E. coli* succinate dehydrogenase from electrophoretic estimations (58,000) and for their molecular weights predicted from the DNA sequence (53,596) [32, 35, 215].

The former study incorporated immunoprecipitation of the enzyme complex solubilized with Lubrol PX in the presence of a cocktail of protease inhibitors and determined $M_x$ values of 71,000, 26,000, 17,000 and 15,000 for four discrete peptides (similar to those of the bovine heart mitochondrial succinate:ubiquinone oxidoreductase) [32]. It was noted that the 71 kDa flavopeptide appeared to dissociate from the complex in the presence of Triton X-100 and that this subunit was readily susceptible to proteolysis. A type-$b$ cytochrome, capable of being reduced by succinate in the Lubrol PX extracts, was associated with the immunoprecipitated complex and assigned to one of the two smallest subunits. Purified cytochrome $b_{556}$ obtained from the Anraku laboratory yielded two polypeptides running in the same region as these smallest subunits when analysed by SDS-polyacrylamide gel electrophoresis [32]. Sequence analysis of the $sdh$ operon indicated that the four genes $CDAB$ code for peptides of molecular weight 14,167, 12,792, 64,268 and 26,637, respectively [35, 215]. Between the two promoter regions closely situated upstream of $sdhC$ is
another region strongly resembling the consensus sequence of the binding site for the catabolite-gene activator protein, providing a possible mechanism for the catabolite repression of succinate dehydrogenase activity that has been reported as well as for the failure of cells grown on glucose to produce the cytochrome $b_{556}$ of elution peak I that was observed in the current study \cite{75, 215}.

In order to obtain sufficient quantities of partially purified peak I cytochrome $b_{556}$ for potentiometric titration a bulk culture of cyd$^+$ strain PLJ01 was grown under high aeration on CYD minimal medium supplemented with succinate and used to produce partially purified peak I cytochrome $b_{556}$ by the standard method. This cytochrome preparation was titrated after adjustment of the pH to 7.0 and was shown to contain a single cytochrome with mid-point potential of $+20$ mV \cite{fig52}. Although this value may be modified by the presence of substantial concentration of Triton X-100 and by the possibility that the active complex has partially dissociated it is in an appropriate range for a component of a dehydrogenase with a substrate couple having a mid-point potential close to zero: that of the succinate/fumarate couple being $+30$ mV at pH 7.0 \cite{145}.

A mid-point potential of $+36$ mV was reported for the cytochrome $b_{556}$ component of the recently purified preparation of cloned succinate dehydrogenase, compared to an earlier value of $-45$ mV for the enzyme complex solubilized and purified in the detergent Sarkosyl \cite{101, 102}. The cloned enzyme complex was solubilized and isolated in Lubrol PX, and retained the capability for the cytochrome to be reduced by succinate whereas neither the Triton solubilized nor Sarkosyl solubilized preparations were reducible by succinate. The divergence of the potentiometric result from the others after solubilization in Sarkosyl suggests a severe alteration of the haem environment occurs under these conditions, ionic detergents often causing more protein denaturation than non-ionic detergents \cite{77, 82}. The recent publication also claims a value of $+35$ mV for the mid-point potential of the succinate dehydrogenase type-$b$ cytochrome measured in membrane suspensions, referring to earlier membrane titrations which were of low resolution and which identified the cytochrome $b_{556}$ by spectral means only, thereby leaving its composition and identity open to conjecture \cite{101, 117}.

(ii) **Peak II: Cytochrome $o$ Complex**

Elution peak II from the ion-exchange fractionation of solubilized membrane extracts through DEAE.BioGel.A was the largest and most intensely red coloured fraction in virtually all separations. Although peak II was generally well separated from the other cytochrome fractions produced by the
Fig. 52: Potentiometric titration of partially purified 'Peak I' cytochrome.

Cells of *cyd* strain PLJ01 (grown in bulk on succinate under high aeration) were used to generate adequate quantities of partially purified peak I cytochrome by the standard method. This cytochrome preparation was titrated in TTE buffer, with pH adjusted to 7.0 and a protein concentration of 0.9 mg mL$^{-1}$. All other titration conditions were standard.

a. Direct plot of Peak I titration data, the curve indicates a theoretical one-component best fit of the data, (—-) one-component fit; $E_m = +19.6$ mV vs. NHE.

b. Nernst plot of data in 'a': $E_m = +21.0$ mV vs. NHE.
fractionation procedure samples from cells grown on the L-proline medium MMP generated a small, mixed cytochrome fraction the leading edge of which overlapped the trailing edge of peak II such that only a minor portion of the latter material was contaminated. The elution and spectrophotometric properties of peak II are provided in Table XI. The ability of this fraction to transfer electrons to dioxygen efficiently from quinol substrates, its content of type-\(b\) cytochromes with an \(\alpha\)-band absorbance in the 560 nm to 565 nm range and the spectral sensitivity of certain of these cytochromes to carbon monoxide suggested the presence of the cytochrome \(o\) terminal oxidase complex. This supposition was subsequently confirmed by several means, including further purification, perturbation spectrophotometry and potentiometry, SDS gel electrophoresis and spectrophotometric comparison with material purified by other laboratories. These experiments are described in detail in section III.C.ii.a+b.

(iii) Peak III: Cytochromes \(b\) Associated with Growth on L-Proline

(a) Control of expression by growth substrate

Peak III was not fully resolved from material included in the trailing edge of the larger peak II during the elution of solubilized membrane extracts from the DEAE.BioGel.A ion-exchange columns (Fig. 46). Yet the spectral properties of the cytochromes contained in peak III were sufficiently distinct that they were known to be present solely in those extracts derived from cells grown on L-proline and not in those from cells grown in any of the other media used in this investigation \((v.i.)\). The proline medium was richer than the standard CYD minimal medium and the former also contained selenium and molybdate supplements that may have contributed to the cells' ability to synthesize additional cytochromes in the later phases of growth (Appendix 'C') \([71, 87, 112]\). The well defined cytochrome redox \(\alpha\)-band spectrum of peak II permitted additional procedures to isolate peak III cytochromes to be assessed conveniently by redox spectrophotometry. Similarly, peak P was recognised as a minor fraction containing cytochrome which eluted immediately after peak III in proline grown cells.

The analysis of the membrane cytochrome complement of \(cyd^+\) cells grown aerobically on L-proline has been described above. The spectrophotometric studies showed that the cytochrome \(o\) complex constituted about 20% of the cytochrome \(b\) absorbance while the potentiometric titration suggested that there were rather greater quantities of low potential cytochromes present in such
**Fig. 53:** DEAE-BioGel.A and BioGel.HTP elution profiles of Triton membrane extracts from cells grown on L-proline.

Cells of strain GR19N were grown under moderate aeration to stationary phase on CYD minimal medium containing L-proline. Washed membranes were prepared and extracted by the standard techniques and the DEAE-BioGel.A was eluted under standard conditions with a KCl gradient. The fractions labelled 'Peak II/III' were pooled, loaded onto a BioGel.HTP column and fractionated with a potassium phosphate gradient in TTE buffer, pH 7.8 as described under Materials & Methods.

**a.** DEAE-BioGel.A fractionation of Triton membrane extracts.

Cytochrome peaks are labelled "I, II/III, P, IV" as described in the text.

1. (-----) Absorbance at 412 nm : \( \Delta A_{412} = 1.000 \).

2. (- - - -) Lowry protein assay : \( \Delta A_{660} = 0.500 ; 1.91 \text { mg mL}^{-1} \) eluate.

**b.** BioGel.HTP fractionation of pooled Peak II/III from 'a'.

Cytochrome peaks are labelled "H₁, H₂, H₃" as described in the text.

1. (-----) Absorbance at 412 nm : \( \Delta A_{412} = 0.060 \).

2. (- - - -) Lowry protein assay : \( \Delta A_{660} = 0.125 ; 302 \mu \text{g mL}^{-1} \) eluate.
(Results & Discussion)

```
Peak # I II/III P IV

a.

b.

ΔA

Conductivity (mS cm⁻¹)

Elution Volume
```
membranes than those grown on glucose, lactate or succinate (sections II.A.ii.a+b; Figs. 25+26 & 33+34). Component cytochromes of solubilized membrane preparations were separated into the standard groups, including all those containing peak III material (termed peak II/III) and those in peak P without any peak III material (termed peak P), based on the absolute absorbance at 412 nm of fractions eluted from a DEAE-BioGel.A column. Fractions constituting peak II/III were further separated by chromatography via a BioGel-HTP column, all cytochromes in this sample initially binding to the hydroxylapatite and eluting under the applied phosphate gradient to yield three major peaks of cytochrome, $H_1$, $H_2$ and $H_3$. The elution profiles from these procedures are shown in Figure 53 and the redox spectra plus derivatized spectra in Figures 54, 55 & 56.

(b) Partial purification and analysis of cytochrome components

The reduced minus oxidized 77 K a-band absorption spectrum of the Triton X-100 membrane extract was fairly broad, asymmetrical and showed a preponderance of cytochrome absorbing between 555 nm and 557 nm (Fig. 54a,1). A small shoulder at 560 nm to 565 nm suggested the presence of cytochrome $o$ complex in the extract. Fourth order derivative analysis clearly indicated a component with an absorption maximum at 555 nm and another at 563 nm, the Triton modified position of the cytochrome $b_{560}^+$ components (Fig. 54b,1). The presence of other type-\(b\) cytochromes with absorption maxima at 557 nm and 560 nm is indicated, but the signal amplitude was small. In comparison the $\alpha$-band absorption spectrum of the unextracted residue was biphasic, showing similar features to those of the extract but with the red shoulder amplified relative to the broad maximum into a distinct, albeit poorly defined, peak (Fig. 54a,2). Absorption maxima derived from the fourth order derivative spectrum correspond closely to those of the cytochrome $o$ complex suggesting that the terminal oxidase complex was a major component remaining with the unextracted material (section III.A.i.a; Fig. 43). The shape and relative height of the maxima of the redox absorption spectra indicate that the cytochromes absorbing between 555 nm and 557 nm partitioned mainly into the detergent, leaving a moderate amount of material in the residue, whereas the reverse was true for the cytochrome $o$ complex of which approximately 20 % was extracted by the standard procedure. Anion exchange chromatography of the extract generated the partially resolved absorption profile depicted in Figure 53a. Redox spectrophotometry of the major peak indicated that it contained cytochrome $o$, corresponding to the standard peak II, but that eluting throughout this region was a large quantity of one or more cytochromes $b$ with an $\alpha$-band absorption maximum at 555 nm (Fig. 54c+d). In addition peak III, merged with the trailing half of peak II, contained cytochromes
Fig. 54: High resolution visible range redox difference spectra of partially purified ‘Peak III’ cytochrome.

Cells of cyd" strain GR19N were grown in bulk on CYD minimal medium containing L-proline to stationary phase. Standard techniques were used to obtain partially purified Peak III cytochrome from Triton extracted membranes by liquid chromatography via DEAE-BioGel A, cytochromes being eluted under standard conditions with a KCl gradient. Sample ‘2’ was resuspended and analysed in 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0 and all others were analysed in 75 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0 containing 25% TTE buffer.

a. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.
   1. Extracted sample as loaded onto column: protein = 1.25 mg mL\(^{-1}\) \(\Delta A = 0.013\),
   2. Unextracted residue: protein concentration = 1.30 mg mL\(^{-1}\) \(\Delta A = 0.032\).

b. Fourth-order finite difference spectra calculated from corresponding curves ‘a’ by four successive first-order derivatizations.

c. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.
   3. Peak II/III (a): protein concentration = 0.37 mg mL\(^{-1}\) \(\Delta A = 0.050\),
   4. Peak II/III (b): protein concentration = 0.98 mg mL\(^{-1}\) \(\Delta A = 0.050\),
   5. Peak P: protein concentration = 1.31 mg mL\(^{-1}\) \(\Delta A = 0.008\).

d. Fourth-order finite difference spectra calculated from corresponding curves ‘c’ by four successive first-order derivatizations.
Fig. 55: High resolution visible range redox difference spectra of partially purified ‘Peak III’ cytochrome fractionated on hydroxylapatite.

Cells of cyd" strain GR19N were grown in bulk on L-proline to stationary phase. Peak III cytochrome was partially purified from Triton extracted membranes by liquid chromatography via DEAE-BioGel A and BioGel HTP columns as described under Materials & Methods. Samples were analysed in 75 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0 containing 25 % TTE buffer.

a. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.
   1. Loaded sample : protein concentration = 0.72 mg mL\(^{-1}\); \(\Delta A = 0.020\),
   2. Peak H1 : protein concentration = 1.49 mg mL\(^{-1}\); \(\Delta A = 0.020\),
   3. Peak H2 : protein concentration = 0.35 mg mL\(^{-1}\); \(\Delta A = 0.020\),
   4. Peak H3 : protein concentration = 0.06 mg mL\(^{-1}\); \(\Delta A = 0.010\).

b. Fourth-order finite difference spectra calculated from corresponding curves ‘a’ by four successive first-order derivatizations.
absorbing at 557.5 nm and 560.0 nm (Fig. 54c+d). Peak P was a minor contributor to the A\textsubscript{412} elution profile, containing cytochromes b\textsubscript{555} and those associated with cytochrome o plus a large proportion of iron-sulphur protein; no other fractions being found to contain significant quantities of this material (Fig. 54c+d; 56b,5).

Fractions contributing to peak III were pooled and fractionated by hydroxylapatite chromatography producing the absorption profile in Figure 53b. The pooled sample contained about one quarter of the b-cytochrome reduced minus oxidized absorbance as cytochrome o complex, judged from the maximum and 560 nm \(\alpha\)-band absorption values (Fig. 55a). This eluted, with most of the protein, early in the phosphate gradient at \(<\ 5\ \text{mS cm}^{-1}\) (Fig. 53b; 55a,2; 55b,2). Two other major cytochrome elution peaks were detected, \(H_2\) and \(H_3\) at 18 mS cm\(^{-1}\) and 35 mS cm\(^{-1}\) respectively, these comprising cytochromes with \(\alpha\)-band maxima at 555.0 nm, 557.5 nm and 560.5 nm, the relative quantity of the cytochrome b\textsubscript{555} being rather greater in peak \(H_2\) (Fig. 53b; 55a,3+4; 55b,3+4).

SDS-polyacrylamide gel electrophoresis of fractions throughout the profile indicated that the cytochromes were among many protein constituents of each fraction (Fig. 57). Subunits of the cytochrome o complex were clearly visible in peak \(H_1\) (\(v.i.\)) and distinct components with \(M_r = 24\ 000\) and 27 000 were eluted in the \(H_2\) peak. It is unknown whether these peptides are related to cytochromes either as apoprotein or holoprotein for haem staining of the low protein content of these gels by the TMBZ peroxidase technique was unsuccessful, possibly because of the small quantities of material present (Materials & Methods).

These results demonstrate that multiple cytochromes b are present in the membranes of \(E.\ coli\) grown aerobically on L-proline. It has been shown above and elsewhere that such cells contain pools of respiratory cytochromes that equilibrate at different rates. Wood and coworkers have shown that oxidation of L-proline by the action of proline dehydrogenase proceeds by electron transfer to oxygen through components of the aerobic respiratory chains \([1, 58]\). Although external electron acceptors could be used to obtain oxidase activity from the dehydrogenase the inability of a strain auxotrophic for 5-aminolaevulinic acid to utilize L-proline as sole carbon/energy source indicated a requirement for porphyrin biosynthesis and therefore cytochromes in the integrated dehydrogenase system \([1]\). The current data suggest that synthesis of specific b-type cytochromes may be required for catabolism of L-proline.
**Fig. 56:** Broad range visible redox difference spectra of partially purified ‘Peak III’ cytochrome.

Cells of *cyd*-' strain GR19N were grown in bulk on CYD minimal medium containing L-proline to stationary phase. Standard techniques were used to obtain partially purified Peak III cytochrome from Triton extracted membranes by liquid chromatography via DEAE-BioGel.A and BioGel.HTP. Samples were analysed in 75 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0 containing 25% TTE buffer.

Dithionite reduced *minus* peroxide oxidized difference spectra obtained at 77 K.

**a.** Samples from extraction procedure:

1. Extracted sample as loaded onto column: protein = 1.25 mg mL\(^{-1}\); \(\Delta A = 0.013\),

2. Unextracted residue: protein concentration = 1.30 mg mL\(^{-1}\); \(\Delta A = 0.032\).

**b.** Samples from DEAE-BioGel.A (anion exchange) fractionation:

3. Peak II/III a: protein concentration = 0.37 mg mL\(^{-1}\); \(\Delta A = 0.050\),

4. Peak II/III b: protein concentration = 0.98 mg mL\(^{-1}\); \(\Delta A = 0.050\),

5. Peak P: protein concentration = 1.31 mg mL\(^{-1}\); \(\Delta A = 0.008\).

**c.** Samples from BioGel.HTP (hydroxlapatite) fractionation:

6. Sample as loaded: protein concentration = 0.72 mg mL\(^{-1}\); \(\Delta A = 0.020\),

7. Peak \(H_1\): protein concentration = 1.49 mg mL\(^{-1}\); \(\Delta A = 0.020\),

8. Peak \(H_2\): protein concentration = 0.35 mg mL\(^{-1}\); \(\Delta A = 0.020\),

9. Peak \(H_3\): protein concentration = 0.06 mg mL\(^{-1}\); \(\Delta A = 0.010\).
Fig. 57: Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis of 'Peak III' cytochrome.

SDS-PAGE of 'Peak III' cytochrome following partial purification by liquid chromatography via DEAE-BioGel.A and BioGel.HTP. The Laemmeli system was used with a 13% acrylamide gel but without heating the sample. Samples run on gel A were prepared by the standard procedure, including treatment with β-mercaptoethanol, those run on gel B were not treated with β-mercaptoethanol. Sample lanes correspond to every third fraction from the phosphate elution gradient; 1, 2 and 3 indicate the major cytochrome-containing fractions H₁, H₂ and H₃. Positions of standard molecular weight markers are shown: see Methods & Materials for details.
(iv) **Peak IV : A Cytochrome b$_{556}$ Hydroperoxidase Expressed at Low pO$_2$**

(a) **Control of expression**

The cytochrome $b_{556}$ separating in anion exchange chromatography as peak IV was well separated from the other eluted peaks absorbing light at 412 nm and was characterized by vigorous hydroperoxidase activity. This fractionated cytochrome peak was observed when the originating cells were grown on all of the growth substrates tested in this study. Nevertheless this late-eluting cytochrome was not found in all preparations: fractionation of membrane extracts from cells harvested in early exponential phase had little, if any of this material whereas all cultures grown to stationary phase showed large quantities in equivalent extracts (Fig. 58). As in the case of expression of the cytochrome $d$ complex, the coordination of the detection of a particular protein with the later stages of growth of a liquid culture may be the result of any of a number of factors. Exhaustion of required nutrients and the generation of waste or toxic products may be associated with the transition of the culture from exponential to stationary growth phase and many control mechanisms govern the observed responses \[4, 27, 56, 87, 157\].

In aerobically grown liquid cultures one of the major restrictions on the rate and extent of growth is the ability of the medium to absorb an adequate supply of dioxygen through its surface to cater to the multiplying quantity of respiring cells \[56, 167, 172\]. Therefore a standard culture of cyd$^{-}$ cells was incubated under differing conditions of aeration to generate either high or low oxygen tensions as the cells grew (see Materials & Methods). As these cultures would also create greater demands for oxygen as the numbers of cells grew the oxygen tension could not be maintained at a constant level throughout the growth of the culture, but by employing the conditions described under Materials & Methods the supply of oxygen could be restricted to one culture throughout from an early stage of incubation and maintained at saturating levels for a greater proportion of the growth period in the other. The growth rate of the cultures (and thus their oxygen requirements) are dependent upon the oxygen supply — especially in the case of cyd$^{-}$ cells which must rely upon the cytochrome $o$ terminal oxidase with its higher $K_m$ for dioxygen \[87, 112, 157\]. However, the use of cyd$^{+}$ strains provided significant advantages in that the cytochrome $d$ complex could not interfere with the cells’ oxygen requirements by being induced at different stages of growth in the two cultures, neither was it able to interfere with the fractionation process (Fig. 44 + 45). A straightforward result was obtained in that the high aeration culture had developed minimal amounts of
Fig. 58: DEAE-BioGel.A elution profiles of Triton membrane extracts from cells harvested at different phases of growth.

Cultures of cyd" strain PLJ01 were grown under high aeration on CYD minimal medium containing succinate to the phase of growth indicated below. Washed membranes were prepared and extracted by the standard techniques. The DEAE-BioGel.A was eluted under standard conditions with a KCl gradient.

a. (———) Absorbance at 412 nm:

1. Cells harvested in early exponential phase; $\Delta A_{412} = 0.150$.
2. Cells harvested in stationary phase; $\Delta A_{412} = 0.080$.

b. (-----) Lowry protein assay:

1. Cells harvested in early exponential phase; $\Delta A_{660} = 0.350$; 283 mg mL$^{-1}$ eluate.
2. Cells harvested in stationary phase; $\Delta A_{660} = 0.125$; 444 mg mL$^{-1}$ eluate.
the peak IV cytochrome when harvested in stationary phase and the culture with the limited oxygen supply contained quantities with total absorbance at 412 nm of about 50% of the absorption of peak II cytochrome α complex by comparison of peak areas (Fig. 59). Intermediate estimations between these maximal and minimal oxygen supplies are not meaningful without a reliable method of controlling the oxygen supply to each culture as a function of the dissolved oxygen concentration.

(b) Redox spectrophotometry

The spectrophotometric properties of the peak IV cytochrome have been described above (section III.A.ii.b; Fig. 47,48). It comprises type-β cytochrome with a single α-band but twin Soret peaks. The redox absorption maxima are provided in Table XI. Although β-cytochromes with hyperporphyrin spectra (biphasic Soret absorbance properties) have been recorded, such characteristics are particularly uncommon, and have been shown to be associated with endogenous cysteinate axial ligation in the procaryotic haemoproteins chloroperoxidase from *Caldariomyces fumago* and cytochrome P-450 from *Pseudomonas putida* [190, 208]. More likely was the possibility that there were two distinct cytochromes in the preparation, either having identical α-band absorption maxima so that the resulting mixed spectrum was symmetrical or that the α-band observed was due principally to one of the two cytochromes and the other provided a minimal contribution to the α-band absorption. In this case the Soret absorbance at 426 nm would probably be associated with the observable redox peak at 556 nm for these values for absorbance maxima correspond to those displayed by other cytochromes b_{556} (Table XI) [87, 112]. Additionally cytochromes known to have high spin haem-β have Soret bands with absorption maxima at longer wavelength than many low spin type-β haemoproteins [112, 216]. One example of a cytochrome β with minimal α-band absorbance has already been described: the high spin iron of bovine heart catalase results in a redox spectrum with a red-shifted Soret absorption in addition to minimal α-band features (Fig. 6) [216].

