THE BIOENERGETICS
OF PARACOCUS DENITRIFICANS

A thesis presented for the degree of
Doctor of Philosophy
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by
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ABSTRACT

This thesis concerns the mechanism of oxidative phosphorylation, with special reference to the bacterium Paracoccus denitrificans.

A new method using affinity chromatography is described for the separation of vesicles of different membrane orientation.

The working hypothesis is the chemiosmotic theory of Mitchell, with its emphasis on the transmembrane proton motive force made up of a membrane potential and a transmembrane pH gradient. The 3 experimental systems used are bovine heart submitochondrial particles, chromatophores from the photosynthetic bacterium Rhodospirillum rubrum and phosphorylating vesicles from P. denitrificans.

From the uncoupling obtained with various ions added to the P. denitrificans particles it is concluded that the transmembrane distribution of weak bases and permeant anions permits a determination of the protonmotive force.

A flow-dialysis method has been developed to measure the steady-state transmembrane distribution of weak bases and anions in a determination of the protonmotive force. This method successfully overcomes problems which arise with the techniques previously used for these measurements.

Under the experimental conditions used to determine the extent of oxidative phosphorylation (the phosphorylation potential) no transmembrane pH gradient was detectable, and the
protonmotive force could be accounted for completely by
the membrane potential. A method using ion-selective
electrodes was developed to provide a continuous determination
of this membrane potential.

In all 3 systems the proton motive force determined by
the above methods was found to be significantly lower than
that required by the chemiosmotic hypothesis. A simple
modification of the chemiosmotic hypothesis is proposed.
This new concept is based on electrodic theory. It is shown
to be useful in explaining results reported in the literature
(from both photosynthetic and respiratory systems) which
appeared to be at odds with the chemiosmotic theory in its
usual form.
ACKNOWLEDGEMENTS

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ABBREVIATIONS

The use of abbreviations in this thesis follows the conventions of the Biochemical Journal (1978) (Biochem. J. 169, 1-27). In addition to the standard abbreviations listed in the Biochemical Journal a number of non-standard abbreviations have been used. These are rendered in their full form at their first appearance.

NON-STANDARD ABBREVIATIONS

DAD 2,3,5,6- tetramethyl- p-phenylene diamine
FCCP Carbonyl cyanide p-trifluoromethoxy phenylhydrazone
HQNO 2- p-heptyl-4-hydroxyquinoline-N-oxide
TMFD N,N,N',N'- tetramethyl p-phenylene diamine
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CHAPTER 1

BIOENERGETICS, MICROBIAL ENERGETICS AND PARACOCCUS DENITRIFICANS
"The flow of energy through a system acts to organise that system".

Morowitz (1968)

Energy transformation via the ATP system

The living cell is a thermodynamically open system which is characterised by a continuous input and output of energy. Each cell is endowed with a system that transforms the chemical and physical energy taken up into biologically useful energy and that utilises the latter to perform work. A universal molecular carrier for biological energy is ATP. Light or chemical energy taken up by the cell is used in oxidative reactions to drive the endergonic synthesis (from ADP and P_i) of ATP, the energy being conventionally considered as 'stored' in the 'high-energy' terminal phosphate bond (Lipmann 1941). Anabolic reactions may then be driven by the phosphorolysis of ATP so that the coupling between anabolism and catabolism is stoichiometric or chemical rather than energetic or physical (Banks & Vernon 1970; Atkinson 1977). Such a relationship is indicated in Fig. 1.1 and values of the standard free energy changes of the reactions of Fig. 1.1 are given in Table 1.1. It should be noted that ATP, ADP, AMP, PP_i and P_i can exist in solution as equilibrium mixtures of several polyanionic species, each of which can form complexes with divalent cations. Therefore the observed free energies of hydrolysis (ΔG_{obs}^{o'}) are functions of the pH and divalent cation concentrations. It may
On Va:

Chemical Energy

Physical Energy

Chemical work

Genetic work

Mechanical work

Heat

Capable

Analysis

Fig 1.1
The figure indicates the central role played by the adenine nucleotide system in the cellular economy. Note that in some cases the ATP system may be bypassed in the sense that an intermediate in the form of a protonmotive force (see later) may act directly to perform osmotic work, active movement, etc. A more detailed consideration of these and other topics is given in the text.
Table 1.1 Free energy changes of reactions of the ATP system

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<th>Reaction</th>
<th>( \Delta G_{\text{obs}}^0 ) (kcal mol(^{-1}))</th>
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<tr>
<td>ATP + H(_2)O = ADP + P(_i)</td>
<td>- 7.60</td>
</tr>
<tr>
<td>ATP + H(_2)O = AMP + PP(_i)</td>
<td>- 9.96</td>
</tr>
<tr>
<td>ATP + AMP = 2 ADP</td>
<td>0.0</td>
</tr>
<tr>
<td>PP(_i) + H(_2)O = 2 P(_i)</td>
<td>- 5.24</td>
</tr>
</tbody>
</table>

Values are obtained from Thauer et al., (1977) (and see also Slater et al., 1973; Wood 1977). \( \Delta G_{\text{obs}}^0 \) values are given for a free Mg\(^{++}\) concentration of 1 mM, an ionic strength of 0.25 and a pH of 7. The value of Slater et al., (1973) for the first reaction, under these conditions, is -6.8 kcal mol\(^{-1}\) (and is the one adopted in later chapters), whilst values as high as -8.7 kcal mol\(^{-1}\) (see Thauer et al., 1977) have been reported by others. However the Table serves to indicate that the hydrolysis of ATP is accompanied by a rather large loss in free energy.
be seen from Table 1.1 that the $\Delta G^{\circ'}$ for the hydrolysis of ATP to produce ADP and $P_i$ is still a matter of some controversy.

The importance to the cellular economy of ATP synthesis is clear from Fig. 1.1. It is therefore appropriate in a bioenergetic study to be concerned with the mechanism of synthesis of ATP, or, more specifically, with the processes by which the release of oxidative energy is coupled to the endergonic synthesis of ATP, and it is to this classical problem that I now turn.

**On the mechanism of ATP synthesis**

The chemotrophic redox processes that occur in nature are characterised by the diversity of substrates that can be used for the production of energy (e.g. Thauer et al., 1977), and the energy required for the synthesis of ATP from ADP and $P_i$ is generally provided by such redox processes. In both chemotrophic and phototrophic organisms the ATP-forming process may be viewed as an exergonic system comprising chains of electron-accepting and -donating carriers together with an ATPase enzyme catalysing the synthesis and hydrolysis of ATP. The electrochemical potential difference $\Delta E$ between redox partners is used to drive the ATPase-catalysed phosphorylation of ADP such that at thermodynamic equilibrium

$$-nF \Delta E = \Delta G_p \quad \text{......... Eq. 1.1}$$

where $n$ is the number of electrons transferred, $F$ is Faraday's constant (23.08 kcal volt$^{-1}$) and

$$\Delta G_p = \Delta G^{\circ'} + 1.38 \log \frac{[ATP]}{[ADP][P_i]} \quad \text{at } 30^\circ \quad \text{......... Eq 1.2}$$
Some reactions catalysed by energy-transducing biological membranes

The relationship between the exergonic reactions of electron transport and the endergonic reactions of ATP synthesis, active solute transport and the 'transhydrogenase' reaction is indicated. The nature of the intermediate between these processes is at present unknown, and investigations concerning the 'high-energy' intermediate constitute a large proportion of the work described in this thesis. The formulation given, consisting of protein conformational changes in equilibrium with a protonmotive force, is probably the most generally acceptable. The endergonic reactions exert a negative feedback (open arrows) on the exergonic or isoenergetic reactions which drive them, leading to a steady state. Uncouplers act to dissipate the build-up of the high energy intermediate as heat energy, and their action is indicated. For further discussion, see text.
Fig 1.2

HEAT

TRANHYDROGENASE

ACTIVE SOLUTE TRANSPORT

ATP SYNTHESIS

PROTEIN CONFORMATION CHANGES

ELECTRON TRANSPORT

\[ \Delta G_H^\circ = \Delta G_Y - 2\Delta pH \]

"HIGH-ENERGY" INTERMEDIATE
Fig 1.3
Fig. 1.3 Some thermochemical components of mammalian mitochondria