The 412 nm absorption profiles of the salt gradient eluate from the anion exchange column generally provided an asymmetric peak IV. While there was some tailing of the elution peaks, this was minimized by the slow elution rate (see Materials & Methods). Samples of peak IV cytochrome were taken from leading and trailing fractions and analysed by high resolution redox spectrophotometry at 77 K. Figure 60 shows that there were no significant differences in the α-bands or their fourth order derivatives from different parts of the elution peak. Interpretation of the Soret region of the leading fractions was obscured by background absorption by non-cytochrome
Fig. 59: DEAE-BioGel.A elution profiles of Triton membrane extracts from cultures grown under different oxygen tensions.

Cultures of cyd$^+$ strain PLJ01 were grown on CYD minimal medium containing succinate under conditions of either high or low aeration to stationary phase. Washed membranes were prepared and extracted by the standard techniques. The DEAE-BioGel.A was eluted under standard conditions with a KCl gradient.

a. Low aeration culture conditions (as described under Materials & Methods),

b. High aeration culture conditions (as described under Materials & Methods).
Absorbance at 412nm (arbitrary units) vs Elution Volume for Peaks I, II, and IV.

Graph shows two curves labeled 'a' and 'b', with 'a' appearing to rise more sharply than 'b'.
Fig. 60: High resolution visible range redox spectra of partially purified 'Peak IV' cytochrome.

Cells of cyd" strain PLJ01 were grown to stationary phase on CYD minimal medium containing glucose. Peak IV cytochrome was partially purified from Triton extracted membranes by the standard methods of membrane preparation, detergent extraction and cytochrome fractionation via DEAE-BioGel A. Samples were analysed in 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0.

a. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.
   1. Peak IV 'leading edge': protein concentration = 1.26 mg mL⁻¹; ΔA = 0.032,
   2. Peak IV 'trailing edge': protein concentration = 1.47 mg mL⁻¹; ΔA = 0.040.

b. Fourth-order finite difference spectra calculated from corresponding curves 'a' by four successive first-order derivatizations.

c. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.
   1. Peak IV 'leading edge': protein concentration = 1.26 mg mL⁻¹; ΔA = 0.100,
   2. Peak IV 'trailing edge': protein concentration = 1.47 mg mL⁻¹; ΔA = 0.100.
material, but the trailing fractions clearly displayed twin Soret maxima of equivalent amplitude and maxima at 426.0 nm + 434.0 nm (Fig. 60c).

(c) **Further purification of cytochrome**

In an attempt to determine whether the cytochrome 6555 from peak IV comprised more than one cytochrome species it was concentrated and subjected to gel exclusion chromatography as described under **Materials & Methods**. This procedure accomplished several functions: it achieved an additional purification step, enabled the detergent concentration to be decreased to 0.2 % (w/v) and the buffer to be exchanged for 100 mM potassium phosphate buffer, pH 7.0, in preparation for potentiometric titration. Calibration of the gel exclusion columns permitted determination of the Stokes’ radius of the eluted chromophores.

Fractionation though Sephacryl S-200 resulted in most of the protein in the sample being excluded from the gel. The bulk of the visible chromophore eluted in the fractions immediately following the void volume, their mobility indicating an $M_r > 100\,000$, and this material, designated peak IVa, was reconcentrated by ultrafiltration and reloaded onto a column of Sephacryl S-300 (Fig. 61a). Some 10-15 % of the coloured material eluted from the S-200 column at a position indicating a Stokes’ radius of $52\,500 \pm 1\,500$ and was termed peak IVb (Fig. 61a).

Fractionation of the coloured material eluting immediately from the S-200 column with $M_r > 100\,000$ by chromatography through the gel exclusion Sephacryl S-300 matrix resulted in a small quantity of chromophore eluting in the void volume (peak IVc : $M_r > 1\,000\,000$) and greater than 95 % as a single, partially included peak termed IVd with Stokes’ radius of $386\,000 \pm 10\,000$. This latter fraction contained the vigorous hydroperoxidase activity noted as a characteristic of the original peak IV from the DEAE-BioGel.A column.

(d) **Spectrophotometric properties of fractions from gel filtration**

The $\alpha$-bands of 77 K reduced minus oxidized difference spectra plus their fourth order derivatives from Sephacryl S-200 peaks IVa and IVb and Sephacryl S-300 peak IVd which all contained $b$-type cytochrome are illustrated in Figure 62. The minor peak IVc which eluted in the void volume from the S-300 column with an apparent molecular weight in excess of one million daltons appeared to contain little cytochrome but had a broad redox absorption peak with a maximum of 418 nm (Fig. 63b,1).
Fig. 61: Gel filtration elution profiles of 'Peak IV' cytochromes from Sephadryl S-200 and S-300 matrices.

Pooled Peak IV cytochrome from the DEAE-BioGelA anion exchange process was further purified via (7.5 x 420)mm columns of Sephadryl S-200 and S-300 gels as described under Materials & Methods. Void elution volumes indicated by arrows.

(-----) Absorbance at 412 nm ; (-----) Lowry protein assay measured at 660 nm.

a. Sephadryl S-200 elution profile : \( \Delta A_{412} = 0.80 ; \Delta A_{660} = 0.40, 4.0 \text{ mg mL}^{-1} \text{ eluate.} \)
   
   Peak IVa : \( M_r > 100 000, \)
   
   Peak IVb : \( M_r = 52 500 \pm 1 500. \)

b. Sephadryl S-300 elution profile : \( \Delta A_{412} = 0.40 ; \Delta A_{660} = 0.40, 2.7 \text{ mg mL}^{-1} \text{ eluate.} \)
   
   Peak IVc : \( M_r > 1 000 000, \)
   
   Peak IVd : \( M_r = 386 000 \pm 10 000. \)
Peak # | IVa | IVb
---|---|---
10 | 14 | 18
22 | 26 | 30

Elution Volume (ml)

ΔA

a.

ΔA

b.

IVc | IVd
Fig. 62: High resolution redox difference spectra of 'Peak IV' cytochrome subfractions separated by gel exclusion chromatography.

Low temperature spectra of Peak IV cytochrome following partial purification by liquid chromatography via DEAE-BioGel A, Sephacryl S-200 (Peaks IVa, IVb) and Sephacryl S-300 (Peak IVd). Samples were analysed in 0.1% (w/v) Triton X-100, 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0.

a. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.

1. **Peak IVa**: (from S-200) protein concentration = 580 μg mL⁻¹; ΔA = 0.010,
2. **Peak IVb**: (from S-200) protein concentration = 450 μg mL⁻¹; ΔA = 0.005,
3. **Peak IVd**: (from S-300) protein concentration = 176 μg mL⁻¹; ΔA = 0.002.

b. Fourth-order finite difference spectra calculated from corresponding curves ‘a’ by four successive first-order derivatizations.
Figure 62 demonstrates that peak IVa, which was largely excluded from the S-200 matrix, displayed a symmetrical cytochrome \(\alpha\)-band with redox absorption maximum at 556 nm. The minor peak IVb contained type-\(b\) cytochrome with a maximum absorption at 556 nm and a closely fused shoulder of almost equal intensity at about 557 nm (Fig. 62a, 62b). The absorption troughs at about 450 nm and 480 nm broad range redox spectra of these two samples showed that both contained large quantities of flavoprotein and/or iron-sulphur protein (Fig. 63a). Subtraction of the peak IVa spectrum from that of IVb resulted in an acceptably level baseline with cytochrome absorption bands of approximately 50% of the intensity of those of IVb (Fig. 63a). This suggests that there may be a compositional distinction between the contents of the two peaks and that peak IVb contained twice as many cytochrome \(b\) molecules per flavoprotein as peak IVa. Since peak IVa had the higher molecular weight and was present in far greater quantity, it was proposed that the complex contained at least two units of both cytochrome \(b\) and flavoprotein, and that peak IVb constituted a fraction in which one of the flavoprotein units had been lost. Although the asymmetry observed in the \(\alpha\)-band reduced minus oxidized spectrum of the type-\(b\) cytochrome of peak IVb may have been caused by a conformational alteration of the cytochrome coinciding with the dissociation of flavoprotein from the complex peak IVa, both elution peaks displayed twin Soret-band absorption upon redox spectrophotometry lending support to the possibility of two separate cytochrome species. The molecular size of the complex in comparison to that of the minor fraction with molecular weight 52.5 kDa suggests that either more peptides may have been lost from the latter in addition to a flavoprotein unit, or that the complex aggregates when that unit has not dissociated. The possibility of aggregation seems most likely for the molecular size of the major cytochrome containing fraction obtained when the complex was fractionated on Sephacryl S-300 was 386 kDa (Fig. 61b).

Gel exclusion chromatography through Sephacryl S-300 produced a narrow elution peak containing virtually all the cytochrome, in association with flavoprotein, that had been loaded onto the column: peak IVd (Fig. 61b). This material provided a redox spectrum strongly resembling that of the loaded sample, peak IVa. It also contained some of the chromophore absorbing light at 420 nm in the 77 K redox spectrum which was also observed in the void volume eluate from this second gel filtration step, peak IVc (Fig. 63b). Subtraction of the latter spectrum from that of the complex generated a spectrum of a cytochrome \(b\) with twin, fused Soret bands and decreased flavoprotein absorbance (Fig. 63b). The redox \(\alpha\)-absorption band of the complex at 77 K is shown to maintain its 556 nm absorption maximum, although the fourth order derivative spectrum indicates a splitting of the absorption peak into two components at 556.0 nm and 558.0 nm.
**Fig. 63:** Broad range visible redox difference spectra of ‘Peak IV’ cytochrome subfractions separated by gel exclusion chromatography.

Low temperature spectra of Peak IV cytochrome following partial purification by liquid chromatography via DEAE-BioGel A, Sephacryl S-200 (Peaks IVa, IVb) and Sephacryl S-300 (Peaks IVc, IVd). Samples were analysed in 0.1 % (w/v) Triton X-100, 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0.

**a.** Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.

1. **Peak IVa:** (from S-200) protein concentration = 580 µg mL⁻¹; \( \Delta A = 0.020 \).
2. **Peak IVb:** (from S-200) protein concentration = 450 µg mL⁻¹; \( \Delta A = 0.020 \).
3. Spectrum #2 minus spectrum #1: \((\text{Peak IVb})-(\text{Peak IVa})\); \( \Delta A = 0.020 \).

**b.** Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.

1. **Peak IVc:** (from S-300) protein concentration = 176 µg mL⁻¹; \( \Delta A = 0.010 \).
2. **Peak IVd:** (from S-300) protein concentration = 763 µg mL⁻¹; \( \Delta A = 0.010 \).
3. Spectrum #2 minus spectrum #1: \((\text{Peak IVd})-(\text{Peak IVb})\); \( \Delta A = 0.010 \).
Stoichiometry of the peak IV cytochrome complex

The narrowness of peak IVd suggests that the molecular aggregate that it may represent corresponds to a defined stoichiometry, although the presence of the ‘420 nm redox chromophore’ complicates interpretation. This complex, $M_r = 386000$, might be composed of a tetramer having monomeric $M_r = 97000$, comprising two units of cytochrome $b$, two units of flavoprotein and an unknown quantity of ‘420 nm chromophore’. With two units of cytochrome plus a flavoprotein creating an entity with $M_r = 52500$ the combined molecular weights of ‘420 nm redox chromophore’ and flavoprotein would amount to 44 kDa. With typical $b$-cytochrome molecular weights in the range of 12-15 kDa (v.i.) this would suggest molecular weights of approximately 25 kDa for the flavoprotein subunit (smaller than many reported flavoproteins) and about 20 kDa for the ‘420 nm redox chromophore’ [189].

SDS-polyacrylamide gel electrophoresis of the partially purified peak IVd complex indicated that it was resistant to disruption by the routine sample preparation procedure of hot SDS treatment [110]. The standard preparation technique resulted in substantial streaking of the sample on the polyacrylamide gel, possibly as a result of re-equilibration of detergents as the gel was running. When the concentration of SDS was reduced to levels appropriate for subsequent haem staining or when the sample was not heated before loading onto the gel (a procedure which improved penetration of the cytochrome $o$ complex into these gels) staining with Coomassie Brilliant Blue indicated that most of the protein remained at the interface between the stacking and main gels, suggesting that aggregation was occurring in the presence of SDS (refer to Materials & Methods, section 't').

Optimal peptide resolution was obtained with 1.0% SDS and $\beta$-mercaptoethanol in the sample buffer, distinct protein bands being visible under these conditions. Faint bands migrated to positions corresponding to $M_r = 100200, 36100$ and 26600. Strongly staining bands indicated that the complex contained peptides with $M_r = 68700, 32700, 29300, 21100$ and 11500. The band corresponding to the smallest peptide (11.5 kDa) stained far more intensely than any other band and was seen in gels of samples which had been treated with low concentrations of SDS even when no other subunits were resolved from the high molecular weight material indicating that this small subunit is more susceptible to dissociation than the other peptides. Haem staining under a variety of conditions in which standard haemoproteins (haemoglobin, myoglobin and cytochrome $c$) produced visible colour reactions in the gels succeeded only in staining the aggregated material at the top of the gel indicating that the haem is easily lost from any cytochrome $b$ components of the complex (refer
(f) **Potentiometric titration of the cytochrome complex**

The combination of partial purification of the peak IV cytochrome complex and high resolution spectrophotometric analyses had been unsuccessful in providing an unequivocal determination of the number of cytochrome $b$ species that the complex contained. Figure 64 illustrates the results of the application of potentiometry to this task. Purification of peak IVd from a bulk culture of *cyd*" strain PLJ01 grown on succinate provided material for the potentiometric titration which resulted in a biphasic curve. In Figure 64 panel ‘a’ shows theoretical single and double component curves optimally fitted to the titration data while panel ‘b’ demonstrates the Nernst plot. The former demonstrates clearly that the single component fit is inappropriate, as does the value of $n = 0.51$ obtained from the linear regression curve through the data of the Nernst plot. The Nernst plot also shows that a double inflection occurs in the plotted data at the potential of half-maximal reduction. By using the potential of 50% total reduction as the demarcation between two theoretical components one may derive the alternative Nernst plots of two independent constituents with mid point potentials of approximately 0 mV and -125 mV, both yielding values of $n$ close to unity. In comparison, BMD:P3R software analysis of these titration data generated the theoretical curves in Figure 64a and calculated mid-point potentials of -2.3 mV and -121.3 mV for the two components, each contributing 57.2% and 42.8% of the total cytochrome respectively. These interpretations assume that the $\alpha$-band absorbance is proportional to the degree of electrochemical reduction and that the two components’ extinction coefficients are equal at the wavelength of the $\alpha$-band maximum. Thus the spectroelectrochemical properties of cytochrome $b_{556}$ of peak IV are analogous to those of cytochrome $b^{NR}$ of nitrate reductase and certain cytochromes $c_4$ in that a single redox $\alpha$-absorbance band is associated with a biphasic potentiometric titration profile. This may indicate that peak IV contains a dihaem cytochrome (section I.ii.b) {111}.

In the titration procedure the percentage of reduced cytochrome was determined from reduced *minus* oxidized $\alpha$-band spectra collected at known potentials which were measured by a platinum combination electrode located in the modified sample cuvette (Materials & Methods). The
**Fig. 64:** Potentiometric titration of partially purified 'Peak IV' cytochrome.

Potentiometric titration of Peak IV cytochrome following partial purification by liquid chromatography via DEAE-BioGel A, Sephacryl S-200 and Sephacryl S-300.

**a.** Peak IV cytochrome at 1.50 mg mL\(^{-1}\) protein in 100 mM potassium phosphate buffer, pH 7.0. The curves indicate theoretical best fits of the data,

(*---*) one-component fit; \(E_m = -32.8\) mVvs.NHE,

(*---*) two-component fit; \(E_m = -2.3\) mVvs.NHE (57.2 % total \(\alpha\)-band \(\lambda_{\text{max}}\) absorbance),
\[\begin{align*}
E_m &= -121.3\ \text{mVvs.NHE} \ (42.8 \% \ \text{total} \ \alpha\text{-band} \ \lambda_{\text{max}} \ \text{absorbance}).
\end{align*}\]

**b.** Nernst plot of data in 'a' showing data for total cytochrome and for a model incorporating two cytochromes of equal absorbance with mid-point potentials estimated from the original data.

\(\text{O-O, total cytochrome : } E_m = -60.0\ \text{mV, slope} = 115.0\ \text{mV, n} = 1.95,\)

\(\text{O-O, high potential cytochrome component; } E_m = +2.0\ \text{mV, slope} = 63.0\ \text{mV, n} = 1.07,\)

\(\text{O-O, low potential cytochrome component; } E_m = -124.5\ \text{mV, slope} = 77.0\ \text{mV, n} = 1.31.\)
double component result indicated that both peak IV cytochromes detected potentiometrically had detectable \( \alpha \)-absorption bands and also that their relative absorption at maximal reduction was approximately equivalent. When coupled with the slightly asymmetrical low temperature redox \( \alpha \)-band spectrum the 57 % + 43 % contributions to the total absorbance measured during the titration suggest that the 556.0 nm component corresponded to the larger contributor and the 557.5 nm component to the lesser contributor (Fig. 62). These results do not conflict with the assumption of stoichiometric quantities of the components since related cytochromes commonly exhibit minor differences in \( \alpha \)-band extinction coefficients \cite{87, 112}. If the 77 K \( \alpha \)-band of the peak IVd cytochrome \( b \) did comprise equivalent contributions from two cytochromes the ratio of maximum \( \alpha \)-band absorbance amplitude to that of maximum Soret band absorbance of each component would have been approximately one fifth, corresponding to that observed in this study for the majority of \( E. coli \) cytochromes \( b \) whether in solubilized isolates, mixtures or membrane preparations.

As the potentiometric titrations were carried out at 305 K without spectral averaging there was no possibility of resolving \( \alpha \)-band components directly from the titration data. Poised potential samples were collected and subjected to high resolution low temperature redox spectrophotometry as described earlier (sections I.iii.c; II.A.ii.d). Unfortunately the combined spectral contributions of electrochemical mediators used in the titration buffer, the '420 nm redox chromophore' and the flavoprotein present in the sample prevented unambiguous interpretation of the data as to which of the twin Soret bands was reduced at higher potential. However, the poised potential spectra indicated that this '420 nm redox chromophore' was reduced at a much higher potential than either of the two \( b \)-cytochromes (Fig. 65). Under the conditions of the experiment this appeared to be fully reduced at +48 mV, although it had minimal redox absorption properties in the 530 nm to 580 nm region and therefore was neither detected by the standard titration procedure, nor did it interfere with it.

Figure 66 illustrates titrations of the peak IVd complex in the presence of ferricyanide ions and with carbon monoxide (following ferricyanide treatment). There is no significant effect of ferricyanide ion on the mid-point potential of either cytochrome component, whereas carbon monoxide alters the lower potential component profoundly. Not only was the \( \alpha \)-band absorbance of this component approximately doubled but the potential range over which it was reoxidized did not correspond to a standard one electron transfer reaction. Either two electrons were involved \( (n = 2) \) or, more probable, the release of CO was occurring as a cooperative mechanism so that the ligand was retained until the potential was raised above a threshold potential \( (\text{apparently centred at } -80 \text{ mV}) \) at which most of it rapidly dissociated from the complex. Interestingly fresh samples without the titration mediators fully reduced by dithionite followed by reaction with carbon monoxide in the dark
Fig. 65: Potentiometrically poised low temperature redox difference spectra of ‘Peak IV’ cytochrome.

Low temperature spectra of partially purified Peak IV cytochrome poised at the indicated electrochemical potentials. Samples were taken from the titration vessel and analysed in titration buffer 0.2% (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0 as described under Methods & Materials. No sucrose was present.

Potentiometrically poised ‘sample’ minus peroxide oxidized ‘reference’ difference spectra obtained at 77K. Protein concentration = 1.5 mg mL$^{-1}$; $\Delta A = 0.01$.

a. Sample poised at +48.5 mV vs NHE: titration mediators present.

b. Sample poised at -105.5 mV vs NHE: titration mediators present.

c. Sample fully reduced with Na$_2$S$_2$O$_4$ (< -350 mV vs NHE): titration mediators absent.
**Fig. 66:** Potentiometric titration of partially purified 'Peak IV' cytochrome following treatment with carbon monoxide.

Potentiometric titration of Peak IV cytochrome at 1.50 mg mL\(^{-1}\) protein in 100 mM potassium phosphate buffer, pH 7.0 following partial purification by liquid chromatography via DEAE-BioGel A, Sephacryl S-200 and Sephacryl S-300. The sample was fully oxidized with small aliquots of \(K_2Fe(CN)_6\) before beginning the reductive titration.

[Graphical representation of titration data]

\[ E_m = -2.0 \text{ mV vs. NHE} \quad (47.4\% \text{ total } \alpha\text{-band } \lambda_{\text{max}} \text{ absorbance}), \]

\[ E_m = -110.5 \text{ mV vs. NHE} \quad (52.6\% \text{ total } \alpha\text{-band } \lambda_{\text{max}} \text{ absorbance}). \]

[Graphical representation of titration data]

\[ E_m = +23.0 \text{ mV vs. NHE} \quad (47.4\% \text{ of the pretreatment total } \alpha\text{-band } \lambda_{\text{max}} \text{ absorbance}), \]

\[ E_m = -80.0 \text{ mV vs. NHE} \quad (94.8\% \text{ of the pretreatment total } \alpha\text{-band } \lambda_{\text{max}} \text{ absorbance}). \]
revealed no alteration of the absorption spectrum (data not shown).

(x) Peaks I & IV: Independence From frd Genes and Fumarate Metabolism

(a) Control of expression

Fumarate reductase catalyses the conversion of fumarate to succinate, the reverse reaction of succinate dehydrogenase, and in E. coli the two enzymes have a high degree of homology between protein composition and genetic sequence. Each comprises four subunits, a large flavoprotein of $M_r = 70,000$, a catalytic subunit of 25,000 and two membrane 'anchor' peptides of about 15,000, one of which appears to be a cytochrome $b_{556}$ in succinate dehydrogenase (section II.B.i.a+b) [31, 87]. No evidence has been obtained to suggest the presence of cytochrome in fumarate reductase to date, although there is strong homology between the genes coding for the small subunits in each of the two enzymes [31, 215]. These two metabolic functions are controlled by the regulation of expression of the enzymes in E. coli since the cell has no ability to compartmentalize these disparate activities. As fumarate may be used as an alternative electron acceptor to dioxygen the fumarate reductase $frd$ operon is expressed under anaerobic conditions in the presence of the substrate.