This figure, modified from Dutton and Wilson (1974), indicates the operating midpoint potential at pH 7.2 of some of the electron transport carriers of the mammalian mitochondrial respiratory chain. The actual redox potential, relative to the standard hydrogen cell, in situ will depend upon the relative proportions of the oxidised and reduced forms of the carriers. Lower case letters represent cytochromes. Other abbreviations: FeS, Iron-sulphur centre; Q, ubiquinone; Fp, flavoprotein; succ, succinate; Cu, Copper atom. The location and pathway of transfer between the carriers are not considered. According to a conventional view energy conservation, in the form of ATP, takes place in certain segments of this respiratory chain, and the 3 energy-conserving segments are considered to be those marked. An alternative conception of this is given in Chapter 7. For details concerning the determination of $E_n$ values, see Dutton and Wilson (1974); Wilson et al., (1974).
(Slater et al., 1973). This coupled process is referred to as oxidative or electron transport phosphorylation (ETP). It takes place on the inner membrane of plant and animal mitochondria, on the plasma membrane of bacteria and on the thylakoid membrane of the chloroplasts of higher plants (Ciba Foundation 1975, Boyer et al., 1977), and is diagrammed, in a network relationship with various energy-consuming reactions, in Fig. 1.2. Compounds exist, known as uncouplers (Hanstein 1976), which dissipate redox energy as heat, and abolish ETP, and their action is also indicated in Fig. 1.2. The redox potential of various components of the mammalian mitochondrial electron transport chain is indicated in Fig. 1.3, together with the thermodynamically possible (and experimentally observed) segments which may be coupled to ATP synthesis. It may be seen that a stoichiometry of 3 ATP per 0 atom reduced with NADH as substrate, and a "P/O ratio" of 2 with succinate as substrate are observed. Such thermodynamically derived data (Dutton and Wilson 1974) are also complemented by kinetic studies (e.g. Chance and Williams 1956). It is not proposed to consider the latter further here.

A very great number of hypotheses have been advanced in an attempt to identify the 'high-energy intermediate' (Fig. 1.2) of ATP synthesis (e.g. Boyer et al., 1977) and to review such theories critically would prove both lengthy and superfluous. Nevertheless, a brief outline of the main currently held hypotheses is warranted.

The chemical hypothesis (Slater 1971; Boyer et al., 1977; Griffiths et al., 1977) assumes that an energy-rich chemical
"X~ I" with a large free energy of hydrolysis is formed as a result of the redox reactions of electron transport, and that the energy conserved in X~ I is transferred to the ATP system, in analogy to the series of glycolytic reactions by which glyceraldehyde-3-phosphate is oxidised to phosphoglycerate, with concomitant formation of ATP from ADP and P_i. A membrane structure is neither required nor excluded by this mechanism.

The conformational hypothesis (Boyer 1974; Boyer et al., 1977) assumes that an "energy-rich" strained conformation of a protein component is induced by the redox reactions of electron transport, and that the strained conformation relaxes in the presence of ADP and P_i, the energy being conserved by the formation of ATP. This view is based on the analogous mechanism of muscular contraction (Huxley, A. 1974), operating here in the reverse direction. In principle, a membrane structure is not required for this mechanism.

The chemiosmotic view of energy coupling which evolved between 1956 and 1966 (Mitchell and Moyle 1958; Mitchell 1961, 1962, 1966a) proposed that energy-yielding reactions generate an electrochemical potential difference of ions across a membrane, which can in turn drive energy-consuming reactions (Fig. 1.2) such as active transport and ATP synthesis. The theory and supporting experimental evidence have subsequently been expounded in detail both by its author (Mitchell 1966b, 1968, 1970 a,b, 1976a,b, 1977a,b) and by others (Greville 1969; Harold 1972; Hamilton 1975; Garland 1977), but the four main postulates may be noted here:

1. The membrane-located ATPase system is a hydro-dehydration system with a terminal specificity for ATP and water. Its
\[ \Delta \mu_{H^+} = \Delta \Psi - \frac{2:3RT}{F} \Delta pH \]

proton motive force

Fig 1.4
The figure indicates the essential nature of the chemiosmotic hypothesis, and leaves out of consideration the question of mechanistic detail. According to the chemiosmotic view, electron transport components, ATPase enzymes and substrate-translocating "porter" molecules, which are embedded in the lipoprotein membrane phase, form, together with membrane phospholipids, an osmotic barrier between two aqueous phases. Catalytic activity of these components is linked to the transmembrane movement of protons from one bulk aqueous phase to the other, in a reversible manner. The direction of the proton movements and the catalytic reactions are determined only by thermodynamic considerations. The vectorial translocation of protons across the osmotic barrier of the membrane results in the build-up of a protonmotive force, consisting of a chemical and electrical component, whose free energy is given by the equation in the Figure. For further details, see text. Abbreviation: S, substrate molecule.
normal function is to couple reversibly the translocation of protons across the membrane to the flow of anhydro-bond equivalents between water and the couple ATP/(ADP + phosphate).

2. The membrane-located oxidoreduction chain systems catalyse the flow of reducing equivalents between substrates of different oxidoreduction potentials, reversibly coupled to the translocation of protons across the membrane.

3. There are present in membranes substrate-specific exchange-diffusion carriers which permit the reversible exchange of substrate molecules across the membrane, tightly coupled to the translocation of protons or hydroxyl ions. They act to regulate the pH and osmotic differential across the membrane and to permit entry and exit of essential metabolites without collapse of the membrane potential.

4. The systems of postulates 1, 2 and 3 are located in a specialised coupling membrane which has a low permeability to protons and to cations and anions generally.

One consequence of moving $H^+$ from the bulk phase on one side of the membrane to the bulk phase on the other (Fig. 1.4) is to set up a transmembrane electrical potential $\Delta \psi$, measured as the electrical potential in the left (L) phase minus that in the right (R) phase (Fig. 1.4). Another consequence is the establishment of a pH difference $\Delta pH$ between the two phases, expressed as the pH in the left phase minus that in the right phase. The total electrochemical difference of protons between the two phases is known as the protonmotive force $\Delta p$ and is given (at $30^\circ$) by:

$$
\Delta \mu_{H^+} = \Delta p = \Delta \psi - 60 \Delta pH \quad \ldots \ldots \text{Eq 1.3}
$$
where $\Delta \varphi$ and $\Delta \psi$ are expressed in millivolts and $\Delta \text{pH}$ in pH units. The relative magnitudes of $\Delta \psi$ and $\Delta \text{pH}$ are governed by the transmembrane mobilities and charges of any counterions present. According to the chemiosmotic hypothesis, if the reaction catalysed by the proton-translocating ATPase is allowed to proceed to equilibrium, the protonmotive force may be equated with the free energy stored in the ATPase reaction (the phosphate potential) $\Delta G_p$, according to the relationship

$$\Delta G_p = -zF \Delta \varphi \quad \text{Eq 1.4}$$

where $z$ is the number of protons translocated across the membrane via the ATPase enzyme for each molecule of ATP synthesised, the $\text{H}^+/\text{ATP}$ ratio.

That the chemiosmotic hypothesis has currently gained such wide (if slow) acceptance may be ascribed to four factors:

1) Vectorial movements of protons and other ions do accompany both electron transport, active transport and ATP hydrolysis (e.g. Harold 1972; Papa 1976).

2) Electrochemical gradients of protons are formed across energy-transducing membranes (e.g. Rottenberg 1975).

3) The action of uncouplers is indicated by their ability to dissipate transmembrane proton gradients, since almost all are lipophilic weak acids (e.g. McLaughlin 1972a; Haydon and Hladky 1972; Hanstein 1976; Cohen et al., 1977; but see Miko and Chance 1975).

4) An artificially generated electrochemical proton gradient is kinetically competent in driving ATP synthesis (Jagendorf and Uribe 1966; Thayer and Hinkle 1975).
Such considerations have led Slater (1977) to the view that "I hope most workers will now use this chemiosmotic framework as the starting point for the presentation of their work".