It was possible that cells grown with succinate as carbon/energy source and harvested in late exponential or stationary phase may have modified their environment substantially. It is known that cultures with high cell population density often approach anaerobiosis, consuming available oxygen at a rate equal to that at which it is able to dissolve in the liquid medium: this may be a primary cause of the induction of the cytochrome $d$ complex [56, 157]. Although the P/O ratios of the procaryotic respiratory chains are still open to dispute, succinate is certainly a less efficient energy source than glucose in mitochondrial systems, thus in late exponential or stationary phase cultures there might be significant intracellular concentrations of fumarate generated as the succinate was depleted. Endogenous substrates might then be consumed under semi-anaerobic conditions using the fumarate as terminal electron acceptor.

This possibility needed to be addressed in the light of a number of earlier reports linking the copurification of cytochrome $b$ with fumarate reductase from E. coli and in order to confirm the conclusions drawn from the experiments described above (sections III.B.i.b+c; III.B.iv.a) [31, 87]. Plasmid pFRD84 is a pBR322 derivative containing the four genes of the $frd$ operon and generating substantial over-expression of the fumarate reductase under appropriate conditions [31,
Samples of strain HB101/pFRD84 were cured of the plasmid by growth at elevated temperature on non-selective media for several culture cycles and cells sensitive to all the antibiotics for which resistance is carried by pBR322 were selected. Figure 67 shows that when strain HB101 was grown under the standard aerobic conditions on succinate a typical $A_{412}$ elution profile was obtained from the initial anion exchange fractionation of the membrane extract: elution peaks I and II are clearly visible, containing cytochrome $b_{556}$ and the cytochrome $o$ complex respectively, and there is a small amount of the peak IV cytochrome $b_{556}$. Yet when HB101/pFRD84 was cultured under the same conditions the membrane extract eluted with a rather different profile: the cytochrome $o$ complex eluted as a distinct peak II but there were no other plainly distinguishable $A_{412}$ features. Thus the plasmid appeared to have an effect upon the expression of cytochromes under these growth conditions, but the peak I cytochrome $b_{556}$ was definitely not related to fumarate reductase since cells containing multiple copies of $frd$ generated less of this product than the wild-type. It has been proposed that the peak I cytochrome $b_{556}$ is part of the succinate dehydrogenase complex, corresponding to the $sdhC$ gene product (section III.B.i.b) \cite{97,144}. Thus the lack of this cytochrome in extracts of the strain carrying the fumarate reductase plasmid may be the result of greater sensitivity of these cells to a transition from aerobic to semi-anaerobic conditions, or to increasing fumarate concentrations as the succinate is consumed. As growth conditions within batch cultures are significantly modified during the course of growth these phenomena would be most accurately investigated with the use of a chemostat, an oxystat being a minimal requirement for analysis of the regulatory mechanisms occurring in these cultures \cite{147,157}.

Expression of the peak IV cytochrome $b_{556}$ was initially observed in cells grown aerobically to stationary phase on succinate and other carbon/energy sources and it was subsequently found to be a result of growth in conditions of low oxygen tension (section III.B.iv.a). It was possible, therefore, that this peak IV cytochrome was associated with the expression of the $frd$ operon as the culture population density grew and decreased the concentration of available dioxygen in the liquid medium. Cultures of HB101 and HB101/pFRD84 were grown on glycerol plus fumarate under anaerobic conditions to test this possibility. Standard membrane extraction and fractionation of these cell samples generated the elution profiles displayed in Figure 68. Extracts from strain HB101 contained a small but detectable quantity of cytochrome $o$ complex as peak II, plus peak $F_1$ eluting at 21 mS cm$^{-1}$, and two large, partially fused peaks of cytochrome $b$ eluting at about 32 mS cm$^{-1}$ and possessing vigorous hydroperoxidase activity, which appeared to correspond to the peak IV of the aerobically grown cultures and were termed peaks $IV_1$ and $IV_2$ (Fig. 68a). The presence of
Fig. 67: DEAE-BioGel.A elution profiles of Triton membrane extracts from cells carrying the fumarate reductase gene on plasmid pFRD84.

Cultures of strains HB101 and HB101/pFRD84 were grown aerobically to late exponential phase on CYD minimal medium containing the carbon/energy sources indicated below. Washed membranes were prepared and extracted by the standard techniques. The DEAE-BioGel.A was eluted under standard conditions with a KCl gradient.

a. Strain HB101 grown aerobically on succinate,
   1. (- - -) Lowry protein assay : $\Delta A_{660} = 0.10$; 125 $\mu$g mL$^{-1}$ eluate,
   2. (-----) Absorbance at 412 nm : $\Delta A_{412} = 0.10$.

b. Strain HB101/pFRD84 grown aerobically on succinate,
   3. (- - -) Lowry protein assay : $\Delta A_{660} = 0.10$; 125 $\mu$g mL$^{-1}$ eluate,
   4. (-----) Absorbance at 412 nm : $\Delta A_{412} = 0.10$. 
Cultures of strains HB101 and HB101/pFRD84 were grown anaerobically on CYD minimal medium containing the carbon/energy sources indicated below. Washed membranes were prepared and cytochromes extracted and fractionated via DEAE-BioGel.A by the standard techniques. Peaks are labelled in standard format, subfractions of Peak IV being observed in addition to a novel Peak ‘F’.

**Fig. 68:** DEAE-BioGel.A elution profiles of Triton membrane extracts from anaerobically-grown cells carrying the plasmid-borne fumarate reductase gene.

a. Strain HB101 grown anaerobically on (glycerol + fumarate),
1. (- - - -) Lowry protein assay : $\Delta A_{660} = 0.20$ ; 500 $\mu$g mL$^{-1}$ eluate,
2. (-----) Absorbance at 412 nm : $\Delta A_{412} = 0.10$ .

b. Strain HB101/pFRD84 grown anaerobically on (glycerol + fumarate).
3. (- - - -) Lowry protein assay : $\Delta A_{660} = 0.20$ ; 667 $\mu$g mL$^{-1}$ eluate,
4. (-----) Absorbance at 412 nm ; $\Delta A_{412} = 0.25$ .
cytochrome $o$ in these static cultures indicated that they were not achieving strict anaerobiosis and that significant quantities of dioxygen must have been present, especially during early phases of the incubation. In comparison to this result, membrane extracts from strain HB101/pFRD84 which carried multiple copies of the complete fumarate reductase operon, eluted from the anion exchange column with a radically different $A_{412}$ profile (Fig. 68b). Virtually no absorbance due to the cytochrome $o$ complex was detectable, the major protein eluted from the column corresponded to a greatly increased 412 nm absorption from the material in peak F$_1$, which was shown to be fused with a smaller trailing peak labelled F$_2$. In comparison with extracts from the wild-type cell membranes, those from strain HB101/pFRD84 contained about one quarter of the material eluting in peaks IV$_1$ and IV$_2$ (Fig. 68b). Thus the peak IV cytochrome $b_{556}$ observed in cells grown 'aerobically' to late exponential or stationary phase was not related to expression of the fumarate reductase genes although it appears to be associated with growth under anaerobic conditions or those approaching anaerobiosis.

(b) Spectrophotometry of fractionated cytochromes

High resolution spectrophotometry of membrane samples from the cultures grown anaerobically with glycerol plus fumarate indicated that those from HB101 displayed a symmetrical reduced minus oxidized cytochrome $\alpha$-band in which fourth order derivative analysis detected components at 556.0 nm and 558.5 nm (data not shown). The presence of the pFRD84 plasmid in the cells brought about a broadening of the cytochrome $\alpha$-band with two component maxima at 555.5 nm and 559.0 nm which were clearly distinguishable by fourth order derivatization of the spectra (data not shown). Redox spectra of resuspended membranes from the cells without the plasmid contained virtually no detectable flavoprotein whereas those from cells bearing the plasmid contained sufficient flavoprotein to generate a redox trough at 450 nm equivalent in size to that of the cytochrome Soret band at 428 nm; more than 95 % of this flavoprotein was extracted by the standard procedure using Triton X-114 (data not shown).

On fractionation of the extracts by anion exchange column chromatography the twin peaks associated with expression of fumarate reductase, F$_1$ and F$_2$, contained very different chromophores. High resolution spectrophotometry showed that peak F$_1$ comprised flavoprotein without detectable cytochrome and peak F$_2$, with considerably smaller 412 nm absolute absorption, contained both flavoprotein and cytochrome $b$ (Fig. 69a). Subtraction of the flavoprotein spectrum of peak F$_1$
Fig. 69: High resolution broad range visible redox difference spectra of partially purified solubilized cytochromes from cells grown anaerobically on glycerol and fumarate.

Strains HB101/pFRD84 and HB101 were grown anaerobically on CYD minimal medium containing (glycerol + fumarate). Washed membranes were prepared and cytochromes extracted and fractionated via DEAE-BioGel A by the standard techniques. Elution peaks are identified by the format indicated in Fig. 66. Samples were analysed in 1.0 M sucrose, 100 mM potassium phosphate buffer, pH 7.0.

a. Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.
   1. \((\text{Spectrum } #2) \text{ minus } (\text{spectrum } #3 \times 0.6)\) : Peak \(((F_2)-(F_1))\) \(\Delta A = 0.030\).
   2. HB101/pFRD84, Peak F2 : protein concentration = 298 µg mL\(^{-1}\) \(\Delta A = 0.030\).
   3. HB101/pFRD84, Peak F1 : protein concentration = 290 µg mL\(^{-1}\) \(\Delta A = 0.030\).

b. 4. HB101/pFRD84, Peak IV1 : protein concentration = 130 µg mL\(^{-1}\) \(\Delta A = 0.030\).
   5. HB101/pFRD84, Peak IV2 : protein concentration = 250 µg mL\(^{-1}\) \(\Delta A = 0.060\).

c. 6. HB101, Peak IV1 : protein concentration = 230 µg mL\(^{-1}\) \(\Delta A = 0.060\).
   7. HB101, Peak IV2 : protein concentration = 310 µg mL\(^{-1}\) \(\Delta A = 0.075\).
from the spectrum of peak $F_2$ permitted accurate analysis of the latter which was shown to be caused by a combination of two cytochromes $b$, fourth order derivatization of the redox spectrum indicating their absorption maxima to be 555.0 nm and 556.0 nm (Fig. 69a, 70a+b). The partially separated peaks $IV_1$ and $IV_2$ generated redox spectra with absorption maxima at 556.0 nm and 557.5 nm respectively (Fig. 70a+b). It was noted that these two type-$b$ cytochromes had different Soret band absorption maxima, peak $IV_1$ displaying twin maxima at 426.0 nm and 434.0 nm and that of peak $IV_2$ at 426.0 nm with a broad shoulder at 445 nm (Fig. 69). The cytochrome $b_{556}$ of peak $IV_1$ eluted at the same ionic strength in the salt gradient as the peak IV cytochrome from cells grown aerobically to stationary phase. Not only did both of these fractions display an unusual twin Soret band with absorption maxima at 426.0 nm + 434.0 nm but each was associated with vigorous hydroperoxidase activity. Nevertheless there is no confirmatory evidence that the peak IV cytochromes $b_{556}$ from the late ‘aerobic’ cultures are equivalent to those from the anaerobic cultures.

(c) **Fumarate reductase**

It has been shown above that membranes of strains with $sdh$ deletions, GVK124 & KW531, have no succinate dehydrogenase activity when grown aerobically on DL-lactate plus succinate, in contrast to $sdh^+$ strains (Table XII). However, when grown under conditions inducing fumarate reductase — anaerobiosis with glycerol and fumarate as carbon source and electron acceptor respectively — membranes from cells of GVK124 are capable of succinate oxidation with the coincident reduction of cytochrome $(P. D. Bragg, personal communication)$. Limited immunological cross-reactivity and similarities in structure between these two enzymes from primary to quaternary levels have provided extensive evidence of their close functional and evolutionary relationship $[31, 87]$. The $frdC$ gene codes for a peptide of similar molecular weight to that of the $sdhC$ gene, $M_t = 15 000$, and the $sdhC$ gene product has been identified as a cytochrome $b_{556}$. Although conservation between the sequences of $frdC$ and $sdhC$ is limited structural similarities exist between the two gene products, including the distribution through the polypeptides of hydrophobic and hydrophilic domains, resulting in similar transmembrane topography being proposed for each $[31, 113, 215]$. Purifications of fumarate reductase have been reported both with and without a cytochrome $b$ component and although there is no evidence to link a type-$b$ cytochrome with any of the four fumarate reductase subunits the small peptides appear to participate in quinol binding by the enzyme $[31, 87]$. In the current study purification of fumarate reductase from cells of
**Fig. 70:** High resolution α-bands of redox difference spectra from partially purified solubilized cytochromes of cells grown anaerobically on glycerol and fumarate.

Strain HB101/pFRD84 was grown anaerobically on CYD minimal medium containing (glycerol + fumarate). Washed membranes were prepared and cytochromes extracted and fractionated via DEAE-BioGel A by the standard techniques. Elution peaks are identified by the format indicated in Figure 66. Samples were analysed in 100 mM potassium phosphate buffer, 1.0 M sucrose, pH 7.0.

**a.** Dithionite reduced minus peroxide oxidized difference spectra obtained at 77 K.

1. Peak F₁ : protein concentration = 298 μg mL⁻¹ ; ΔA = 0.010,
2. Peak F₂ : protein concentration = 290 μg mL⁻¹ ; ΔA = 0.003,
3. Peak IV₁ : protein concentration = 130 μg mL⁻¹ ; ΔA = 0.008,
4. Peak IV₂ : protein concentration = 250 μg mL⁻¹ ; ΔA = 0.010.

**b.** Fourth-order finite difference spectra calculated from corresponding curves 'a' by four successive first-order derivatizations.
HB101/pFRD84 grown anaerobically on glycerol+fumarate by the method of Weiner (Materials & Methods) resulted in a product with which much flavoprotein and a small amount of cytochrome $b_{556}$ was associated (data not shown). The relationship between this cytochrome and the fumarate reductase activity was unknown and since fumarate reductase had been shown to be unrelated to fractionation peaks I or IV these investigations were not pursued.

The *frdC* gene product is known to act in association with the *frdD* product as a membrane anchor for the catalytic subunits of the enzyme \(31, 113\). Further investigation of the structural characteristics of the *frdC* protein would be of relevance to studies of haemoprotein function for its similarity to the cytochrome \(b_{556}\) of succinate dehydrogenase suggests that it may be a cytochrome suffering from a facility for haem loss during preparation. Alternatively its function and structure may have both evolved so that it uses no prosthetic group. Consequently these comparable protein subunits of two closely related enzymes offer promising targets for investigations of haemoprotein evolution, haem and apoprotein function and possibly of the effects of apoprotein structure on the stability of haem binding.

\{C\} **Cytochrome \(o\) Terminal Oxidase Complex**

The cytochrome \(o\) terminal oxidase of *E. coli* has been identified with each of a number of different cytochrome preparations, including cytochromes \(b_{555}, b_{562}\), a combination of these two and a high spin cytochrome \(b\) with minimal absorbance in the \(\alpha\)-band region (Introduction, section B.iv) \(69, 95, 97, 162, 117, 159, 173, 174\). In the current study potentiometry, reduction with high potential reductants such as duroquinol and spectral perturbation by carbon monoxide were selective procedures that had provided insights into specific properties of this oxidase complex in the cytoplasmic membrane of *E. coli* (section II.C.ii) \(129, 164, 165, 183, 214\). However, even when investigations combined the use of mutant cells, high resolution redox spectrophotometry, potentiometric titration and kinetic analyses the multiplicity and similarity of the type-\(b\) cytochromes in these membranes had rendered the available procedures insufficient to decipher the organizational complexity of the bacterium’s aerobic respiratory chains. Moreover, these methods had been unable to reveal the nature of the constituents of the cytochrome \(o\) complex or of their integration with other respiratory components.

The experimental approach that was pursued was to solubilize and isolate the cytochrome \(o\) complex from other cytochromes \(b\) and then subject it to biophysical and kinetic analyses. This
would serve as a preliminary study for future investigations reconstituting the cytochrome into phospholipid vesicles with individual components of the bacterial respiratory chains. In this manner the interactions of the various branches of the aerobic electron transport pathways might be investigated; the coexistence of these inducible branches being a complicating factor of many procaryotic respiratory systems which is partly responsible for the complexities to which reference was made above.

(i) Purification of the Cytochrome \( o \) Complex

(a) Standard isolation procedure

The cytochrome \( o \) complex was purified from \( cyd^+ \) and wild type cells grown aerobically on a variety of carbon/energy sources. The presence of the cytochrome \( d \) complex yields beige cells and olive green membrane preparations whereas its absence from \( cyd^+ \) cells or from \( cyd^- \) cells harvested in mid-exponential phase results in cells which are a very pale beige from which reddish brown membranes are prepared, much of this colour being due to the cytochromes of the cytochrome \( o \) complex.

The standard procedure for the fractionation of cytochromes from membranes solubilized and extracted with Triton X-114 had been shown to produce a fraction containing a complex group of type-\( b \) cytochromes which eluted from the DEAE.BioGel.A ion-exchange matrix in the KCl gradient at about 18 mS cm\(^{-1}\) and was recognised as the 'peak II' fraction when the absorbance of the eluate was monitored at 412 nm to generate an elution profile (sections III.A.i+ii, Fig. 44,45,46). This peak was the only fraction eluted that contained cytochrome with a reduced minus oxidized \( \alpha \)-band absorption spectrum with a maximum at wavelengths greater than 560 nm (Fig. 47+48). The cytochrome \( o \) complex had been associated with cytochrome \( \alpha \)-bands of this type and the ability of this fraction to transfer electrons from duroquinol to dioxygen, plus its interaction with carbon monoxide suggested that it contained the terminal oxidase (sections III.C.ii.a,c,d) \( \{112,216\} \). As described above, the addition of Triton detergents to washed membrane preparations resulted in a loss of distinction of the three cytochrome \( \alpha \)-band absorption maxima at 560.5 nm, 563.0 nm and 565.0 nm as they merged into a single peak at 563.0 nm. The duroquinol oxidase activity of the peak II fraction justified the continued use of this method of solubilization during the preparation of the cytochrome \( o \) complex. The Triton series of non-ionic detergents was the most efficient at
extracting cytochromes from washed membranes of *E. coli* (data not shown) and have been promoted as less disruptive to the integrity of intrinsic membrane proteins than single or mixed ionic detergents (152, 166). Extrinsic proteins were removed from the washed membranes before detergent extraction in cytochrome-o preparations by sequential urea and cholate washes as described under Materials & Methods. Absorption profiles of the column eluate monitored at 412 nm were unchanged indicating that these procedures did not affect subsequent extraction and anion exchange fractionation of membrane cytochromes and that all the cytochromes b investigated in this study were true intrinsic proteins.

The overall extraction efficiency of type-b cytochromes by Triton X-114 solubilization was estimated at less than 35 %. However, the distinctively convoluted redox α-band of the peak II fraction strongly resembled that of the unextracted residue from solubilized membrane samples, suggesting that the efficiency of extraction of the cytochrome-o complex was much lower than the average for b-cytochromes. Similarly, wild type cell membranes yielded a low percentage of their cytochrome-d content: the distinct, high wavelength α-band absorption resulting from the chlorin in this terminal oxidase indicated that in a typical extraction from stationary phase wild type cell membranes 72 % of cytochrome-d remained in the unextracted residue (data not shown). Thus both terminal oxidases appear to be particularly difficult to solubilize.

Fractionation of the extracted cytochromes entailed both anion exchange and gel exclusion chromatography. These procedures were interspersed with salt precipitation and ultrafiltration steps as described under Materials & Methods. The separations obtained from the initial DEAE-BioGel.A anion exchange column have been discussed in detail above (section III.A+B). The cytochrome-o complex was associated with elution peak II which the absolute absorption profile at 412 nm indicated to be the predominant cytochrome fraction of extracts from cells of cyd- strains grown under virtually all conditions tested in this study (section III.A.ii). Gel exclusion chromatography of the peak II material was routinely performed through a matrix of Sephacryl S-300 which removed (NH₄)₂SO₄ from the single band of chromophore containing the cytochrome-o complex. Passage through a calibrated column of Sephacryl S-300 gel provided a value for the relative molecular weight of the cytochrome-o complex of 516 000 (±10 000) (Fig. 71). Subsequent chromatographic purification steps included further anion exchange chromatography in which the cytochrome-o complex was eluted by a pH gradient indicating that in this solubilized state it has a pI of approximately 6.05 (data not shown).

SDS-polyacrylamide gel electrophoresis of samples taken from stages of the isolation procedure demonstrates that all but five peptides were progressively removed as the cytochrome-o complex was purified (Fig. 72). These polypeptides migrated with $M_r = 55 000, 32 000, 31 000,
**Fig. 71:** Gel filtration elution profiles: calibration of the Sephacryl S-300 matrix and elution of 'Peak II' cytochromes.

Pooled Peak II cytochrome from the DEAE-BioGel.A anion exchange process was further purified as described under **Methods & Materials** before being passed through a calibrated (7.5 x 420) mm column of Sephacryl S-300 in 0.2 % (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0. Fractions of 900 mL were collected.

( ) Absorbance at 412 nm ; ( - - - ) Lowry protein assay measured at 660 nm.

**a.** Calibration of Sephacryl S-300 column, plus elution position of Peaks II and IVd:

- T. thyroglobulin standard : $M_r = 669\,000$,
- F. ferritin standard : $M_r = 440\,000$,
- C. catalase standard : $M_r = 232\,000$,
- A. aldolase standard : $M_r = 158\,000$.

**b.** Elution profile of partially purified cytochrome o complex via Sephacryl S-300:

$$\Delta A_{412} = 0.40 ; \Delta A_{660} = 0.40, \quad 2.7 \text{ mg protein mL}^{-1} \text{ eluate.}$$

Interpolated absorption maximum corresponds to $M_r = 516\,000$.

(o)$_4$ Reference peak; thought to correspond to tetramer (see text, section III.C.i.a),

(o)$_2$ Predicted elution point of dimer, based upon reference peak position and column calibration,

(o)$_1$ Predicted elution point of monomer, based upon reference peak position and column calibration.
**Fig. 72**: SDS-Polyacrylamide gel electrophoresis of samples from stages during the purification of cytochrome $o$.

The gels comprised 13 % polyacrylamide and were stained with Coomassie Brilliant Blue.