It is appropriate here to draw the reader's attention to an independently derived version of the chemiosmotic hypothesis known as the 'proton-in-the-membrane' theory. Whereas Mitchell postulated the existence of a protonmotive force between the two bulk phases on either side of the membrane, Williams (1961, 1969, 1977, 1978a,b) considered that the reaction

\[ \text{ADP} + P_i + n \text{H}^+ = \text{ATP} + \text{H}_2\text{O} \]

would be driven to the right (i.e. in the direction of ATP synthesis) if it took place within the hydrophobic energy-transducing membrane, where \( [\text{H}^+] \) would be high and \( [\cdot\text{H}_2\text{O}] \) would be low. Thus the theories of both Mitchell and Williams stress the importance of proton gradients in biological energy transduction, but while Williams considers the 'high-energy intermediate' to consist of localised, membrane-associated protons, Mitchell believes that the functional proton movements occur between the bulk aqueous phases on either side of the membrane (see further discussion in Chapter 7).

Mitchell himself was the first to measure electron transport-linked proton movements into the bulk aqueous phase external to rat-liver mitochondria, using a glass electrode (Mitchell and Moyle 1967b), but found that the presence of membrane-permeable, charge-neutralising ions was required to
observe a maximal $\text{H}^+ / 0$ ratio (the number of protons translocated per oxygen atom reduced) of 6 with NADH as electron donor. Brand (1977) has reviewed current controversy regarding the validity of this stoichiometry (see also Chapters 4 and 7). This requirement for permeant counterions is explained, according to the chemiosmotic view, by a respiratory control resulting from a large initial membrane potential feeding back (cf. Fig. 1.2) to depress respiration and hence further proton ejection, consistent with the ability of uncouplers to release such respiratory control (see e.g. Mitchell 1968; Scholes and Mitchell 1970b). This explanation, however, has been challenged by a series of experiments (Gould and Cramer 1977) which studied the effect of permeant counterions on the measured $\text{H}^+ / 0$ ratio of *Escherichia coli* cells oxidising endogenous substrate. The results (Gould and Cramer 1977), which are reviewed briefly in Chapter 7, are more consistent with the notion that the functional current of protons involved in *E. coli* membrane energy transduction is confined within the cytoplasmic membrane. Thus the action of the high (50-100 mM) concentrations of permeant ions such as thiocyanate used in such experiments is viewed as being more than a simple electrophoretic neutralisation of vectorial proton movements between two bulk phases.

Although many of the concepts described above have been tested with mitochondria and chloroplasts of higher organisms, it was from experiments with microorganisms that Mitchell's ideas about vectorial metabolism and ion currents first evolved (Mitchell 1977b; Harold 1978). Therefore I will turn to a
consideration of the role of microbial systems in advancing our understanding of bioenergetic phenomena.

**Microbial energetics**

The above title was given to a recent microbiological symposium (Haddock and Hamilton 1977), and a perusal of this volume indicates both the paradigm status (Kuhn 1970) of the chemiosmotic view and the informative results provided by the application of these principles to microbial cells. Three notable advantages offered by bacteria in general over mitochondria and chloroplasts as experimental tools are:

1) a uniform population of cells may be grown at small cost and in large quantities under precisely defined conditions, on a continuous basis if required (Tempest 1970)

2) mutants with specific deletions in the bioenergetic apparatus may readily be obtained (Cox and Gibson 1974; Haddock 1977)

3) the effects of changes in genotype or environment are directly reflected in the rate and/or efficiency of microbial growth (e.g. Stouthamer 1977).

Additionally, particular types of bacteria offer particular advantages. Chromatophores from photosynthetic bacteria may be energised by short flashes of light applied in succession such that single turnovers of the electron transport chain and associated rapid changes in pH can be followed (e.g. Jackson et al., 1975). The halophile *Halobacterium halobium* provides us with a light-driven proton pump called bacteriorhodopsin which can power ATP synthesis in the absence of electron transport (Oesterhelt et al., 1977). Thermophilic
bacteria provide a source of membrane-bound enzymes which are much more stable, when isolated, than the corresponding mitochondrial or chloroplastic enzymes (Kagawa et al., 1976). Bacteria which live under unusually alkaline conditions (Brock 1969) can be used to determine the importance of the transmembrane proton gradient for energy conservation (Haddock 1977). Correspondingly, the acidophile *Thiobacillus ferrooxidans* has been demonstrated (Ingledew et al., 1977) to conserve respiration-generated free energy by an apparently direct chemiosmotic system.

Most aerobic bacteria have respiratory chains which differ from the mitochondrial (Fig. 1.3) respiratory chain (Garland and Haddock 1977; Haddock and Jones 1977), but there are at least two bacteria which, when grown aerobically, have a respiratory chain which is quite similar to that of mitochondria. These bacteria are *Rhodopseudomonas spheroides* and *Paracoccus denitrificans* (John and Whatley 1975). Vesicles derived from the plasma membrane of *P. denitrificans* (Chapter 2) are of special interest since they show (John and Hamilton 1970) the tight coupling between respiration and ATP synthesis observed in carefully isolated rat-liver mitochondria (Chance and Williams 1956). The numerous similarities between *P. denitrificans* and mammalian mitochondria have been explained on the basis of an evolutionary origin for mitochondria from a prokaryotic ancestor resembling *P. denitrificans*, and it is for reasons such as the above that much of the work described in this thesis has been performed with *P. denitrificans*. I shall therefore conclude this
chapter with a summary of our knowledge of the energetics of intact cells of _P. denitrificans_ (John and Whatley 1977a,b).

Oxidative phosphorylation in intact cells of _P. denitrificans_ (previously _Micrococcus denitrificans_) (Davis et al., 1969) can grow either autotrophically with hydrogen and carbon dioxide or heterotrophically with a wide variety of carbon compounds. It can grow aerobically or it can adapt to grow anaerobically if nitrate, nitrite or nitrous oxide are available as terminal electron acceptors. It is incapable of fermentative growth (Davis et al., 1969). During autotrophic growth the reductive pentose phosphate cycle is operative (Kornberg et al., 1960). During heterotrophic growth glucose is oxidised via the Entner-Doudoroff pathway, by the hexose monophosphate pathway or by a combination of these two pathways; the glycolytic pathway is absent (Forget 1968; Slabas and Whatley 1974). Pyruvate, succinate and malate are oxidised to carbon dioxide via the tricarboxylic acid cycle (Forget and Pichinoty 1965). Acetate can be metabolised via the glyoxylate cycle (Kornberg et al., 1960), and when glycollate is the sole carbon and energy source the β-hydroxyaspartate pathway (Kornberg and Morris 1965), a pathway apparently unique to _P. denitrificans_, is operative. When nitrate is added as terminal electron acceptor to anaerobic cells it is reduced to dinitrogen via nitrite and nitrous oxide, while cells grown with adequate aeration are unable to use nitrate as a terminal electron acceptor, since the synthesis of the respiratory nitrate reductase is repressed by oxygen (Pichinoty 1965). Cells which have been grown anaerobically
Fig. 1.5  The respiratory chain of *Paracoccus denitrificans*

Adaptive components appear in lower case letters while the constitutive components appear in underlined upper case letters. The redox potentials are not known, but are assumed to be similar to those of mammalian mitochondria (Fig. 1.3). For further details see John and Whatley (1975, 1977), and the text.
with nitrate are able to use oxygen, since the oxidase is a constitutive feature of the cells. Indeed, oxygen is used in preference to nitrate by whole cells (John 1977), although membrane vesicles derived from \textit{P. denitrificans} do not possess this regulatory mechanism for 'switching' from nitrate to oxygen as terminal electron acceptor (John 1977). The nature of the 'switching' mechanism is at present unknown.

The components of the electron transport chain of \textit{P. denitrificans} are indicated in Fig. 1.5. It may be seen that they are indeed similar to those found in the mammalian mitochondrial respiratory chain (Fig. 1.3), with additional inducible components that allow the oxidation of alternative substrates or the reduction of alternative terminal electron acceptors. By analogy with mitochondria, it might be expected that the stoichiometry of oxidative phosphorylation (the P/O ratio referred to above) would be three with NADH and two with succinate. However there is still much uncertainty regarding the P/O ratio in intact cells of this organism. This is largely because in intact cells direct measurements of ATP synthesis have proved to be difficult, partly because of the impermeability of the plasma membrane to adenine nucleotides (see John and Whatley 1977a) and partly because of the rapid turnover time of the adenine nucleotide pool (John and Whatley 1977b). In cell-free systems which have been developed to obviate these problems of work with intact cells, the P/O ratios observed are in general unreasonably low (Gel'man \textit{et al.}, 1967). However, a procedure developed by John for \textit{P. denitrificans} (see John and Whatley 1977b) results in a membrane vesicle preparation giving a high P/O
ratio. Some properties of these vesicles are described in the next chapter.