A. Standard Laemmli sample preparation, $\beta$-mercaptoethanol, + 8 minutes’ boiling.

B. Sample preparation performed as in (A), $\beta$-mercaptoethanol, but without boiling.

C. Sample preparation performed as in (A), without $\beta$-mercaptoethanol, without boiling.

**Lanes**:

1. Triton X-114 extract,

2. Triton X-114 extract + Triton X-100 as loaded onto the initial DEAE-BioGel.A column,

3. pooled Peak II from the initial DEAE-BioGel.A column,

4. resuspended (NH$_4$)$_2$SO$_4$ precipitate as loaded onto Sephacryl S-300 column,

5. cytochrome peak pooled from S-300 step, loaded onto second DEAE-BioGel.A column,

6. cytochrome $o$ complex from second DEAE-BioGel.A column : pH elution gradient,

7. cytochrome $o$ complex from second DEAE-BioGel.A column : KCl elution gradient.

Running positions of standard molecular weight markers are indicated:

$M_r$ — 94 000, 67 000, 43 000, 30 000, 20 100, 14 400 (see Methods & Materials).
21 000 and 16 000. Silver staining confirmed these molecular weights and showed that no other significant bands were present in the purified sample.

Reduced minus oxidized spectrophotometry of the purified cytochrome o complex and of its pyridine haemochromogen derivative indicated that all haem in the complex was of type-\(b\) and that the isolated complex had a haem content of 9.6 nmol (mg protein)\(^{-1}\). The determination of haem from the pyridine haemochromogen in reduced minus oxidized difference spectra at ambient temperature for the absorbance difference between the wavelength pair 557 nm and 541 nm was by the method of Falk (\(\epsilon_{(557-541)} = 20.7 \text{ cm}^{-1} \text{ mM}^{-1}\)) (49). A summary of the purification procedure is provided in Table XIII: values of about 4.1 nmol (mg protein)\(^{-1}\) were obtained for the specific content of the cytochrome o complex using the modified ambient temperature extinction coefficient of proposed by Kita et al. (\(\epsilon_{(560-580)} = 18.7 \text{ cm}^{-1} \text{ mM}^{-1}\) in reduced minus oxidized difference spectra) (97). The alternative extinction coefficient determined by these workers (\(\epsilon_{(416-430)} = 145 \text{ cm}^{-1} \text{ mM}^{-1}\) in reduced plus carbon monoxide minus reduced difference spectra) is almost double the traditional value used for the purification of cytochrome o by Matsushita et al. (\(\epsilon_{(416-430)} = 80 \text{ cm}^{-1} \text{ mM}^{-1}\)) (97, 129). It is noteworthy that the former group prepared a soluble complex with two peptides visible in SDS-PAGE analyses whereas the latter group reported four constituent peptides (v.i.). One difficulty in estimating cytochrome o concentrations from CO-binding spectra of detergent solubilized preparations is the possibility of detergent denaturation of other type-\(b\) cytochromes which may bind carbon monoxide as a result of haem exposure with the loss of an internal axial ligand. Thus a stoichiometric haem content cannot be assigned to the complex purified by the current technique using published extinction coefficients. The availability of relatively large quantities of cloned material should enable precise estimations of the oxidase’s haem content to be obtained from future studies — as well as providing material for the quantitation of individual subunit stoichiometry for the estimation of the oxidase’s total molecular mass. A value of 2.3 was obtained for the number of haems per cytochrome o complex using highly purified uncloned material in this study.

(b) Comparison with alternative procedures

Methodologies for the purification of the cytochrome o complex have now been published by the laboratories of Anraku and of Kaback and Gennis (97, 99, 130). These two groups of authors differ on the number of different peptides comprising the complex and on their approach to the purification process. The former has relied mainly upon liquid chromatographic separation of
| Purification step                        | Protein (mg) | Cytochrome o<sup>a</sup> (nmol) | Specific Content<sup>b</sup> (nmol mg<sup>-1</sup>) | Recovery (%) | Purification
<table>
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</thead>
<tbody>
<tr>
<td>1. Washed, crude membranes</td>
<td>4957</td>
<td>337</td>
<td>0.068</td>
<td>100.0</td>
<td>1.0 x</td>
</tr>
<tr>
<td>2. Urea washed inner membranes</td>
<td>1563</td>
<td>236</td>
<td>0.151</td>
<td>80.4</td>
<td>2.2 x</td>
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<tr>
<td>3. Cholate-stripped inner membranes</td>
<td>1437</td>
<td>239</td>
<td>0.166</td>
<td>70.1</td>
<td>2.4 x</td>
</tr>
<tr>
<td>4. Triton X-114 extraction &amp; Triton X-100 dilution</td>
<td>1064</td>
<td>92.6</td>
<td>0.087</td>
<td>27.0</td>
<td>1.3 x</td>
</tr>
<tr>
<td>5. DEAE-BioGel.A; KCl gradient peak II</td>
<td>98.6</td>
<td>59.8</td>
<td>0.606</td>
<td>17.7</td>
<td>8.9 x</td>
</tr>
<tr>
<td>6. Resuspended (NH₄)₂SO₄ precipitate</td>
<td>71.7</td>
<td>45.0</td>
<td>0.627</td>
<td>13.4</td>
<td>9.2 x</td>
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<tr>
<td>7. Sephacryl S-300 cytochrome peak</td>
<td>46.1</td>
<td>40.1</td>
<td>0.87</td>
<td>11.9</td>
<td>12.8 x</td>
</tr>
<tr>
<td>8. DEAE-BioGel.A; pH gradient cytochrome peak</td>
<td>5.9</td>
<td>24.5</td>
<td>4.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.3</td>
<td>61.1 x</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determination of cytochrome o was from a combination of reduced minus oxidized and reduced plus carbon monoxide minus reduced difference spectra using the extinction coefficients provided in references {97,104} (see Table V).

<sup>b</sup> Specific Content values may be low: values almost twice as high would be provided by utilization of the extinction coefficients suggested by the laboratory of Kaback (Table V).

<sup>c</sup> Haem content of the purified material is discussed in Results (section III.C.i.a).

---

**Table XIII:** Major steps in the purification of the cytochrome o complex.

Cytochrome o was purified from cells of strain PLJ01 following growth under high aeration on CYD minimal medium supplemented with disodium succinate.
solubilized proteins, and the latter upon stripping the membranes before a simpler anion exchange procedure, with a secondary gel exclusion step if required to remove 'minor impurities' \(^{99, 130}\). The current study employed stripping of extrinsic proteins with urea and with cholate, selective ammonium sulphate precipitation and rigorous fractionation employing high resolution liquid chromatographic techniques.

Sephacryl S-300 gel exclusion chromatography of the solubilized cytochrome \(o\) complex after the initial fractionation of the membrane extract via DEAE-BioGel.A indicated a molecular size of the complex of 516 000 (±10 000) \(^{\text{III.C.i.a}}\; \text{Fig. 71}\). This separation was carried out at a Triton X-100 concentration which had been lowered to 0.2 % (w/v), and represents the status of the cytochrome \(o\) complex prior to analysis by potentiometric titration. Kaback and coworkers have characterised the cytochrome \(o\) complex after solubilization and purification in octylglucoside \(^{129}\). This detergent solubilized preparation was subjected to density gradient centrifugation which resulted in two peaks of ubiquinol-1 oxidase activity: one with \(M_r = 140 000\) and a somewhat smaller peak of \(M_r = 240 000\). It was suggested that these represented the monomeric form of the oxidase in which one copy of each of the four subunits was present \(\nu.i\), and the dimeric form, containing two copies of each subunit, respectively. In comparison with other types of solubilizing agent the Triton series of detergents are known to extract hydrophobic proteins from membranes efficiently in a largely lipid-free state, but are relatively poor at dissociating protein-protein interactions \(^{82, 166}\). Consequently multimeric aggregates are frequently isolated from Triton solubilized preparations \(^{82}\). On the basis of the gel exclusion data it is proposed that the form isolated in the current study was probably tetrameric, containing four units of the cytochrome \(o\) complex. The shape of the chromophore peak eluting from the Sephacryl S-300 column was broad in comparison to those of the calibrating standards and of a preparation of solubilized peak IVd, suggesting that the sample was of heterogeneous size at this stage of the isolation procedure, although whether this was caused by aggregation with detergent or with proteins other than units of the cytochrome \(o\) complex is unknown \(\text{section III.B.iv.e}\; \text{Fig. 61b, 71}\).

The complex purified in this study comprised four main sizes of subunit, one of which was split into a doublet \(\text{section III.C.i.a}\). Anraku has reported two peptide constituents, \(M_r = 55 000\) and 33 000, whereas Kaback and Gennis have both observed four distinct subunits with \(M_r = 55 000, 35 000, 22 000\) and 17 000, noting that the largest subunit is hydrophobic and probably has a true molecular weight of 66 000 and that the smallest subunit stains poorly with Coomassie Brilliant Blue. Thus SDS-polyacrylamide electrophoretic gels of the cytochrome \(o\) complex prepared by the technique described in this study resembled those observed by Kaback and coworkers, the smallest subunit providing by far the faintest band with Coomassie Brilliant Blue, and
the stoichiometry of the subunits remaining unclear (Fig. 73) \{129\}. In contrast the 33-35 kDa band was resolved into twin bands running at 31 kDa and 32 kDa. No evidence was found for the monomeric form running in the gel as reported by Kaback's group with their octylglucoside solubilized preparation, although the high temperature incubation of the sample after addition of SDS was found to cause excessive deposition of protein at the top of the polyacrylamide gels during electrophoresis and was therefore omitted \{110, 129\}. Identification of the five bands as peptides of the cytochrome \(\alpha\) terminal oxidase complex was subsequently confirmed by gels on which were run samples of the oxidase prepared in the laboratory of R. B. Gennis, suggesting that the observation of twin bands at 31 kDa and 32 kDa was due to superior resolving power of the SDS-PAGE procedure employed in the current study rather than being a caused by differences in preparative technique (Fig. 73). Nevertheless, when purifying type-\(b\) haemoproteins an SDS-PAGE band split by such a size differential must be suspected of indicating haem loss from the larger peptide to generate the smaller of the two bands. The sample of cytochrome \(\alpha\) complex provided from the Gennis laboratory was derived from a \(cyo^+\) strain bearing a plasmid containing the cloned \(cyo\) operon, so these results also support the identity and completeness of the cloned material, which is discussed in greater detail below. The resolution of just two component peptides in the Anraku preparation may have been a result of exchanging the solubilizing detergent from Triton X-100 to Sarkosyl part way through the procedure \{97, 99\}. It thus appears that the association of the component peptides of the cytochrome \(\alpha\) complex is particularly susceptible to detergent perturbation.

Detection of haem in peptides separated by electrophoresis on SDS-polyacrylamide gels was unsuccessful, although several methods were attempted incorporating both peroxidase and hydroperoxidase tests with substrates of \(H_2O_2\), TMPD and TMBZ (Materials & Methods). It has been reported that the octylglucoside preparation of the cytochrome \(\alpha\) complex stains adequately for both peroxidase and TMPD oxidase activities in its monomeric state after electrophoresis under related conditions but that the individual peptides do not, only the largest peptide showing a faint, positive response to haem-catalysed peroxidase activity \{129\}. In this study the undissociated monomeric form of the oxidase complex was not observed after electrophoresis, possibly because in the presence of Triton X-100 larger aggregates predominated, especially the tetramer as was shown by gel exclusion chromatography and suggested by the failure of significant quantities of material to enter the gel after heating in the presence of SDS \(v.s.\).
**Fig. 73:** SDS-Polyacrylamide gel electrophoresis of purified samples of uncloned and of plasmid encoded cytochrome $o$.

Uncloned cytochrome $o$ complex was prepared from strain PLJ01 by the standard techniques, plasmid encoded cytochrome $o$ complex was purified and provided by R. B. Gennis and coworkers. The gels comprised 13% polyacrylamide and were stained with Coomassie Brilliant Blue. Running positions of the protein subunits of each preparation are indicated:

1. $M_r = 55,000$,
2. $M_r = 33,000$ (+ 32,000),
3. $M_r = 22,000$,
4. $M_r = 17,000$.

**Gel A** Uncloned cytochrome $o$ complex.

**Gel B** Plasmid encoded cytochrome $o$ complex.
(ii) Spectrophotometric Analyses of Natural and Cloned Cytochrome o Complex

(a) Spectrophotometry at ambient temperature

The pyridine haemochromogen derivative of the cytochrome o complex displayed a reduced minus oxidized spectrum with 295 K absorption maxima at 422.0 nm, 526.0 nm and 556.5 nm (420.0 nm, 524.5 nm and 555.0 nm at 77 K) indicating that only type-b haem was present in the oxidase (Fig. 74). Haems with either high or low spin iron would provide equivalent pyridine haemochromogen spectra as illustrated by the spectrum of the product derived from the haem b of catalase which contains high spin iron (Fig. 6).

Features of the absolute reduced and absolute oxidized spectra are shown in Fig. 74 and Table XIV. The Soret maxima are 428 nm and 412 nm respectively, and the reduced α-band maximum is a broad peak centred at approximately 560 nm. The oxidized absorption spectrum was virtually featureless between 500 nm and 700 nm, there being no apparent difference between the ferricyanide oxidized and the hydrogen peroxide oxygenated forms (data not shown).

(b) High resolution spectrophotometry

High resolution reduced minus oxidized spectra of the purified cytochrome o complex are illustrated in Figures 75 + 76. A comparison is provided between cytochrome o complex purified by the procedure described in the current study and that of the cloned material purified and provided by R. B. Gennis. Subsequently the strain RG167/pRG110 was made available and preparations of the cloned material were carried out by the procedures described under Materials & Methods. All preparations of the complex exhibited a multiphasic redox α-band spectrum with three major absorption maxima. The cloned material always produced a spectrum in which the lowest wavelength α-band peak, relative to the higher wavelength α-band peaks, exhibited approximately double the absorption of the low wavelength α-band peak of the 'uncloned complex' (Fig. 76 a). Fourth order derivative spectra showed that this absorption maximum was shifted slightly to the blue in the cloned material from 554.5 nm to 553.5 nm, probably because of the decreased percentage overlap of this lower wavelength peak brought about by the greater absorption intensity (Fig. 76 b). Subjecting the complex to many different fractionation techniques and alternative isolation procedures
Fig. 74: Absolute absorption spectra of cytochrome o measured at ambient temperature.

Purified, solubilized cytochrome o complex was resuspended to a protein concentration of 0.13 mg mL\(^{-1}\) in 0.25 % (w/v) Triton X-100, 1.0 M sucrose, 100 mM potassium phosphate buffer, pH 7.0. Spectrophotometry was performed at 295 K. \(\Delta A = 0.004\).

1. Absolute spectrum of dithionite reduced complex.
3. Dithionite reduced minus H\(_2\)O\(_2\) oxidized difference spectrum of the complex.
4. Dithionite reduced minus ferricyanide oxidized difference spectrum of the pyridine haemochromogen derivative of the complex.
### Unclonated cytochrome o complex:

<table>
<thead>
<tr>
<th>Spectrum</th>
<th>Temperature (K)</th>
<th>( \lambda_{\text{max}}^{\gamma} ) (nm)</th>
<th>( \lambda_{\text{min}}^{\gamma} ) (nm)</th>
<th>( \lambda_{\text{max}}^{\beta} ) (nm)</th>
<th>( \lambda_{\text{max}}^{\alpha} ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced</td>
<td>295</td>
<td>(405), 428</td>
<td></td>
<td>531</td>
<td>560</td>
</tr>
<tr>
<td>Oxidized</td>
<td>295</td>
<td>412</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced + CO</td>
<td>295</td>
<td>(415), 428</td>
<td></td>
<td>531</td>
<td>560</td>
</tr>
<tr>
<td>Reduced - oxidized</td>
<td>295</td>
<td>430</td>
<td></td>
<td>531</td>
<td>561</td>
</tr>
<tr>
<td>Reduced - oxidized</td>
<td>77</td>
<td>428</td>
<td></td>
<td>(526) 529,537</td>
<td>554.5,557.5,563.5</td>
</tr>
<tr>
<td>(Reduced + CO) - reduced</td>
<td>295</td>
<td>416, 430</td>
<td></td>
<td></td>
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</table>

### Plasmid encoded cytochrome o complex:

<table>
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<tr>
<th>Spectrum</th>
<th>Temperature (K)</th>
<th>( \lambda_{\text{max}}^{\gamma} ) (nm)</th>
<th>( \lambda_{\text{min}}^{\gamma} ) (nm)</th>
<th>( \lambda_{\text{max}}^{\beta} ) (nm)</th>
<th>( \lambda_{\text{max}}^{\alpha} ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced - oxidized</td>
<td>295</td>
<td>430</td>
<td></td>
<td>531</td>
<td>560</td>
</tr>
<tr>
<td>Reduced - oxidized</td>
<td>77</td>
<td>428</td>
<td></td>
<td>(524) 528,537</td>
<td>553.5,557.5,564.0</td>
</tr>
<tr>
<td>(Reduced + CO) - reduced</td>
<td>295</td>
<td>416, 430</td>
<td></td>
<td></td>
<td></td>
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</table>

**Table XIV:** Absorption maxima of cytochrome o complex from natural and cloned sources.
resulted in no significant spectral changes. Spectra indistinguishable from that displayed by the cytochrome $o$ complex obtained from strain PLJ01 (Fig. 76 a) have been produced by samples of uncloned oxidase purified from cells of all genetic backgrounds tested, amongst which were several unrelated strains including strain RG132 from which the plasmid carrying the $cyo$ genes was originally cloned by R. B. Gennis and coworkers \(6\). Consequently the complicated $\alpha$-band was taken to indicate the presence of multiple type-$b$ cytochromes in the complex itself rather than a failure to remove them during the purification procedure. This supposition was confirmed by the SDS-PAGE data described above. Table XIV provides the observed absorption maxima of the two types of preparation. One interpretation of the existence of the two distinct spectral forms was that the cloned material contained an extra copy of a cytochrome $b$ with an $\alpha$-band absorption maximum at 554.5 nm. However, the SDS-PAGE results did not show a dramatic increase in intensity of any of the bands between the natural and cloned oxidase preparations and it is difficult to envisage a mechanism permitting attachment of an additional subunit sufficiently tightly to allow its copurification through such a variety of fractionation procedures. The implications of the different spectral properties of the natural and cloned material are discussed below (section III.C.vi).

Kaback had reported that the largest subunit of the complex contained haem, yet the haem detection methods applied in this study were unsuccessful, as discussed above (section III.C.i.b). Consequently techniques were required to distinguish between the three major absorption peaks in order to determine how many cytochromes-$b$ were responsible for producing them and the nature of each of those haemoprotein components. Single peptides containing multiple haems had been detected in *E. coli* and the gene for a single apoprotein had spectrally been shown to generate two distinct mitochondrial $b$-type cytochromes \(111, 142\). Thus, although the relative intensities of the three redox $\alpha$-band absorbance maxima remained approximately constant in every preparation of the uncloned terminal oxidase complex methods of perturbing this $\alpha$-band spectrum were sought in order to record any divergence or linkage in the response of individual spectral features.

No alteration was observed between reduced minus oxidized difference spectra when the sample was reduced with duroquinol, with NADH or ascorbate in the presence of the electrochemical mediator PMS, or when dithionite was used (Fig. 34 +77). This not only suggested that the cytochrome $o$ complex had a relatively high mid-point potential since the duroquinol-duroquinone redox couple has a potential of approximately 0 mV but also that there were no lower potential cytochromes copurifying with the preparation.
**Fig. 75:** High resolution broad range redox difference spectra of uncloned and of plasmid encoded cytochrome o.

Plasmid encoded cytochrome o complex was purified and provided by R. B. Gennis and coworkers. It was analysed at a protein concentration of 34 µg mL⁻¹. Uncloned cytochrome o complex was prepared from strain PLJ01 by the standard techniques and resuspended to a protein concentration of 80 µg mL⁻¹. The buffer was 0.25 % (w/v) Triton X-100, 1.0 M sucrose, 100 mM potassium phosphate buffer, pH 7.0. Spectrophotometry was performed at 77 K.

1. Dithionite reduced *minus* H₂O₂ oxidized difference spectrum of the plasmid encoded cytochrome o complex. \( \Delta A = 0.10 \).

2. Dithionite reduced *minus* H₂O₂ oxidized difference spectrum of the uncloned cytochrome o complex. \( \Delta A = 0.10 \).

3. Dithionite reduced plus carbon monoxide *minus* H₂O₂ oxidized difference spectrum of the uncloned cytochrome o complex. \( \Delta A = 0.075 \).

4. Dithionite reduced plus carbon monoxide *minus* dithionite reduced difference spectrum of the uncloned cytochrome o complex. \( \Delta A = 0.020 \).
Fig. 76: High resolution redox $\alpha$-absorption spectra of uncloned and of plasmid encoded cytochrome $o$.

Plasmid encoded cytochrome $o$ complex was purified and provided by R. B. Gennis and coworkers. It was analysed at a protein concentration of 34 $\mu$g mL$^{-1}$. Uncloned cytochrome $o$ complex was prepared from strain PLJ01 by the standard techniques and resuspended to a protein concentration of 80 $\mu$g mL$^{-1}$. The buffer was 0.25 % (w/v) Triton X-100, 1.0 M sucrose, 100 mM potassium phosphate buffer, pH 7.0.

Spectrophotometry was performed at 77 K as indicated below.

**a.** Dithionite reduced minus H$_2$O$_2$ oxidized difference spectra: $\alpha$-absorption bands.
1. Plasmid encoded cytochrome $o$ complex. $\Delta A = 0.01$.
2. Uncloned cytochrome $o$ complex. $\Delta A = 0.01$.
3. Spectrum #a.1 minus spectrum #a.2. $\Delta A = 0.01$.

**b.** Fourth order derivative spectra.
1. Fourth order derivative of spectrum #a.1.
2. Fourth order derivative of spectrum #a.2.

**c.** Dithionite reduced plus carbon monoxide minus dithionite reduced difference spectrum.
1. Carbon monoxide binding spectrum of the uncloned cytochrome $o$ complex at 77 K: $\alpha$-band absorption region. $\Delta A = 0.001$. 
(c) **Perturbation spectrophotometry**

The isolated cytochrome $o$ complex had a distinct spectroscopic response to the quinone analogue HOQNO accompanying the kinetic effects of this respiratory inhibitor which are described in section III.C.iii.b. During the dual wavelength kinetic studies samples were removed for high resolution spectrophotometric analysis at identifiable stages of reduction in the presence and absence of HOQNO. Figure 78 demonstrates the changes in the redox difference spectrum caused by reduction of the oxidase complex with the inhibitor present. The two $\alpha$-band peaks at 554.5 nm and 557.0 nm were largely unaffected while the longer wavelength $\alpha$-band peak underwent a red shift from 564.0 nm to 564.8 nm. This suggests that the site of action of the quinol analogue may be close to a haem responsible for this feature of the absorption spectrum and that it has less effect on the environments of any haem causing the shorter wavelength $\alpha$-band absorption peaks.

The action of carbon monoxide upon the dithionite-reduced cytochrome $o$ complex at ambient temperature is shown in Figures 74 and 79. The main spectral effect of this ligand is in the Soret region where the absorption band of the reduced form undergoes a shift from 428 nm to 415 nm, similar to that seen after the interaction of carbon monoxide with reduced membrane preparations derived from cyd$^+$ cells (section II.C.ii.c). The absolute reduced spectrum demonstrates that the 415 nm absorption peak appears as a blue shoulder on the 428 nm 'reduced' peak after reaction with CO, the intensity of the reduced minus oxidized Soret band decreasing to about 80% of its original value when measured in reduced plus carbon monoxide minus oxidized difference spectra at 295 K, as indicated qualitatively by the reduced plus carbon monoxide minus reduced difference spectrum (Fig. 74, 79). The low temperature reduced plus carbon monoxide minus reduced difference spectrum of the solubilized cytochrome $o$ complex maintained the Soret absorption maximum at 415 nm and the minimum at 428 nm observed at ambient temperatures but showed some $\alpha$-band detail that was not apparent at 295 K — a sharp minimum at 558 nm and a broader, overlapping maximum at 567 nm (Fig. 76). The intensity of these $\alpha$-band features was low but they indicate that perturbation of the $\alpha$-band by carbon monoxide involves the inhibitor's interaction with a cytochrome component with $\alpha$-band absorbance at wavelengths greater than 558 nm, a suggestion in contrast to that proposed by Anraku and coworkers [97].
Fig. 77: Reduction of the cytochrome o complex by various reductants: high resolution redox α-absorption spectra.

Purified, solubilized cytochrome o complex was resuspended to a protein concentration of 0.13 mg mL\(^{-1}\) in 0.25 % (w/v) Triton X-100, 1.0 M sucrose, 100 mM potassium phosphate buffer, pH 7.0. Spectrophotometry was performed at 77 K as indicated below. \(\Delta A = 0.004\).

\textbf{a.} Comparison of spectral effects of reduction by various reductants.
1. Dithionite reduced \textit{minus} \(\text{H}_2\text{O}_2\) oxidized difference spectrum of the complex.
2. NADH+PMS reduced \textit{minus} \(\text{H}_2\text{O}_2\) oxidized difference spectrum of the complex.
3. Ascorbate+PMS reduced \textit{minus} \(\text{H}_2\text{O}_2\) oxidized difference spectrum of the complex.
4. Duroquinol reduced \textit{minus} \(\text{H}_2\text{O}_2\) oxidized difference spectrum of the complex.
5. Difference spectrum of complex treated with NADH in the absence of PMS \textit{minus} \(\text{H}_2\text{O}_2\) oxidized complex.

\textbf{b.} Comparison of spectral effects of oxygenation and oxidation.
1. Dithionite reduced \textit{minus} \(\text{H}_2\text{O}_2\) oxidized difference spectrum of the complex.
2. Dithionite reduced \textit{minus} ferricyanide oxidized difference spectrum of the complex.

\textbf{c.} Comparison of spectral effects of oxygenation and oxidation.
1. Fourth order derivative of spectrum \#b.1.
2. Fourth order derivative of spectrum \#b.2.
**Fig. 78:** Effects of HOQNO upon high resolution redox α-absorption spectrum of solubilized cytochrome o.

Cytochrome o was solubilized, purified by the standard technique and resuspended to a protein concentration of 0.43 mg mL\(^{-1}\) in 0.5 % (w/v) Triton X-100, 1.0 M sucrose, 100 mM potassium phosphate buffer, pH 7.0. Cytochrome reduction was monitored by dual wavelength spectrophotometry at 559 nm \textit{minus} 590 nm following addition of 20 μL 80 mM NADH plus 2μL 15 mM phenazine methosulphate. Samples were withdrawn during the anaerobic steady state and rapidly frozen to 77 K before low temperature redox difference spectrophotometry \textit{versus} reference samples oxidized with hydrogen peroxide. \(\Delta A = 0.004\).

1. Reduction in the presence of 20 μM HOQNO.

2. Control.

3. Fourth order derivative of spectrum #1.

4. Fourth order derivative of spectrum #2.
(d) Photolysis of the oxidase-carbon monoxide complex

The carbon monoxide binding experiments were carried out in total darkness. Irradiation of the samples with an incandescent light source at ambient temperature caused little change in the α-band region but resulted in a further decrease in absorbance intensity of the 428 nm peak of the absolute reduced plus carbon monoxide spectrum with a corresponding increase in the intensity of a previously minor peak with maximum absorption at 405 nm (Fig. 79). Because of the overlap between these various Soret features modification of the 415 nm absorption peak induced by CO binding could not be monitored. It is apparent that at ambient temperatures the effect of irradiating the carbon monoxide derivative of the Triton solubilized cytochrome o complex is not a simple reversal of the binding reaction. These samples had been reduced with dithionite, were oxygen free and sealed: the formation of oxygenated derivatives should not have interfered with photolysis and release of carbon monoxide.

(iii) Kinetic Analyses of Reduction of the Cytochrome o Complex

(a) Response to various reductants

As stated above, no spectrophotometric alteration was observed between reduced minus oxidized difference spectra when the sample was reduced with any of a variety of reductants indicating that the oxidase complex was being fully reduced in each case. The complex was reduced directly by dithionite and by duroquinol, but required the addition of an electrochemical mediator before reduction could be achieved with NADH, ascorbate or other biological reductants. Reoxidation could be brought about by ferricyanide, ammonium persulphate or hydrogen peroxide but not by fumarate or nitrate, even in the presence of PMS (data not shown). These observations supported the identification of the isolated product as a quinol oxidase and the proposals that the cytochrome o complex has a relatively high mid-point potential and that there were no dehydrogenase or other oxidases copurifying with the preparation. It has been reported that dithionite acts on biological material as a reductant by reducing dissolved dioxygen to water and that caution should be exercised in its use since several minutes may be required to bring about full reduction of a biological sample in a cuvette after the buffer has been made anoxic in this way [90]. Moreover many biological membranes have been reported to be impermeable to dithionite ions [90]. The current results show
**Fig. 79:** Ambient temperature carbon monoxide binding spectra of solubilized cytochrome o.

Purified, solubilized cytochrome o complex was resuspended to a protein concentration of 0.13 mg mL\(^{-1}\) in 0.5 % (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0. Treatment of dithionite reduced cytochrome with carbon monoxide was achieved by the method described under Materials & Methods.

Spectrophotometry was performed as indicated below. \(\Delta A = 0.004\) unless otherwise indicated.

1. Absolute spectrum of dithionite reduced complex.
2. Absolute spectrum of dithionite reduced complex after gassing with carbon monoxide.
3. Absolute spectrum after treatment (2) plus illumination with a 150 W incandescent light source at 5 cm for 150 s.
4. Dithionite reduced minus \(\text{H}_2\text{O}_2\) oxidized difference spectrum of the complex.
5. Dithionite reduced plus carbon monoxide minus \(\text{H}_2\text{O}_2\) oxidized difference spectrum of the complex. The CO gassing procedure was as for spectrum (2).
6. Dithionite reduced plus carbon monoxide minus dithionite reduced difference spectrum of the complex. The CO gassing procedure was as for spectrum (2). \(\Delta A = 0.001\).
that dithionite reduces the solubilized cytochrome $o$ complex directly, for it rapidly reduced the oxidized complex in the absence of any other reductants when added to an oxidase sample in either aerobic or anaerobic solution. Numerous examples of rapid reduction by dithionite had been observed in dual wavelength and split beam difference spectroscopy throughout these studies: in the case of membrane suspensions full reduction was attained within seconds of adding aliquots of fresh, buffered Na$_2$S$_2$O$_4$ whether or not endogenous substrates had been previously exhausted, and titrations of membrane suspensions or purified solubilized cytochromes were immediately reduced by dithionite under conditions in which oxygen had previously been exhaustively removed (sections II.A.ii, II.C.i+ii, III.B.iv.f; Materials & Methods).

Certain reductants produced aerobic steady state reduction levels in a standard solution of the uncloned oxidase complex during dual wavelength studies yet dithionite produced full reduction at a rate that was effectively instantaneous in 'slow' kinetic measurements (Fig. 80). This absence of a steady state upon reduction by dithionite and the extended delay before slow reduction of the cytochrome $o$ complex by duroquinol indicated that the reaction of duroquinol with the oxidase was far slower than the reduction of available oxygen by the cytochrome $o$ complex. Interestingly an aerobic steady state reduction level of 37% was observed during duroquinol reduction at a higher concentration of the oxidase complex (0.68 mg mL$^{-1}$protein). These studies were carried out at wavelengths of 559.0 nm minus 590.0 nm in 0.2% (w/v) Triton X-100 and were the basis for an investigation of the inhibitory action of HOQNO and also for analysis of the rapid kinetics of reduction of the complex by dithionite using stopped-flow apparatus (v.i).

(b) Inhibition of reduction and oxidation

Dual wavelength spectrophotometric analysis of the redox kinetics of the cytochrome $o$ terminal oxidase showed a complex response to the quinone analogue HOQNO. Figure 81 illustrates that addition of a moderate excess of NADH to the solubilized oxidase containing the redox mediator PMS achieved an aerobic steady state reduction level of 44% which progressed rapidly to full reduction upon exhaustion of dissolved dioxygen. Addition of an oxygen supply, in the form of 4 µL aqueous hydrogen peroxide containing approximately 3.5 µmol H$_2$O$_2$, resulted in transient reoxidation of the cytochrome $o$ complex until anaerobiosis was reestablished by the continued transfer of electrons from the NADH and the oxidase complex was rereduced.

When these operations were performed in the presence of the quinol analogue HOQNO the rapid reoxidation of the NADH-reduced oxidase was observed upon addition of H$_2$O$_2$ to the sample
Fig. 80: Reduction kinetics of the solubilized cytochrome o complex.

Cytochrome o was solubilized, purified by the standard technique and resuspended in 0.5 % (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0. Type-b cytochrome reduction was monitored in by dual wavelength spectrophotometry at 559 nm minus 590 nm following oxidation of the 2.0 mL samples by adding 2 μL 3 % (v/v) H₂O₂.

P, addition of 2 μL 15 mM phenazine methosulphate.

N, addition of 20 μL 80 mM NADH.

A, addition of 10 μL 400 mM ascorbate.

D, addition of 20 μL duroquinol at 10 mg mL⁻¹ in ethanol.
**Fig. 81:** HOQNO effects upon the kinetics of reduction and reoxidation of solubilized cytochrome o complex.

The net redox state of the total b-cytochrome in samples of solubilized cytochrome o complex was measured by dual wavelength spectrophotometry at 559 nm relative to 590 nm. Samples comprised 2.0 mL solubilized cytochrome complex at a protein concentration of 0.43 mg mL$^{-1}$ in 0.5 % (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0.

Samples contained HOQNO at the following concentrations:

1. 0.0 mM,
2. 12.5 mM,
3. 25.0 mM,
4. 50.0 mM.

S, addition of 20 μL 80 mM NADH and 2 μL 15 mM phenazine methosulphate.

O, addition of 3.5 μmol H$_2$O$_2$ in 4 μL aqueous solution.
although subsequent rereduction of the cytochrome to the fully reduced state occurred after a shorter delay. Since the redox state of a dynamic equilibrium was being measured, in that the cytochrome was undergoing simultaneous reduction by the NADH/PMS and oxidation from available oxygen, the observations indicated that the rates of cytochrome reduction and oxidation had been affected differentially by HOQNO such that the rate of net reduction had been increased. That this was caused by HOQNO inhibition of the rate of cytochrome oxidation by $\text{O}_2$ was indicated by additional observations showing that HOQNO inhibits the direct reduction of cytochrome $o$ complex by NADH/PMS: at HOQNO concentrations of 50 mM the proportion of cytochrome reduced in the aerobic steady state was decreased to about 36% of the total, the duration of the aerobic steady state was extended by 67% and the rate of subsequent reduction to the fully reduced, anaerobic state was slower than in the absence of the inhibitor (Fig. 81). Also reports from the laboratories of Anraku and Kaback have shown that this quinone analogue inhibits cytochrome $o$ activity during reduction of the oxidase by quinols {97,129}. In addition to quinol solubility limitations the current study found that the rate of reduction of the oxidase complex by duroquinol was sufficiently slow to make impractical 'reoxidation' experiments similar to those described above but using duroquinol as reductant.

Implications of the inhibition of both reduction and oxidation of the cytochrome $o$ terminal oxidase complex by a quinol analogue are discussed below in section III.C.vi.

The relatively recent availability of substantial quantities of cytochrome $o$ complex in the form of cloned material will permit a thorough investigation of the kinetics of inhibition of the oxidase activities of the cytochrome $o$ complex by HOQNO. These results may then be compared to data obtained from critical comparisons with experiments using the restricted quantities of material available from uncloned sources in order to detect whether the factors responsible for the spectrophotometric and potentiometric differences between these two products may be correlated with distinct kinetic properties.

(c) Stopped-flow analysis of reduction kinetics

Rapid kinetic analysis of the reduction of the cytochromes of the cytochrome $o$ complex was carried out with an Aminco-Morrow stopped-flow apparatus coupled to the SLM/Aminco DW-2c spectrophotometer operated in dual wavelength mode (560 nm minus 575 nm) as described under Materials & Methods. Dithionite was added to the sample of oxidase as an equal volume of essentially oxygen-free solution at 80 mM in 100 mM potassium phosphate buffer. Although the
stopped-flow apparatus was certified by the manufacturer to be in correct working order its functional
dead time was determined to be approximately 100 ms, an order of magnitude greater than the
instrument’s specifications and a period during which about 65 % of the sample was reduced under the
experimental conditions. In spite of signal strength and noise constraints which precluded further
dilution of the oxidase complex the data obtained demonstrated two phases of reduction (Fig. 82).
The first phase that was measurable had a first order rate constant in excess of $0.4 \text{s}^{-1}$ and the second,
accounting for the final 35 % of the total cytochrome reduced, exhibited a value for $k_1 = 0.03 \text{s}^{-1}$.

This multiphasic reduction behaviour, measured at 560 nm, indicates that the complexity of the
$\alpha$-band redox difference spectrum is caused by multiple haems. Repetition of the experiments with
equipment enabling data to be gathered within the initial 100 ms of the reaction should indicate
whether the number of components contributing significantly to the reduced $\alpha$-band spectrum
number more than two.

(iv) Potentiometric Titration of the Cytochrome $\alpha$ Complex

(a) Titration of the complex isolated from natural sources

Titration of the solubilized cytochrome $\alpha$ complex was carried out under standard conditions
with the addition of 0.2 % (w/v) Triton X-100 to the 100 mM potassium phosphate titration buffer at
pH 7.0. The samples were oxidized, and presumably oxygenated, in their endogenous state,
with an electrochemical potential of about +320 mV. The solubilized cytochrome $\alpha$ preparation
titrated as a complex of three type-$b$ cytochromes with mid-point potentials of -58 mV, +127 mV,
and +260 mV contributing 29 %, 49 % and 22 % respectively to the absorbance change at the $\alpha$-band
redox absorption maximum (Fig. 83).

This preparation of cytochrome $\alpha$ complex was then titrated in the presence of carbon
monoxide as described under Materials & Methods: the reduced sample was gassed with CO
and titrated under an atmosphere of this oxidase inhibitor, microlitre additions of a saturated solution
of K$_3$Fe(CN)$_6$ being used to oxidize the sample at potentials above +150 mV. Under these
conditions not only was ferricyanide ($E_m = +430 \text{mV}$) unable to oxidize the sample completely but
the mid-point potentials of each of the three components were modified sufficiently for the theoretical
curve providing the best fit of the data to be displaced significantly (Fig. 83). The $E_m$ values
obtained for the three components after treatment with carbon monoxide were -92 mV, +51 mV and
Fig. 82: Rapid kinetics of reduction of solubilized cytochrome o.

Solubilized cytochrome o complex in 0.5 % (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0 was mixed with an equal volume of 80 mM Na$_2$S$_2$O$_4$ in 100 mM potassium phosphate buffer, pH 7.0 in the reaction chamber of an Aminco-Morrow stopped-flow apparatus coupled to the DW-2c spectrophotometer operating in dual wavelength mode, yielding a final protein concentration of 2.5 mg mL$^{-1}$. The redox state of cytochrome b present in the sample was monitored at 560 nm relative to 575 nm. The line represents a first order rate constant of 0.03 s$^{-1}$. 
Fig. 83: Potentiometric titration of solubilized cytochrome o: the effect of carbon monoxide.

The cytochrome o complex, partially purified by liquid chromatography via DEAE-BioGel A anion exchange and gel filtration through Sephacryl S-200 and S-300 matrices was titrated in a solution of 0.2 % (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0 at a protein concentration of 500 µg mL⁻¹. Where appropriate an intermittent flow of carbon monoxide was supplied as described in ‘Materials & Methods’. Theoretical curves for three component fitting of each data set are illustrated. The mid-point potentials of these components are indicated and their individual contributions to the total type-b cytochrome are expressed as percentages of the α-band λ_max absorbance of the sample:

O—O, Data for standard reductive titration of cytochrome o: (———), theoretical curve,

• - - • , Data obtained following carbon monoxide treatment: ( — — — ), theoretical curve.

<table>
<thead>
<tr>
<th>Standard Titration</th>
<th>Treatment with CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ 260.1 mVvs.NHE</td>
<td>+ 436.5 mVvs.NHE</td>
</tr>
<tr>
<td>+ 127.0 mVvs.NHE</td>
<td>+ 51.0 mVvs.NHE</td>
</tr>
<tr>
<td>- 58.3 mVvs.NHE</td>
<td>- 92.1 mVvs.NHE</td>
</tr>
</tbody>
</table>
+437 mV, their contributions to the \( \alpha \)-absorbance band at the peak maximum being 31\%, 54\% and 15\%. The data obtained in the presence of carbon monoxide indicated that the mid-point potential of the +260 mV component had shifted dramatically to higher potential, although only an estimate of this 'CO-treated' potential can be obtained from these data because of the inability to oxidize this component sufficiently (Fig. 83). This large shift to higher potential, indicating the formation of a stable carbonmonoxy derivative suggests that this component of the redox \( \alpha \)-absorption band was caused by an exposed haem capable of reacting with oxygen \{112, 216\}. Thus the component responsible for this feature of the titration profile may have been cytochrome \( \text{o} \) itself \{8, 117\}. The two lower potential components of the complex also underwent shifts of potential which were smaller in magnitude and to lower potentials. These shifts were not consistent with previously reported effects of carbon monoxide binding to cytochrome haem and since previous evidence had been obtained for potentiometric perturbations being brought about in resuspended membrane samples by the addition of specific reagents (sections II.A.ii.c, II.C.i.b, II.C.ii.b) these effects were investigated further \{117,216\}.

Suspecting that certain components of the solubilized cytochrome \( \text{o} \) complex were susceptible to perturbation by exogenous reagents, a similar preparation of the complex was titrated after treatment with ferricyanide. A comparison between the results obtained from this titration and the untreated sample shows that the potential of the +260 mV component was effectively unchanged at +273 mV but that the middle potential component behaved with \( E_m \) lowered about 60 mV to +68 mV, similar to the modification observed in the presence of carbon monoxide, and the low potential component underwent a change of \( E_m \) to -118 mV, again resembling that observed in the presence of CO (Fig. 84). The percentage contribution of each component to the total \( \alpha \)-band absorbance was modified by the decrease in the size of the low potential cytochrome absorption after the ferricyanide treatment. When plotted as the reduced minus oxidized \( \alpha \)-band absorption change it is apparent that the magnitude of absorption by each of the two higher potential components was unaffected (Fig. 84).

Thus the +260 mV component of the cytochrome \( \text{o} \) complex reacted with carbon monoxide with an increase of mid-point potential by some 180 mV, but was not sensitive to ferricyanide. The +127 mV component underwent a decrease of mid-point potential of some 65 mV when treated with either CO or \([\text{Fe(CN)}_6]^3-\). The -58 mV component was also affected by both reagents: carbon monoxide caused a decrease in mid-point potential of some 35 mV, without modifying the absorption intensity of the \( \alpha \)-band as this component was reduced and ferricyanide shifted the \( E_m \) about 60 mV lower, substantially decreasing the \( \alpha \)-band absorption change.
Fig. 84: Potentiometric titration of solubilized cytochrome o: the effect of ferricyanide.

The cytochrome o complex, partially purified by liquid chromatography via DEAE-BioGel A anion exchange and gel filtration through Sephacryl S-200 and S-300 matrices was titrated in a solution of 0.2% (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0 at a protein concentration of 500 µg mL⁻¹. Theoretical curves for three component fitting of each data set are illustrated. The mid-point potentials of these components are indicated and their individual contributions to the total type-b cytochrome are expressed as percentages of the α-band $\lambda_{max}$ absorbance of the sample:

O—O, Data for standard reductive titration of cytochrome o: (________), theoretical curve,

bullet—bullet, Data following sample oxidation with [Fe(CN)$_6$]$_3^{3-}$: ( — — — ), theoretical curve.

<table>
<thead>
<tr>
<th>Standard Titration</th>
<th>Treatment with [Fe(CN)$_6$]$_3^{3-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ 260.1 mV vs. NHE : 22.3 %</td>
<td>+ 272.6 mV vs. NHE : 31.9 %</td>
</tr>
<tr>
<td>+ 127.0 mV vs. NHE : 49.0 %</td>
<td>+ 68.1 mV vs. NHE : 58.6 %</td>
</tr>
<tr>
<td>- 58.3 mV vs. NHE : 28.7 %</td>
<td>- 118.0 mV vs. NHE : 9.5 %</td>
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**Fig. 85:** Potentiometric titration of solubilized cytochrome o: comparison of perturbation effects.

The cytochrome o complex, partially purified by liquid chromatography via DEAE-BioGel.A anion exchange and gel filtration through Sephacryl S-200 and S-300 matrices was titrated in a solution of 0.2 % (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0 at a protein concentration of 500 µg mL⁻¹. Before titration the sample was fully oxidized with small aliquots of concentrated K₂Fe(CN)₆: the CO-treated sample was titrated under an intermittent flow of carbon monoxide as described in Materials & Methods. Theoretical curves for three component fitting of each data set are illustrated. The mid-point potentials of these components are indicated and their individual contributions to the total type-b cytochrome are expressed as percentages of the α-band λ_max absorbance of the sample:

- •••, Data following sample oxidation with [Fe(CN)₆]³⁻: (-----), theoretical curve,
- ~ ~ ~, Data following { [Fe(CN)₆]³⁻ + CO } treatments: ( ---- ---- ), theoretical curve.

<table>
<thead>
<tr>
<th>Treatment with [Fe(CN)₆]³⁻</th>
<th>Treatment with [[Fe(CN)₆]³⁻ + CO]</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ 272.6 mV vs.NHE</td>
<td>+ 420.1 mV vs.NHE</td>
</tr>
<tr>
<td>31.9 %</td>
<td>27.0 %</td>
</tr>
<tr>
<td>+ 68.1 mV vs.NHE</td>
<td>+ 72.3 mV vs.NHE</td>
</tr>
<tr>
<td>58.6 %</td>
<td>62.7 %</td>
</tr>
<tr>
<td>- 118.0 mV vs.NHE</td>
<td>- 52.2 mV vs.NHE</td>
</tr>
<tr>
<td>9.5 %</td>
<td>10.3 %</td>
</tr>
</tbody>
</table>
The effect of carbon monoxide was tested on a sample of the oxidase complex that had been pretreated with ferricyanide. Thus the effect of carbon monoxide upon the reactive haem was observed without interference from cytochrome components undergoing perturbations of response caused by sensitivity to other exogenous reagents (Fig. 85). Comparing the response of the solubilized oxidase complex when titrated in the presence of carbon monoxide after pretreatment with ferricyanide with that after ferricyanide pretreatment alone, the theoretical best-fit curves show that the major effect of exposure to CO was to shift the mid-point potential of the '+260 mV component' from +273 mV to +420 mV (in the presence of ferricyanide). The magnitude of the α-band redox absorption change due to reduction of each component remained constant between these two sets of conditions, as did the $E_m$ of the mid potential component. Treatment of the complex with ferricyanide decreased the size of the low potential component's α-band redox absorption so that it was unclear whether the lowering of this component's mid-point potential by CO was a significant change (Fig. 85).

Since poised potential high resolution spectra had indicated that the +260 mV 'component' of cytochrome $o$ comprised a biphasic α-band this region of the titration profile was inspected separately with readings taken at intervals of approximately 5 mV (Fig. 86). The reduction data derived from the α-band absorption maximum were best fitted by a single component curve, and a Nernst plot indicative of a single electron transfer, suggesting that a single haem group might be responsible for the twin α-band feature or that two haems were present with $E_m$ values too similar to be differentiated (Fig. 86). The low mid-point potential value obtained in this titration, 229 mV, in comparison with others of +260 mV for this component, plus the greater scatter in the data observed below +230 mV reflect the difficulty in determining the maximal reduction of this component in the presence of the other constituents of the complex.

(b) Titration of the cloned complex

Preparations of the cloned cytochrome $o$ complex from RG167 pRG110 were subjected to potentiometric titration under the standard conditions and under conditions perturbed by ferricyanide and/or carbon monoxide. Representative data for these reductive titrations are shown in Figures 87 and 88. Data for the standard titration were fitted best by a four component, rather than a three component theoretical curve (RMS = 3.53 and 5.73 respectively) and indicated mid-point potentials of +55 mV, +153 mV and +273 mV, in addition to the component at about -100 mV which was generally present in small quantities (c. 10 %) but was not observed in all preparations. Percentages
**Fig. 86:** Potentiometric titration of the high potential component of solubilized cytochrome $o$.

The cytochrome $o$ complex, partially purified by liquid chromatography via DEAE-BioGel A anion exchange was titrated in a solution of 1.0 % (w/v) Triton X-100, 1.0 mM EDTA, 10 mM Tris-HCl buffer, pH 7.0 at a protein concentration of 2.50 μg mL$^{-1}$. The high-potential component of the complex was investigated in detail and the data is shown in (a) direct and (b) Nernst plots.

Linear regression analysis of the Nernst plot data indicates a linear correlation of 0.920 for a single component with $E_h = +229.1$ mV vs. NHE, slope = 71.7 mV, $n = 1.20$. 
Fig. 87: Potentiometric titration of the solubilized, plasmid encoded cytochrome o.

The cloned cytochrome o complex, partially purified by liquid chromatography was titrated in a solution of 0.2% (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0 at a protein concentration of 500 μg mL⁻¹. Theoretical curves for three and four cytochrome components are illustrated. The mid-point potentials of these components are indicated and their individual contributions to the total type-b cytochrome are expressed as percentages of the α-band λ_{max} absorbance of the sample.

(— — —) Three Component Fit
+ 178.0 mV vs. NHE : 57.5 %  
+ 76.9 mV vs. NHE : 30.0 %  
- 90.6 mV vs. NHE : 12.5 %

(— — —) Four Component Fit
+ 228.1 mV vs. NHE : 12.3 %  
+ 157.4 mV vs. NHE : 54.3 %  
+ 56.8 mV vs. NHE : 22.0 %  
- 100.2 mV vs. NHE : 11.3 %
of the three major components were 13\%, 56\% and 31\% respectively.

Oxidative titration with ferricyanide in the presence of carbon monoxide revealed just two components at potentials above 0 mV: the high potential component with $E_m$ shifted to +459 mV (20\%) and a component with a mid-point potential of +71 mV (80\%) displaying an abrupt transition from reduced to oxidized forms.

One interpretation of these data is that the cloned oxidase complex contains an extra cytochrome subunit which is observed as a supplemented 554 nm $\alpha$-band peak in high resolution redox difference spectra and as an additional component in the potentiometric titrations. However, it is difficult to postulate a mechanism by which an 'extra' subunit might be bound stoichiometrically to the complex throughout the purification procedure which incorporates separatory procedures exploiting various chemical and physical properties of the oxidase. No differences were observed in SDS-PAGE gels of the two types of material, although disparity in the subunits' affinity for Coomassie Brilliant Blue stain hindered quantitation. Inspection of the titrations carried out under conditions perturbed by ferricyanide or carbon monoxide suggests an alternative explanation of the results. Figures 83 and 85 demonstrate that ferricyanide and carbon monoxide are both capable of perturbing the mid-point potentials of the -58 mV and +127 mV components in the solubilized complex. Ferricyanide pretreatment also decreases the $\alpha$-band reduced minus oxidized absorption of the low potential component by approximately 70\%. Thus these components appear to be capable of existing in the solubilized complex in at least two forms, the higher potential form of each being stabilized in standard oxidase preparations and a shift to the lower potential, 'perturbed' form occurring upon treatment with ferricyanide or carbon monoxide. Both forms are recognised in the standard titrations of oxidase preparations derived from cells expressing the cloned form of the complex and the -100 mV component appears to have its absorption decreased in a manner analogous to that brought about by ferricyanide treatment of the uncloned material (Fig. 87).

Although the high potential, +260 mV component is unaffected by ferricyanide and undergoes the CO perturbation characteristic of the terminal oxidase \cite{117} the shift of potential observed in the lower potential components, +125/+60 mV and -60/-100 mV may be indicative of conformational alterations resulting from the transition between oxygenated and oxidized states which would be generated by the sequential reduction and reoxidation phases of the titration when treating with ferricyanide. Since this technique of ferricyanide perturbation has been developed procedures have been described by Gennis for studying spectral effects of ferricyanide upon cytochrome $d$, similar to those described in section II.C.i.a \cite{116}. However, these effects appear to be due to oxidation of the cytochrome $d$, which may be distinguished spectrally from the results of oxygenation or peroxidation which occur with several commonly used oxidants, and no permanent modification of
Fig. 88: Potentiometric titration of solubilized, cloned cytochrome o: the effect of carbon monoxide.

The cloned cytochrome o complex, partially purified by liquid chromatography was titrated in a solution of 0.2 % (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0 at a protein concentration of 500 μg mL⁻¹. Theoretical curves fitting each data set are illustrated. The mid-point potentials of these components are indicated and their individual contributions to the total type-b cytochrome are expressed as percentages of the α-band λ_max absorbance of the sample.

a. Standard titration: theoretical curve shown is for three components with $E_m$ above 0 mV.

b. Where appropriate an intermittent flow of carbon monoxide was supplied as described in Materials & Methods: theoretical curve shown is for two components with $E_m$ above 0 mV.

<table>
<thead>
<tr>
<th>Standard Titration</th>
<th>Treatment with ${(Fe(CN)_6)^{3+} + CO}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$+ 272.5$ mV vs. NHE</td>
<td>$+ 459.2$ mV vs. NHE: $18.2%$</td>
</tr>
<tr>
<td>$+ 152.7$ mV vs. NHE</td>
<td>$+ 71.4$ mV vs. NHE: $81.8%$</td>
</tr>
<tr>
<td>$- 55.3$ mV vs. NHE</td>
<td>$- 55.3$ mV vs. NHE: $13.1%$</td>
</tr>
</tbody>
</table>
this oxidase was detected following the ferricyanide treatment \cite{116}.

As in all studies of solubilized membrane proteins the disruptive effects of the solubilization procedure itself may cause major shifts in electrochemical properties and this may explain the presence in the cytochrome \(o\) complex of the low potential component with an \(E_m\) of \(-60\) mV to \(-100\) mV — values far below those normally associated with oxidase activities \cite{87,112,157}. The potentiometric studies of cytochrome components of the purified cytochrome \(d\) complex carried out by Gennis and co-workers indicate that the mid-point potential of cytochrome \(b_{558}\) is particularly susceptible to detergent effects, shifting the \(E_m\) by as much as 100 mV \cite{119}. Thus the potentiometric values obtained in this study should be compared only with preparations obtained by equivalent procedures.

(v) Correlation of Potentiometric and Spectrophotometric Analyses

(a) High resolution spectrophotometry at kinetically poised potentials

Steady state poised potential experiments used the PE-356 spectrophotometer in dual wavelength mode to monitor the reduction state of the cytochrome \(o\) complex. The technique described in Materials & Methods was used to remove samples at the partially reduced steady state level of reduction in which electrons were being transferred to oxygen dissolved in the reaction buffer and also to remove them at the fully reduced state after the reaction buffer had become anaerobic. Poising was achieved by rapid freezing to 77 K in liquid nitrogen and the DW-2c spectrophotometer was used to obtain high resolution reduced \(\text{minus}\) oxidized difference spectra. No significant absorption differences were observed between the \(\alpha\)-band absorption peak intensities displayed by samples taken at the aerobic steady state or fully reduced phases of reduction (data not shown). Neither were any spectral alterations observed when the sample was analysed by repeated spectral scanning as soon as possible after rapid freezing in liquid nitrogen, although a delay of at least five minutes is required between freezing and the initiation of data collection so that the sample holder may cool sufficiently for the rate at which nitrogen boils in the sample Dewar to drop to a level at which a reasonable signal to noise ratio can be obtained from the spectrophotometer. The dual wavelength kinetic traces measuring absorbance changes in the \(\alpha\)-band region (580 nm \(\text{minus}\) 560 nm) indicate that there are increases in absorbance as the steady state is attained and again when the sample becomes anaerobic (section III.C.iii.a; Fig. 80). Yet only ‘fully reduced’ \(\text{minus}\)
Fig. 89: Response of the redox $\alpha$-absorption maximum of solubilized cytochrome $o$ to the electrochemical potential of the titration buffer.

The relative intensity of absorption at each of two redox $\alpha$-band wavelengths. Reduced minus oxidized difference absorption was measured at 305 K over the indicated potential range during standard titration of solubilized cytochrome $o$ complex in a solution of 0.2% Triton X-100, 100 mM potassium phosphate buffer, pH 7.0 at a protein concentration of 500 $\mu$g mL$^{-1}$. Data sets of absorbance relative to the spectral baseline were collected at 565 nm and 558 nm (ambient temperature $\alpha$-band absorbance maxima of postulated components of the complex) and compared with that at the wavelength of maximal $\alpha$-band absorbance. In each case theoretical values from the non-linear regression analyses of the data sets have been compared and illustrated.

$\text{0}--\text{0}$, $\Delta A$ at 558 nm as a percentage of $\Delta A$ at $\lambda_{\alpha_{\text{max}}}$.

$\bullet--\bullet$, $\Delta A$ at 565 nm as a percentage of $\Delta A$ at $\lambda_{\alpha_{\text{max}}}$.

a. Uncloned cytochrome $o$ complex prepared from PLJ01 cyd$^+$ cells by partial purification utilizing liquid chromatography via DEAE-BioGel.A anion exchange and gel filtration through Sephacryl S-200 and S-300 matrices. Protein concentration = 500 $\mu$g mL$^{-1}$.

b. Plasmid encoded cytochrome $o$ complex prepared from RG167/p110 cyd$^+$ cells by partial purification utilizing liquid chromatography via DEAE-BioGel.A anion exchange and HPLC matrices. Protein concentration = 500 $\mu$g mL$^{-1}$. 
proportionally greater absorption at 558 nm associated with the sample of cloned cytochrome o was expressed throughout the potential range of the titration (Fig 89b).

Thus the +260 mV component appears to exhibit a split α-band with a major 77 K redox absorption maximum at 563.0 nm and a lesser peak at 556 nm (565 nm and 558 nm at 305 K), and both the +127 mV and +58 mV components have redox absorption maxima at 556 nm. This finding corresponds to those described earlier in which membrane vesicles prepared from cyd~ cells demonstrated a twin redox α-band absorbance peak in spectra recorded at high potentials during potentiometric titration at 305 K (section II.C.ii.d).

(c) High resolution poised potential spectrophotometry

Poised potential high resolution spectrophotometry was performed during certain potentiometric titrations of preparations of the cytochrome o complex from both wild-type and cloned sources. The results of these poised potential measurements are subject to the interpretive precautions listed above with respect to the precise potentials at which they are assumed to represent (sections I.ii.e, II.C.i.e, II.C.ii.e). Nevertheless, although absorption at 558 nm relative to 558 nm increased somewhat at lower potentials, both types of preparation presented absorption profiles which were very similar to their respective fully reduced absorption profile in redox difference spectra poised at all potentials tested (Fig. 90). These results indicate that in each case an abnormal reduction mechanism was occurring since the standard soluble cytochromes and also cytochromes in membrane preparations had previously yielded poised redox spectra which corresponded to increasing reduction of individual components at characteristic potentials (Fig. 14, 15, 28).

The quantitative behaviour of both cloned and uncloned forms of cytochrome o complex in poised potential high resolution spectrophotometric analyses was that of progressive reduction as the electrochemical potential of the sample was lowered, although insufficient sample could be prepared and manipulated to enable sufficient data points to be obtained for a reduction curve to be plotted accurately at these poised potentials. This phenomenon was observed throughout the range over which the complex was seen to be reduced, at whichever α-band wavelength was chosen for the estimate of percentage cytochrome reduced. Thus the constituent cytochromes appeared to behave as if the complex was being reduced as a single entity, in that the relative intensities of their absorbance maxima were approximately constant throughout the experimental potential range resulting in little change in the shape of the combined absorbance profile (Fig. 90).

An interesting and possibly related result following reconstitution of the cytochrome o
Fig. 90: Poised potential high resolution redox difference spectra of solubilized cytcochrome o from uncloned and from plasmid encoded sources.

Low temperature spectra of solubilized preparations of cytochrome o poised at the indicated electrochemical potentials. Samples were taken from the titration vessel and analysed in titration buffer, 0.2 % (w/v) Triton X-100, 100 mM potassium phosphate buffer, pH 7.0, as described under Methods & Materials. No sucrose was present.

**a.** Uncloned cytochrome o : protein concentration = 500 µg mL⁻¹,
1. -475.0 mV vs. NHE, ΔA = 0.005,
2. -174.0 mV vs. NHE, ΔA = 0.005,
3. -135.0 mV vs. NHE, ΔA = 0.005,
4. -13.0 mV vs. NHE, ΔA = 0.005.

**b.** Plasmid encoded cytochrome o complex : protein concentration = 500 µg mL⁻¹,
1. -307.0 mV vs. NHE, ΔA = 0.020,
2. -180.0 mV vs. NHE, ΔA = 0.020,
3. -101.0 mV vs. NHE, ΔA = 0.020,
4. -31.0 mV vs. NHE, ΔA = 0.020,
5. +49.5 mV vs. NHE, ΔA = 0.020,
6. +126.0 mV vs. NHE, ΔA = 0.004,
complex into liposomes with ubiquinone-8 and the photosynthetic reaction centre was noted by Moser et al. in which kinetic reduction of the oxidase could be induced by flash illumination of the preparation: the reaction centre generated a brief pulse of reduced quinol enabling the reduction of the oxidase complex to be studied at applied potentials — a ‘functional potentiometric titration’ (140). Under these conditions the flash-induced reduction of cytochrome o yielded the same redox difference spectrum as that of the fully reduced complex, the cytochrome o reduction kinetics at various wavelengths indicated single component behaviour and flash-induced reduction of cytochrome o titrated with monophasic dependence on applied redox potential. One of the explanations offered for these data was that the haems of the oxidase complex may be in rapid, submillisecond equilibrium with each other.

(d) Comparison of poised potential spectrophotometry at 77 K and 305 K

Whereas the titration procedure itself entailed the collection of single-scan $\alpha$-band spectra at 305 K in order to determine the percentage reduction of the sample at regular intervals in the potentiometric range, the low temperature poised potential analyses required the withdrawal of samples from the reaction chamber at selected potentials during a potentiometric titration and the maintenance of that potential as the sample was rapidly frozen to 77 K in liquid nitrogen.

In the former analyses the cytochrome components of each type of oxidase complex appeared to be being reduced sequentially at individually characteristic potentials, as shown by the coupled potentiometric and 305 K spectrophotometric data; but the latter, poised analyses indicated that the complex was being reduced as a unit with its cytochrome components in equilibrium at mutually equivalent stages of reduction. Whether these apparently anomalous results are the result of rapid equilibration during the freezing process associated with the poising procedure or whether they are indicative of an equilibration mechanism of the complex such that as each component is reduced the effect is shared across the entire complex is unknown. These phenomena may be related to the perturbation effects of ferricyanide on the constituents’ mid-point potentials noted above in section III.C.iv.a: under titration conditions the pre-reduction form of the complex, which in the case of cytochrome d is now known to be oxygenated (116), may enforce different electrochemical constraints on the subunits than the reduced plus ferricyanide reoxidized form.
(vi) **Synopsis of Results Relating to the Solubilized Cytochrome \( \alpha \) Complex**

The solubilized and isolated cytochrome \( \alpha \) complex comprised four major peptide subunits, one behaving as a doublet in SDS-PAGE as described in detail above. The \( \alpha \)-absorption band of the oxidase complex exhibited three peaks in high resolution redox spectra at 77 K, the one with maximal absorbance of 563.5 nm appearing to be a detergent induced fusion of three incompletely resolved redox absorption maxima observed in membrane suspensions at 560.5 nm, 563.0 nm and 565.0 nm which may themselves represent portions of the complex in different environmental domains (section II.A.i.b.3). The redox \( \alpha \)-band peak of the solubilized cytochrome \( \alpha \) complex observed at 554.5 nm is of markedly greater intensity in preparations of the complex encoded from plasmid pRGl10 than in that from uncloned sources.

Potentiometric titration of the uncloned oxidase indicated that it contained three titratable cytochromes \( b \), with mid-point potentials of -58 mV, +127 mV and +260 mV. In redox difference spectra obtained at 305 K the high potential component was associated with a major redox \( \alpha \)-band absorbance peak at 565 nm and a less intense \( \alpha \)-band peak at about 558 nm and it displayed \( E_m \approx +420 \) mV in oxidative titrations after carbon monoxide had bound to the reduced form. The middle \( E_m \) value of +127 mV exhibited by the uncloned complex was sensitive to ferricyanide which altered its mid-point potential to about +60 mV. In the cloned oxidase preparations a proportion of both of these states appeared to be present before ferricyanide treatment enabling the component with mid-point potential of +127 mV/+60 mV to be identified with the redox \( \alpha \)-band absorption maximum at 554.5 nm.

The central \( \alpha \)-band absorption maximum of 557.0 nm observed in redox difference spectra at 77 K may correspond to part of a complex \( \alpha \)-band of one of the other two components. Alternatively it may be associated with a third haem having \( E_m = -50 \) mV to -100 mV providing a minor contribution to the 558 nm absorption of the complex at 305 K in both uncloned and cloned forms of the complex, an absorption feature being largely eliminated by ferricyanide.

The profound modification by carbon monoxide of the mid-point potential of the high potential haem and the correlation of this component with the 563.5 nm redox \( \alpha \)-band absorption maximum identifies it as cytochrome \( \alpha \) and obviates any requirement to postulate the existence of a spectrum with minimal \( \alpha \)-band absorbance to account for the minor spectral changes observed in this region upon CO binding to the oxidase. Such low intensity redox \( \alpha \)-band absorbance is an indication but not a necessary consequence of high spin haem iron. Both high spin and low spin
haem iron atoms have been detected in the cytochrome \( o \) complex by Anraku and coworkers \(^{74, 202}\), although the nature of the haem environments generating each of the components of the \( \alpha \)-band spectrum are unknown, as are the reasons for the abnormally small qualitative changes in the \( \alpha \)-band which accompany major Soret band alterations when the CO adduct is formed. Structural investigations may provide clues to the mechanism of the oxidase activity besides determining the physical interactions and functions of the other components of the complex.

Previous estimates of the mid-point potential of the haem components of the purified cytochrome \( o \) complex have produced single values, although few titrations have been extended over the range that was tested in the current investigation. Kita \textit{et al.} have proposed a value of +125 mV but provide no data above +200 mV indicating that their titration may have been restricted to the lower potential, ferricyanide sensitive component \(^{97}\). After reconstitution of purified cytochrome \( o \) into proteoliposomes Moser \textit{et al.} determined a single functional reductive mid-point potential of 160 mV to 185 mV for both haems whereas in membranes from \( \text{cyd}^+ \) strains grown aerobically the cytochrome \( o \) is recognisable as the high potential type-\( b \) cytochrome and titrates with \( E_m = +165 \text{ mV} \) in a simplistic two-component fit of the data \(^{117, 140}\). In the current study a minor contribution to the total cytochrome reduced was recorded with a high potential value of +225 mV and attributed to cytochrome \( o \) whereas \( b \)-cytochrome components exhibiting the mid-point potentials measured at lower values could not be assigned individual identities (sections II.A.ii.b; II.C.ii.d). Thus although the haems of the cytochrome \( o \) complex were capable of being titrated independently in both membrane preparations and in the solubilized complex under the conditions used in the current study, this may not be the case under 'functional' circumstances \((v.i.)\).

Kinetic studies of the cytochrome \( o \) complex have displayed more than one component. Stopped-flow experiments showed that rapid reduction of the cytochrome \( o \) complex by dithionite was at least biphasic, comprising a very rapid and a relatively slow rate of reduction. Nevertheless, these experiments could provide no information about the spectral identity or redox state of individual component cytochromes during the process (section III.C.iii.c). However, low temperature poising of the solubilized complex in aerobic and anaerobic steady states during this reduction ('kinetically poised potentials') provided redox difference spectra corresponding to the fully reduced oxidase in each case (section III.C.v.a) as did low temperature poising of samples withdrawn from the potentiometric titrations. A possible explanation for this anomalous behaviour is that the haems may act independently in the presence of redox mediators or certain powerful reductants such as dithionite whereas during 'functional reduction' by quinols they may act as an obligately coupled entity. Preliminary experiments suggest that this may be related to the rate-limiting step of such
functional reduction being the initial reduction of cytochrome by quinol as indicated by the lack of an aerobic steady state followed by monophasic reduction of the cytochrome complex in standard kinetic analyses employing reduction of the oxidase by excess duroquinol (Table XV, section III.C.iii.a). However, the apparent electronic equilibration in partially reduced samples of the oxidase withdrawn from potentiometric titrations at 305 K and poised by rapid freezing to 77 K yielding similar α-band profiles in redox difference spectra remains unexplained. In contrast to this behaviour independent reduction of membrane cytochrome components was observed in samples poised in his manner from titrations of cytochromes in resuspended membrane preparations from several sources. As the solubilizing detergent would be expected to increase independent properties of constituent haems in the complex this anomalous behaviour of the cytochrome o complex appears to be a characteristic peculiar to this oxidase, as postulated earlier for reconstituted preparations by Moser et al. [140].

There have been other reports of purified preparations of the cytochrome o complex being reconstituted into sealed proteoliposomes either by itself or with other respiration linked components such as D-lactate dehydrogenase or the lac carrier protein for functional studies [126, 128, 171]. Since minimal electron transport systems may be constructed in such instances, supplying the oxidase with an exogenous or substrate-reduced supply of ubiquinol-8 with which it is able to generate an electrochemical proton gradient, it is pertinent to ask how the cytochrome o complex interacts with other respiratory components and whether this may provide information regarding the composition and operation of the aerobic respiratory chains in the membrane. The results described above with regard to the inhibition of both reduction and subsequent reoxidation of the cytochrome o oxidase by the quinonoid compound HOQNO suggest that the quinol oxidase activity of the complex may be achieved by means of a more complicated interaction than that of straightforward quinol reduction. These results are in agreement with previously reported conclusions from investigations of cytochrome pools in membranes prepared from aerobically grown cyd+ strains of E. coli in which the quinol analogue was found to inhibit cytochrome o activity at a stage between cytochrome b564 and dioxygen [214].

High resolution redox difference spectrophotometry of samples ‘kinetically poised’ in the presence of the quinol analogue HOQNO demonstrated a modification of the wavelength of the 563.5 nm α-band absorption maximum to 564.8 nm suggesting that the binding site for this inhibitor of the cytochrome o quinol oxidase activity is in the proximity of the haem responsible for this spectral characteristic (section III.C.ii.e). Other data have shown that this haem is associated with the high potential $E_m$ of the complex and with carbon monoxide binding, indicating that it is
the cytochrome *o* b-haem (*v.s.*). It thus appears that rather than acting 'between' the cytochrome *b*~564*~ and dioxygen, HOQNO acts on the cytochrome *b*~564*~ which is the component of the complex that reduces oxygen.

These results raise questions concerning the function of the other *b*-cytochrome component or components of the complex and the implications of the inhibition by HOQNO of both the reduction of the complex and also its reoxidation by exogenously introduced oxygen. There may be a relationship between the answers to these queries in that the dual inhibitory rôle of HOQNO is suggestive of the involvement of a 'Q-cycle' functioning in conjunction with the cytochrome *o* complex in its native membrane (*Introduction*, section A.iv). Thus quinol would reduce the complex and an electron carrier — possibly another cytochrome *b* of the complex and the resulting uncharged quinone would diffuse across the lipid bilayer. At the interior surface of the membrane the quinone would undergo reduction to the quinol by accepting one electron each from the carrier and the the substrate oxidases of the respiratory chain plus a proton from the substrate or from the soluble-phase (Fig. 1b i,ii). These proposals are discussed in greater detail in the following chapter in relation to the possible architecture of the aerobic respiratory chains of *E. coli* (*Conclusion*).

These suggestions for the arrangement and interactions of cytochrome *o* with other constituents of the oxidase complex are necessarily simplistic for the reduction of dioxygen requires the donation of two pairs of electrons through a system comprising type-*b* cytochromes which are presumably capable of only single electron transfer. Moreover the rôle of the copper atoms reported to be present in the complex has not been considered, although resonance Raman studies have suggested that they probably have a stabilizing effect upon the carbon monoxide adduct of cytochrome *o* (97, 202). Further investigations into the structure and physical interactions of the peptide constituents of the complex, coupled with studies of their topographical arrangement within the membrane will provide insights into the function of this complicated enzyme. Most importantly it would appear that with the availability of relatively large quantities of purified material from the cloned source the preliminary kinetic studies described herein may be readily extended to yield a wealth of information regarding the functional reduction and reoxidation of the complex in both solubilized and reconstituted forms as well as enabling thorough investigations of the oxidase's ligand binding properties to be performed and compared with results from the uncloned material. In view of the different spectral and electrochemical properties of the uncloned and plasmid encoded forms of the cytochrome *o* complex, and since the DNA sequence has still not been reported after a considerable delay, it is suggested that an attempt to reclone the oxidase genes may prove fruitful for future comparison with the genetic sequence and physical properties of the form that is currently available.
CONCLUSION

The combination of procedures employed in this study enabled respiratory cytochromes of *E. coli* to be distinguished and investigated both *in situ*, by means of whole cell suspensions, and also in washed preparations of everted cell membranes. However, the analytical techniques were most effective with those samples containing a simplified cytochrome complement which were obtained through detergent solubilization, extraction and fractionation of membrane cytochromes.

Spectral and potentiometric analyses of everted membrane vesicles showed that in cells grown aerobically on defined media under conditions designed to minimise respiratory chain diversity the cytochrome content is more complex than that suggested by accepted models {4, 42, 95, 126}. Solubilization and fractionation of the multiple *b*-type cytochromes supported this contention and enabled several cytochromes *b*$_{556}$ to be partially purified and characterized. Purification of the cytochrome *o* complex permitted an electrochemical investigation of its type-*b* cytochrome components. Although the interaction of these multiple respiratory cytochromes with each other and with other components of the aerobic electron transport pathways remains undetermined the observations are related to recent proposals and models of respiratory chain structure in this bacterium as discussed below.

A. Cytochrome Pools in Membranes of Aerobically Grown *E. coli*

As described in the Introduction the supply of oxygen to cultures of *Escherichia coli* not only represses expression of the components of the anaerobic respiratory pathways but the availability of dioxygen also determines the extent of the organism's reliance on each of the two aerobic electron transport chains, one of which terminates in the cytochrome *o* oxidase, the other with cytochrome *d* {3, 4, 17, 18, 71, 157, 181}.

Both of these oxidase complexes are present under most conditions of aerobic growth and harvest as shown by the carbon monoxide binding spectra of wild-type cells in this and other studies {3, 216}. Investigations of the respiratory capability of double *chl* mutants under anaerobic growth conditions have indicated that production of the cytochrome *o* respiratory chain may be constitutive unless expression is either specifically repressed or induced by a sophisticated regulatory mechanism.
Interpretation of the composition of the aerobic respiratory apparatus is further complicated by R. K. Poole's proposal that another cytochrome o or an alternative form of this oxidase is produced under conditions of low aeration, support for which has been obtained from carbon monoxide binding studies of cells grown anaerobically with TMAO as electron acceptor (P. D. Bragg, personal communications) (160). Thus further investigations of the mechanism and spectral effects of carbon monoxide binding and release from the cytochrome o b-type haem are warranted.

In order to minimise these complexities the current study employed cells of cyd— strains to provide a simpler and more readily resolved complement of cytochromes under aerobic conditions of growth. Nevertheless, a previously unrecognised multiplicity of cytochromes b was observed following aerobic growth on each of the substrates tested in the current study. Under these conditions both potentiometric and kinetic analyses of cyd— cells' membranes demonstrated the presence of at least four type-b cytochromes (Results & Discussion, sections II.A.i+ii). Membranes of cyd— cells grown aerobically with L-proline as carbon/energy source were used for combined spectral, potentiometric and kinetic analyses as described in section II.B.i of the Results & Discussion and published elsewhere (214). The type-b cytochromes produced in these membranes could be grouped into two sets, distinguishable in dual wavelength kinetic studies by the rate of their reduction by a variety of electron donors (214). One third of the total complement of cytochromes b was reduced in the initial 'rapid phase' of reduction and included the cytochromes of the cytochrome o complex. The 'slow phase' cytochromes exhibited redox α-band absorption maxima at approximately 556 nm and constituted two groups with distinct oxidative properties, approximately one half being oxidizable by fumarate and including fumarate dehydrogenase activity (Fig. 91).

Succinate and formate dehydrogenases were also present, each containing a cytochrome b556 component and recognised by their respective oxidase activities. However, removing and excluding oxygen from the samples throughout the experiments and performing reduction by NADH and formate in the presence of KCN indicated that these slowly reduced cytochromes were functionally independent of the cytochrome o complex. Nevertheless, when using ascorbate with PMS as a source of slowly donated electrons, unable to reduce the slow-phase cytochromes fully, KCN addition resulted in their immediate and complete reduction indicating that electron flow was occurring between these cytochromes and the cytochrome o complex, albeit at a relatively slow rate. Thus the number of individual cytochromes detected is greater than would be predicted from the sole presence of the cytochrome o electron transport chain and separate pools of cytochrome exist within the membrane which are capable of undergoing selective reduction and oxidation (Results & Discussion, section II.B.i; 214). Therefore the concept of multiple respiratory pathways comprising alternative routes of electron transport created by large quantities of quinone linking the
Fig. 91: Model of cytochrome pools in the membrane of aerobically-grown cyd' cells of *E. coli*.

Two types of cytochrome pool have been found in cyd' cells of *E. coli* grown aerobically on L-proline. Through the activity of their respective dehydrogenases the substrates NADH, succinate and formate donate electrons to the pool of ubiquinone-8 (UQ) in the membrane. Succinate dehydrogenase incorporates cytochrome b$_{59}$ and formate dehydrogenase contains cytochrome b$_{67}$. The cytochrome o terminal oxidase oxidizes the ubiquinol-8 and also the artificial quinol duroquinol (DQH$_2$), (as does cytochrome d if present). Cytochromes in this pool are reduced during the ‘fast phase’ of cytochrome reduction whereas cytochrome b$_{ferri}$ (oxidized by ferricyanide but not by fumarate) and cytochrome b$_{fr}$ (oxidized by fumarate and by ferricyanide) constitute the ‘slow phase’ pool and are reduced by succinate and formate as a result of interacting with the ‘fast phase’ pool of cytochromes by undetermined mechanisms. NADH is able to reduce cytochrome b$_{fr}$ more directly, probably through reduction of menaquinone-8 [87]. Based upon a proposal from reference [214].
redox activities of different dehydrogenases and oxidases is suggested to be an oversimplification. Although the individually defined electron transfer systems of *E. coli* may be constitutionally simpler than that of the mitochondrion the ability of the former to interact contributes to the organism's adaptability to alterations in its environment.

B. The Aerobic Respiratory 'Cytochrome $b_{556}'$

Low temperature redox spectrophotometric studies have led several laboratories to suggest that a cytochrome $b_{556}$ is associated with the cytochrome $o$ respiratory pathway of *E. coli* as an intermediate electron carrier between the dehydrogenases and the terminal oxidase \{18, 42, 71, 87, 112, 180, 181\}.

Anraku and coworkers have isolated a cytochrome $b_{556}$ from *E. coli* and identified it as the *sdhC* gene product by amino acid and nucleotide sequence analyses, indicating that it is a constituent of the particularly labile succinate dehydrogenase of this bacterium \{143, 144\}. Subsequent publications originating from the same laboratory and also from other sources have implied that this cytochrome $b_{556}$ is the sole cytochrome $b_{556}$ of aerobic respiration \{3, 4, 144\}. In the current study a new technique of fractionating solubilized $b$-cytochromes demonstrated that haemoproteins of this type with redox $\alpha$-absorption maxima of 556 nm at 77 K are expressed under all conditions of aerobic growth, whether or not succinate is a major growth substrate. Type-$b$ cytochromes with the same $\alpha$-absorption maximum were also detected in cells incapable of expressing the *sdhC* gene product (Results & Discussion, sections III.B.i+v).

The investigations described in this thesis have shown that independent cytochromes $b_{556}$ were expressed during growth on particular carbon/energy sources. Each of these type-$b$ cytochromes may be associated with a particular dehydrogenase. Comparison of cells producing the *sdhC* gene product with *sdhC*$^{-}$ mutants showed this cytochrome $b_{556}$ to have $M_r = 52 500$ by gel exclusion chromatography and a mid-point potential of $+20$ mV when solubilized in Triton X-100. Growth on DL-lactate generated at least one and possibly two other cytochromes $b_{556}$ (Results & Discussion, sections III.B.i+v). At least one other cytochrome $b_{556}$ was readily extractable under the solubilization conditions employed in these investigations, although this product, a hydroperoxidase, was associated with growth under conditions of low but significant aeration. This cytochrome possesses twin mid-point potentials of $-2$ mV and $-121$ mV and a split Soret absorbance band at 77 K with maxima at 426.0 nm and 434.0 nm (Results & Discussion, section
III.B.iv). Its physiological function remains undetermined.

The direct involvement of a cytochrome \( b_{556} \) between the dehydrogenases and cytochrome \( a \) remains unproven and has not been confirmed by the solubilization and fractionation techniques employed in this study. However, incomplete extraction of type-\( b \) cytochromes from membrane preparations of \( E. coli \) has been reported by many investigators in this field and is a disadvantage of solubilization and fractionation procedures [Results & Discussion, section III.A.i; 112]. A cytochrome \( b_{556} \) may be present in the membranes which possesses the integral membrane properties to be expected of an electron transfer protein participating in a chemiosmotic 'half-loop', thereby providing it with greater resistance to solubilization than a cytochrome associated with a dehydrogenase activity.

C. The Cytochrome \( d \) Aerobic Terminal Oxidase

Robert Gennis and coworkers have confirmed and extended several of the findings relating to the spectral responses and electrochemical properties of the components of the cytochrome \( d \) complex that are described in this thesis. The redox \( \alpha \)-absorption bands of cytochrome \( b_{595} \) and of cytochrome \( d \) are spectrally distinguishable from other aerobic respiratory cytochromes. Mid-point potential values of +125 mV (cytochrome \( b_{595} \)) and +187 mV (cytochrome \( d \)) were obtained in the current study for these two cytochrome constituents of membrane-associated cytochrome \( d \) complex. The terminal oxidase displayed extreme hysteresis during redox titration, being resistant to oxidation by ferricyanide and undergoing oxidation by oxygen over a particularly narrow potential range (Results & Discussion, section II.C.i.d). Mid-point potentials obtained by the laboratory of R. B. Gennis, measured after exhaustive deoxygenation of the solubilized complex, are +140 mV, +160 mV and +260 mV for cytochromes \( b_{558}, b_{595} \) and \( d \), respectively \([74,117,118,119]\). The differences between these estimates of mid-point potential for the components of the cytochrome \( d \) complex may be attributed to the disruption in the environment of an integral membrane protein upon solubilization, the marked influence of specific detergents on these cytochromes' \( E_m \) values having been recorded \([105,119]\). This oxidase transfers electrons between its multiple cytochrome components in the order \( b_{558}, b_{595}, d \) \([62,74,118]\). Extensive physical analyses of the purified complex have demonstrated its exceptionally high affinity for dioxygen (\( K_m = 0.38 \) mM as purified) and the existence of several intermediates incorporating bound oxygen including spectrally distinct oxidized and peroxo compounds (Results & Discussion,
sections II.C.i.a+b) \{74,105,115,116, 118,119, 205\}.

D. The Cytochrome o Aerobic Terminal Oxidase

Growth under conditions of high aeration causes the cytochrome o complex to be expressed as an alternative terminal oxidase to that containing cytochrome d. The known structural properties of the cytochrome o complex have been reviewed in several recent articles \{3,4,87,97,129,216\}. A summary of this information and the additional data obtained during the current investigation is provided in section III.C.vi of the Results & Discussion.

Under conditions of Triton X-100 solubilization the purified complex appears to behave as a tetramer exhibiting $M_r = 516,000$ during gel exclusion chromatography. Peptide detection following SDS-PAGE revealed components having $M_r = 55$ kDa, 32 kDa, 31 kDa, 21 kDa and 16 kDa. The splitting of the bands with $M_r = 32$ kDa and 31 kDa, indicating that the complex comprises five distinct peptides, has not been previously reported. Type-b cytochromes of the solubilized complex displayed three mid-point potentials: -58 mV, +127 mV and +260 mV, and $\alpha$-band redox absorption maxima at 554.5 nm, 557.0 nm and 563.5 nm. The complex underwent a rapid and unusual reduction pattern since each peak was reduced equivalently during poised potential low temperature spectrophotometric analyses (Results & Discussion, section III.C.iv+v). Carbon monoxide binding studies suggested that the high potential component was cytochrome o itself, undergoing a shift of potential to +420 mV in the presence of CO. Although this inhibitor had little effect upon the $\alpha$-band absorption spectrum, potentiometric titrations provided some evidence that the redox absorption spectrum may incorporate a split $\alpha$-band at 305 K, with lower potential components exhibiting $\alpha$-absorption maxima below 560 nm. Comparison of data from cloned cytochrome o material suggested that another type-b cytochrome component of the complex may exist in more than one form, with varying extinction coefficients at 554 nm and possibly a shift in absorption maximum between 554 nm and 557 nm as the potential of the solubilized complex is artificially lowered. Significantly, an additional constituent with $E_m$ of intermediate potential was detected during reductive potentiometric titration of the plasmid encoded material. Mid-point potential values were recorded at -100 mV, +57 mV, +157 mV and +228 mV, the titration profile indicating that little of the lowest potential component was present. Since this result was simulated by treatment of the solubilized, uncloned complex with ferricyanide it is suggested that a perturbation effect was being observed in both cases (Results & Discussion,
Purified preparations of each of the two terminal oxidase complexes have been reconstituted into sealed proteoliposomes to form minimal electron transport chains \( \{72,126,127, 128,130\} \). These functional respiratory systems generate an uncoupler sensitive transmembrane electrochemical proton gradient of \(+115 \text{ mV} \) to \(+180 \text{ mV} \) (interior negative and alkaline). Such experiments have provided evidence that both aerobic terminal oxidases of \( \textit{E. coli} \) are not only quinol oxidases but that they serve as respiratory ‘coupling sites’ able to support the performance of physiological work as in the transport of lactose into proteoliposomes against a concentration gradient \( \{128\} \).

Catalytic turnover of the purified cytochrome \( \text{o} \) complex reconstituted in proteoliposomes generates vectorial electron flow (measured by means of the membrane potentials created) but not vectorial proton translocation (monitored by pH changes upon reduction by either ubiquinol-8 or TMPD) \( \{73,129\} \). Consequently R. B. Gennis has proposed that the cytochrome \( \text{o} \) oxidase complex acts in a manner analogous to that suggested for the cytochrome \( \text{d} \) complex. Thus the 180 mV transmembrane potential observed in reconstituted proteoliposomes, which become negatively charged and alkaline inside, would be generated by the oxidation of quinol at the external face of the membrane to release protons, with electrons being transferred through the oxidase complex to the inner membrane surface for the reduction of dioxygen \( \{3,4,104\} \). Consumption of protons by dehydrogenases would normally, but not necessarily occur at the internal membrane surface (Fig. 92) \( \{3,4\} \). This model suggests that the diffusion of quinol within the bilayer may be a rate limiting step. Quinol acting as a diffusible molecular link between the dehydrogenases and oxidases would provide an explanation for the adaptability of the respiratory apparatus of \( \textit{E. coli} \). A rationale is also provided for the observation that certain purified dehydrogenases may be added to either surface of liposomes containing the cytochrome \( \text{o} \) quinol oxidase and still participate in the generation of a functional respiratory chain creating an electrochemical proton gradient \( \{72\} \). This arrangement of respiratory components would obviate any requirement to invoke a proton-pumping mechanism as has been proposed for projected linear electron transport chains in \( \textit{E. coli} \) \( \{72\} \). Yet a very recent report from M. Wikström’s laboratory suggests that the cytochrome \( \text{o} \) complexes of both \textit{Paracoccus denitrificans} and \( \textit{E. coli} \) are, indeed, proton pumps \( \{171\} \). The \( \text{H}^+/\text{e}^- \) ratios observed in spheroplasts indicated that cytochrome \( \text{o} \) from each of these organisms catalyses translocation of up to four protons per electron pair crossing the cytoplasmic membrane during succinate oxidation.

Another model for the aerobic respiratory architecture that is consistent with the reconstitution results is one incorporating a protonmotive ‘Q-cycle’ (Fig. 93; also see Introduction, section A.iv, Fig. 1b) \( \{87,138\} \). In addition to the extra coupling site between NADH dehydrogenase and lactate or succinate dehydrogenases the latter reduce a membrane pool of
**Fig. 92:** Model of terminal oxidase action in *E. coli* aerobic respiratory chains.

Ubiquinol-8 is generated from ubiquinone-8 by substrate specific dehydrogenases acting in parallel. The ubiquinol-8 diffuses through the lipid bilayer of the cytoplasmic membrane to the terminal oxidases. Each of the aerobic oxidase complexes catalyzes scalar release of two protons from ubiquinol at the outer membrane surface, vectorial transfer of two electrons across the membrane and utilization of two protons at the inner surface during the reduction of dioxygen. Thus each oxidase acts as a coupling site for oxidative phosphorylation by creating a transmembrane proton gradient in this manner. Based upon proposals in references {4, 126}.
OUTSIDE MEMBRANE

\[ \text{cyt. } b \xrightarrow{2e^-} \text{cyt. } d \]

\[ \text{cyt. } b_{558} \]

\[ 2H^+ \rightarrow \]

\[ \text{QH}_2 \leftarrow \text{Q} \leftarrow \text{Q} \leftarrow \text{QH}_2 \leftarrow \]

\[ \Delta \rightarrow \text{S} \rightarrow \text{SH}_2 \]

\[ \text{Q} \leftarrow \text{Q} \leftarrow \text{QH}_2 \leftarrow \]

\[ \text{cyt. } b_{562} \]

\[ 2e^- \xrightarrow{Cu^{2+}} \text{cyt. } o \]

\[ \Delta \rightarrow \frac{1}{2}O_2 + 2H^+ \rightarrow \]

\[ \text{H}_2\text{O} \]

INSIDE

\[ \text{cyt. } d \xrightarrow{2e^-} \text{cyt. } d \]

\[ \frac{1}{2}O_2 + 2H^+ \rightarrow \text{H}_2\text{O} \]

\[ \text{Cytochrome } \alpha \]

\[ \text{complex} \]

\[ \text{Cytochrome } \alpha \]

\[ \text{complex} \]
**Fig. 93:** Model of cytochrome $o$ interaction with a protonmotive Q-cycle in *E. coli* membranes.

Substrate specific dehydrogenases reduce a ubiquinone pool. Electron transfer from the NADH dehydrogenase generates an extra coupling site for oxidative phosphorylation than that from either succinate or lactate dehydrogenases, and may involve a cytochrome $b$ — possibly a cytochrome $b_{556}$. Reduction of the cytochrome $o$ complex by ubiquinol-8 results in vectorial electron transfer plus scalar utilization and consumption of protons at the membrane’s exterior and interior surfaces respectively. The Q-cycle mechanism increases the efficiency of generation of the proton electrochemical gradient through rereduction of quinone at the inner membrane surface by electron transfer from one of the components of the complex with incorporation of intracellular protons to form ubiquinol, thereby enabling translocation of a further two protons. This oxidation of certain components of the complex by ubiquinone would be linked to and ‘driven by’ the terminal oxidase activity — the reduction of dioxygen to water. Interference with the the reactions of ubiquinol-8 by quinonoid analogues might therefore be expected to inhibit both reduction and reoxidation reactions of the terminal oxidase complex. Based, in part, upon proposals in references {42, 138, 216} and Jones, C. W. “Aerobic respiratory systems in bacteria” in “Microbial Energetics”, ed. Haddock, B. A. & W. A. Williams, pp. 23-59, 27th. Symp. of the Society of General Microbiology, pub. Cambridge University Press, Cambridge, U.K., as well as upon observations described in the current work.
CONCLUSION

OUTSIDE MEMBRANE INSIDE

NADH + H⁺

NAD⁺ + 2H⁺

lactate/succinate

pyruvate/fumarate

1/2 O₂ + 2H⁺

H₂O

2H⁺ ← QH₂ ← 2H

2e⁻

2H⁺ ← Q ← 2H

2e⁻

2H⁺ ← Q ← 2H

2e⁻

2H⁺ ← QH₂ ← 2H

2e⁻

NADH + H⁺

NAD⁺ + 2H⁺
ubiquinone-8 through which electrons are 'cycled' and are able to interact with the cytochrome o complex. Not only does this scheme account for Wikström's observation of four protons per electron pair, it also requires that components of the terminal oxidase complex are both reduced and reoxidized by the quinol/quinone pool, thereby explaining the inhibitory action of the quinol analogue HOQNO upon both reduction and oxidation of the solubilized cytochrome o complex (Results & Discussion, section III.C.iii.b). A more complicated system of electron transfer would be required than in the alternative schemes. Analogy with the mitochondrial system and the results of the current investigation (section III.C.) suggest that type-b cytochromes may participate in these activities (Fig. 1b, 93). However, the implementation of such a mechanism would appear to raise the theoretical H+/O ratios to values that have not been recorded with these bacterial systems. Similar double inhibition sites relating to the action of quinol in the aerobic respiratory chains of E. coli have been noted in other laboratories leading to proposals that there are two distinct sites at which quinols act, separated by one or more cytochromes {42, 87, 95} (Fig. 93).

It is apparent that many aspects of the composition and integration of the aerobic respiratory chains of E. coli remain to be determined, especially with regard to the electron transport pathway terminating in cytochrome o. Structural definition of the cytochrome o complex will aid in the development of an accurate understanding of the operation of this oxidase and of the compatibility of the two aerobic respiratory systems which are frequently present within the same membrane preparation. Answers to the questions raised may also shed light on evolutionary possibilities in that the cytochrome d oxidase may have evolved a respiratory function from an ancestral oxygen scavenging enzyme required by the obligate anaerobes of an earlier epoch. If a respiratory rôle developed in this manner, what advantage was provided by the ability to express a second aerobic terminal oxidase, and what were the evolutionary origins of this enzyme and alternative oxidases employed by other procaryotes? How do the properties of the second oxidase confer their presumed advantages? Understanding these problems will not only provide information relating to those procaryotes with aerobic respiratory systems similar to those of E. coli but will also create a basis for detailed comparison of function and efficiency with those expressing electron transport pathways which are similar to the respiratory chain present in the eucaryotic mitochondrion.
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APPENDIX 'A'

Spectral Characteristics of Cytochromes

The optical spectra of porphyrin derivatives are due in large part to the inner 16-membered ring with 18 π electrons, with the four-fold symmetry of the planar ring and the highly conjugated π-electron system being perturbed by chemical modifications to the chemical structure (Fig. 3). In general, substituents of the ring able to integrate with the resonance structure will have a greater effect upon this electron distribution (65). The nature of ligands at the fifth or sixth coordination positions of the iron atom in haems and, in the case of the haemoproteins the environment created for the haem by the enveloping polypeptide chain may have significant effects upon the electronic structure of the prosthetic group (2).

In protoporphyrin IX and other free base porphyrins only two-fold planar symmetry exists since the metal is absent and replaced with two protons. Electronic transitions are polarized along each of the non-equivalent axes of the plane, x and y, each having its own range of vibrational energy levels or vibronic envelope. Consequently four fairly broad absorption bands are observed in the visible region between 500 nm and 650 nm in an alkaline pyridine plus water solution, and a third, more intense band in the violet region of the spectrum. Ferrous protohaem IX possesses four-fold planar symmetry and transitions of the conjugated π-electron system are polarized in the plane of the porphyrin ring causing them to be doubly degenerate and resulting in two absorption bands in the visible region (the Q bands) and an intense violet-range band (the B band).

These absorption bands increase in energy from high to low wavelength and are referred to throughout this thesis by the standard biophysical terms: α-, β-, and Soret or γ-absorption bands (2, 112).

The α-absorption band (Q₀ or Q (0,0) band) of cytochromes and other metalloporphyrins corresponds to the lowest energy porphyrin π-π* excited singlet state. The β-absorption band (Qᵥ or Q (1,0) band) represents the next highest energy level and includes one mode of vibrational excitation. This is a combination of multiple vibrations and the β-absorption band usually exhibits greater complexity than the α-band. The Soret absorption band originates with the second excited singlet state, B (0,0) (2, 65).

The ligation and formal charge on the iron affects the energy levels and occupation of the d orbitals which are oriented in the plane of the porphyrin ring. The position of the iron relative to the
plane of the ring, which is affected by oxidation and reduction, and also the spin state of the iron will influence the absorption properties of the cytochrome through effects upon the \( \pi \)-electron energy states \( \{2, 11\} \). Nevertheless, the over-riding spectral characteristics of these haemoproteins in the \( \alpha \)-absorption region are caused by the symmetry of the inner 16-membered ring of the haem with minor modification due to substituents. Thus the \( \alpha \)-bands of type-\( b \) cytochromes in a mixture will also be generally symmetrical, since the width of the absorption band reflects its energy range, with the possibility of distinguishing between individual cytochromes if the resolution of the detection apparatus attains a level at which the minor influences on the \( \pi \)-electrons become apparent. At lower temperatures the energy differences between permitted electronic transitions is restricted resulting in narrower vibronic envelopes and absorption bands of decreased wavelength range. High resolution spectrophotometry exploiting low temperatures may allow detection of distinct \( \alpha \)-absorption features resulting from perturbations of the immediate haem environment by the enveloping peptide chain which in extreme cases may restrict the conjugated \( \pi \)-electron system sufficiently to result in twin \( \alpha \)-absorption spectra. Such spectral characteristics will be constant in relative proportion for a particular cytochrome but must be shown to be associated with a single molecular source by other techniques, as described for this study under Results & Discussion.

Among those factors that might be expected to cause significant deviations from the ‘general’ behaviour and result in altered \( \alpha \)-band spectra would be a change of spin state by the iron atom, the lack of a ligand at one coordination site as in the terminal oxidases or the replacement of a ligand as in dioxygen or carbon monoxide binding to a terminal oxidase or in CO binding to a partially denatured cytochrome.
### APPENDIX ‘B’

**Bacterial Strain Characteristics**

<table>
<thead>
<tr>
<th>Strain</th>
<th>CGSC#</th>
<th>Sex</th>
<th>Genetic Characteristics</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0103</td>
<td></td>
<td>F−</td>
<td>gal, kan&lt;sup&gt;R&lt;/sup&gt;—Δcyd, rpsL, thi−1.</td>
<td>R.B. Gennis</td>
<td></td>
</tr>
<tr>
<td>GR17N</td>
<td></td>
<td>F−</td>
<td>gal-33, kdpABL5, lacZ82, rha-4, thi-1.</td>
<td>R.B. Gennis</td>
<td>(60)</td>
</tr>
<tr>
<td>GR19N</td>
<td></td>
<td>F−</td>
<td>cydA1, cyd&lt;sup&gt;can&lt;/sup&gt;, gal-33, kdpABL5, lacZ82, rha-4, thi−1.</td>
<td>GR17N</td>
<td>(60)</td>
</tr>
<tr>
<td>GVK124</td>
<td></td>
<td>F−</td>
<td>Δ(lac-pro)&lt;sup&gt;3&lt;/sup&gt;, hsd Δ5, recA, sdhC:: kan&lt;sup&gt;R&lt;/sup&gt;, thi.</td>
<td>R.B. Gennis</td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td></td>
<td>F−</td>
<td>gal, hsdRM, lacZ4, leuB6, proA2, recA13, rpsL, supE44, (su2&lt;sup&gt;+&lt;/sup&gt;), thi−1.</td>
<td>J. Wiener</td>
<td>(37)</td>
</tr>
<tr>
<td>KW107</td>
<td></td>
<td>F−</td>
<td>cyd&lt;sup&gt;can&lt;/sup&gt;, gal-33, kdpABL5, lacZ82, rha-4, thi−1.</td>
<td>PLJ01</td>
<td>Hinc&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KW202</td>
<td></td>
<td>Hfr</td>
<td>Δ(gpt-lac)&lt;sup&gt;S&lt;/sup&gt;, nadA, relA1, spoT1, thi−1.</td>
<td>NK6033</td>
<td>Hinc</td>
</tr>
<tr>
<td>KW531</td>
<td></td>
<td>F−</td>
<td>cyd&lt;sup&gt;can&lt;/sup&gt;, gal-33, kdpABL5, lacZ82, rha-4, sdh:: kan&lt;sup&gt;R&lt;/sup&gt;, thi−1.</td>
<td>PLJ01</td>
<td>Hinc</td>
</tr>
<tr>
<td>ML308-225</td>
<td></td>
<td></td>
<td>lacIZ, thi.</td>
<td>K.R. Kaback</td>
<td>(188)</td>
</tr>
<tr>
<td>MR43L</td>
<td></td>
<td></td>
<td>gal, lac, recA.</td>
<td>R.B. Gennis</td>
<td>(109)</td>
</tr>
<tr>
<td>NK6033</td>
<td>6180</td>
<td>Hfr</td>
<td>Δ(gpt-lac)&lt;sup&gt;S&lt;/sup&gt;, nadA50:: Tn10, relA1, spoT1, thi−1.</td>
<td>B. Bachman</td>
<td>(10)</td>
</tr>
<tr>
<td>PA2-18</td>
<td>5176</td>
<td>F−</td>
<td>argA52, gal-6, lacY1, malA1, met-2, nadA19, rpsL85, supE44, thi−1, tonA2, xyl-7, λ&lt;sup&gt;R&lt;/sup&gt;, λ−.</td>
<td>B. Bachman</td>
<td>(10)</td>
</tr>
<tr>
<td>PLJ01</td>
<td></td>
<td>F−</td>
<td>cyd&lt;sup&gt;can&lt;/sup&gt;, gal-33, kdpABL5, lacZ82, rha-4, thi−1.</td>
<td>GR19N</td>
<td>(89)</td>
</tr>
<tr>
<td>PLJ04</td>
<td></td>
<td>F−</td>
<td>argA, cydA1, gal, lacY, malA, metI, rpsL, supE, thi, tonA1, xyl-7, λ&lt;sup&gt;R&lt;/sup&gt;, λ−.</td>
<td>PA2-18</td>
<td>(89)</td>
</tr>
<tr>
<td>PLJ07</td>
<td></td>
<td>F−</td>
<td>argA, cydA1, gal, lacY, malA, metI, rpsL, supE, thi, tonA1, xyl-7, λ&lt;sup&gt;R&lt;/sup&gt;, λ−.</td>
<td>PA2-18</td>
<td>(89)</td>
</tr>
<tr>
<td>Strain</td>
<td>CGSC#</td>
<td>Sex</td>
<td>Genetic Characteristics</td>
<td>Source</td>
<td>Ref.</td>
</tr>
<tr>
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<td>------</td>
<td>-----</td>
<td>------------------------------------------------------------------------------------------</td>
<td>---------------</td>
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<tr>
<td>RG167</td>
<td></td>
<td>F'</td>
<td>$\text{cydA2, cyo, gal, lon-100, nadA, recA, srl-300::Tn10, rpsL, thi.}$</td>
<td>R.B. Gennis</td>
<td>(6)</td>
</tr>
<tr>
<td>RK4353</td>
<td></td>
<td></td>
<td>$\text{araD, gyrA, lacU, non, rpsL, thi.}$</td>
<td>V. Stewart</td>
<td>(194)</td>
</tr>
<tr>
<td>TK3D11</td>
<td></td>
<td>F'</td>
<td>$\Delta(\text{gal-att}^-\text{bio})2134, \Delta(\text{kdpD-gluA})D01, \text{rha, thi,}$</td>
<td>W. Epstein</td>
<td>(177)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\text{trkA405, trkD1.}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS9A</td>
<td></td>
<td></td>
<td>Hfr $\text{chlC}^{\text{LH}}, \text{thi.}$</td>
<td>J. DeMoss</td>
<td>(39)</td>
</tr>
</tbody>
</table>

|   |   |   | a $\Delta(\text{lac-pro})$ : $\text{lacI8, lacZ} \Delta M15, \text{pro}^+$. |   |   |
|   |   |   | b $Hinc$ : This study.                                                       |   |   |

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size</th>
<th>Genetic Characteristics</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRG110</td>
<td>5.7 kb</td>
<td>$\text{Sphl-Sall}$ fragment carrying cyo cloned into pBR322</td>
<td>R.B. Gennis</td>
<td>(6)</td>
</tr>
</tbody>
</table>
APPENDIX 'C'

Bacterial Growth Media

**CYD Medium:**  [Ref. 30, 117]

- KH$_2$PO$_4$ \(13.6 \text{ g L}^{-1}\)
- (NH$_4$)$_2$SO$_4$ \(2.0 \text{ g L}^{-1}\)
- FeSO$_4$$7\text{H}_2\text{O}$ \(9.0 \text{ mg L}^{-1}\) dissolved in c. 20 mL boiling water,

Adjust pH to 7.0 with solid KOH, adjust volume and autoclave.

Supplement with sterile solutions to final concentrations of:

- MgSO$_4$ \(1.0 \text{ mM}\)
- CaCl$_2$ \(0.1 \text{ mM}\)
- thiamine.HCl \(10.0 \text{ mg L}^{-1}\)
- carbon/energy source \(5.0 \text{ g L}^{-1}\)

**MMP Medium:**

- K$_2$HPO$_4$ \(7.0 \text{ g L}^{-1}\)
- KH$_2$PO$_4$ \(3.0 \text{ g L}^{-1}\)
- (NH$_4$)$_2$SO$_4$ \(1.0 \text{ g L}^{-1}\)
- Na$_3$ citrate \(0.5 \text{ g L}^{-1}\)
- MgSO$_4$ \(0.2 \text{ g L}^{-1}\)

Add stock supplements:

- ferric citrate \(0.46 \text{ mL L}^{-1}\) of 26.7 mM stock solution for 12 mM final concentration,
- Mo/Se \(1.00 \text{ mL L}^{-1}\) of 1.0 mM stock solution for 1 mM final concentration,

(Mo/Se stock contains \(1.24 \text{ g L}^{-1}\) (NH$_4$)$_6$Mo$_7$O$_{24}$4H$_2$O + 0.13 g L$^{-1}$ H$_2$SeO$_3$)

Adjust pH to 7.0 and autoclave.

**M9K Medium:**

- Na$_2$HPO$_4$ \(7.0 \text{ g L}^{-1}\)
- KH$_2$PO$_4$ \(3.0 \text{ g L}^{-1}\)
- NH$_4$Cl \(1.0 \text{ g L}^{-1}\)
- NaCl \(0.5 \text{ g L}^{-1}\)

Adjust pH to 7.2, adjust volume and autoclave.

Supplement with sterile solutions:

- carbon/energy source \(5.0 \text{ g L}^{-1}\) (continued overleaf)
- ferric citrate \(11.0 \text{ mg L}^{-1}\)
- thiamine.HCl \(10.0 \text{ mg L}^{-1}\)
- MgSO$_4$ \(1.0 \text{ mM}\) (final concentration)
- CaCl$_2$ \(0.1 \text{ mM}\) (final concentration)
NR Medium:  

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$HPO$_4$3H$_2$O</td>
<td>9.16 g L$^{-1}$</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>2.00 g L$^{-1}$</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>1.00 g L$^{-1}$</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.10 g L$^{-1}$</td>
</tr>
<tr>
<td>KNO$_3$ (reagent grade)</td>
<td>10.00 g L$^{-1}$</td>
</tr>
<tr>
<td>Na$_3$ citrate.2H$_2$O</td>
<td>0.40 g L$^{-1}$</td>
</tr>
<tr>
<td>Bacto yeast extract</td>
<td>0.25 g L$^{-1}$</td>
</tr>
<tr>
<td>Bacto tryptone</td>
<td>0.50 g L$^{-1}$</td>
</tr>
</tbody>
</table>

Dissolve nutrients in 500 mL distilled water and add:
ferrous ammonium sulphate 1.0 mg L$^{-1}$
thiamine.HCl 5.0 mg L$^{-1}$
Mo/Se 1.0 mL L$^{-1}$ (1.0 mM stock solution; see MMP medium)

Add 350 mL distilled water and warm to dissolve nutrients.
Adjust to pH 7 and autoclave with sparging apparatus (if required).
Add supplements, each in 50 mL:
glucose 5.5 g L$^{-1}$ (autoclaved)
KHCO$_3$ 10.0 g L$^{-1}$ (filter sterilized)

Adjust pH to 7.0 with c.HCl by aseptically removing aliquots of medium for testing.
Sparge vigorously with 95 % N$_2$, 5 % CO$_2$.

GF Medium:  

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>5.44 g L$^{-1}$</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>10.49 g L$^{-1}$</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>2.00 g L$^{-1}$</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>50.0 mg L$^{-1}$</td>
</tr>
<tr>
<td>MnSO$_4$.4H$_2$O</td>
<td>5.0 mg L$^{-1}$</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.93 mg L$^{-1}$</td>
</tr>
</tbody>
</table>

Dissolve in half total volume in narrow-necked vessel and autoclave.
Supplement with sterile solutions of:
CaCl$_2$ 0.50 mg L$^{-1}$
casamino acids 0.50 g L$^{-1}$
glycerol (0.04 M) 3.68 g L$^{-1}$
Na$_2$ fumarate (0.04 M) 5.52 g L$^{-1}$

All other supplements, antibiotics, etc. that were required for the growth of specific bacterial strains were filter sterilized and used at concentrations recommended by Miller [134].
APPENDIX ‘D’

Buffer Solutions

The pH of each buffer solution was adjusted at the temperature at which it was to be used, this being 0°C or 4°C in most cases.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTE-Buffer</td>
<td>0.2 % (w/v) Triton X-100, 10.0 mM Tris-HCl, 1.0 mM EDTA (free acid)</td>
<td>7.8 (unless otherwise noted)</td>
</tr>
<tr>
<td>TP-Buffer</td>
<td>10.0 mM Tris-HCl, 0.1 mM dithiothreitol</td>
<td>8.0 with NaOH</td>
</tr>
<tr>
<td>TPGA-Buffer</td>
<td>10.0 % glycerol, 5.0 mM Tris-HCl, 0.5 mM dithiothreitol, 20.0 mM e-aminocaproic acid, 6.0 mM p-aminobenzamide-2HCl</td>
<td>7.5 with NaOH</td>
</tr>
<tr>
<td>TPGB-Buffer</td>
<td>10.0 % glycerol, 1.0 mM Tris-HCl, 0.1 mM dithiothreitol, 0.5 mM EDTA</td>
<td>7.5 with NaOH</td>
</tr>
<tr>
<td>TE-Buffer</td>
<td>10.0 mM Tris-HCl, 1.0 mM EDTA (free acid)</td>
<td>7.8 (unless otherwise noted)</td>
</tr>
<tr>
<td>TM-Buffer</td>
<td>50.0 mM Tris-H$_2$SO$_4$, 10.0 mM MgSO$_4$</td>
<td>7.8 (unless otherwise noted)</td>
</tr>
<tr>
<td>TX-Buffer</td>
<td>10.0 mM Tris-HCl, 10.0 mM MgCl$_2$</td>
<td>7.8 (unless otherwise noted)</td>
</tr>
<tr>
<td>TD-Buffer</td>
<td>10.0 mM Tris-HCl, 0.1 mM dithiothreitol</td>
<td>8.0 with NaOH</td>
</tr>
<tr>
<td>TDGB-Buffer</td>
<td>10.0 % glycerol, 1.0 mM Tris-HCl, 0.1 mM dithiothreitol, 0.5 mM EDTA</td>
<td>7.5 with NaOH</td>
</tr>
<tr>
<td>TTE-Buffer</td>
<td>1.0 % (w/v) Triton X-100, 10.0 mM Tris-HCl, 1.0 mM EDTA (free acid)</td>
<td>7.8 (unless otherwise noted)</td>
</tr>
<tr>
<td>TTX-Buffer</td>
<td>10.0 % (w/v) Triton X-114, 10.0 mM Tris-HCl, 10.0 mM MgCl$_2$</td>
<td>7.8 (unless otherwise noted)</td>
</tr>
</tbody>
</table>