The following three methods have been used for estimating P/0 ratios in intact cells of *P. denitrificans*: (a) \( \text{H}^+ / \text{O} \) ratios, (b) adenine nucleotide changes during an oxygen pulse, (c) molar growth yields. Such studies are now reviewed.

(a) The rationale behind the use of the glass pH electrode in the presence of charge-neutralising permeant ions to measure the stoichiometry of respiration-driven proton movements in mitochondria (Mitchell and Moyle 1968) has been indicated above. The \( \text{H}^+ / \text{O} \) ratio is related to the P/0 ratio by the relationship

\[
P/0 = \frac{\text{H}^+/\text{O}}{\text{H}^+/\text{ATP}}\quad \text{......... Eq 1.5}
\]

and thus if the \( \text{H}^+/\text{ATP} \) ratio is known, the P/0 ratio may be calculated from \( \text{H}^+/\text{O} \) measurements. In the case of mitochondria, there is currently much controversy regarding the \( \text{H}^+/\text{ATP} \) ratio (see Brand 1977), which is found to be 2 or 3 using kinetic methods in the presence of high concentrations of permeant counterions (Moyle and Mitchell 1973a; Thayer and Hinkle 1973; Brand 1977), but appears to be at least 3 using thermodynamic analyses (e.g. Nicholls 1977; Wiechmann et al., 1975; Rottenberg and Gutman 1977). In addition, measurements in intact mitochondria are severely complicated by the presence of an energy-requiring adenine nucleotide translocase (Klingenberg and Rottenberg 1977). In bacteria, which do not possess the adenine nucleotide translocase, it has been common practice (e.g. Haddock and Jones 1977) to assume an \( \text{H}^+/\text{ATP} \) ratio of 2.
### Table 1.2 → $H^+/O$ ratios for intact *P. denitrificans* cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$H^+/O$ ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>6-8</td>
<td>Scholes and Mitchell (1970b)</td>
</tr>
<tr>
<td>Various</td>
<td>4-8</td>
<td>Lawford <em>et al.</em>, (1976)</td>
</tr>
<tr>
<td>Endogenous</td>
<td>7-8</td>
<td>Meijer <em>et al.</em>, (1977a)</td>
</tr>
<tr>
<td>Endogenous</td>
<td>6</td>
<td>van Verseveld <em>et al.</em>, (1977)</td>
</tr>
<tr>
<td>Endogenous</td>
<td>8</td>
<td>Edwards <em>et al.</em>, (1977)</td>
</tr>
<tr>
<td>Various</td>
<td>6-9</td>
<td>Lawford (1978)</td>
</tr>
</tbody>
</table>

The purpose of Table 1.2 is to indicate the experimental importance of the oxygen pulse method as a tool for studying the efficiency of energy transduction in intact *P. denitrificans* cells. Without a detailed discussion of the exact conditions of growth and resuspension no comparison should be made between different experimenta. → $H^+/O$ ratios measured using cells grown under sulphate-limited conditions have not been included (see Meijer *et al.*, 1977b; Lawford 1978) owing to a current controversy on this point. A knowledge of the → $H^+/ATP$ ratio (see text) is all-important in calculating the P/O ratio for intact cells from the observed → $H^+/O$ ratios.
\textbf{P. denitrificans} was the first bacterium for which an accurate determination of $\Delta \text{H}\,/\,O$ ratios was made. The high ratio of $8.0 \pm 0.1$ obtained for cells oxidising endogenous substrates (Scholes and Mitchell, 1970b) in the presence of 17.5 to 100 mM thiocyanate has also been obtained in mitochondria treated with N-ethyl maleimide, a treatment that results in the anaerobic accumulation of NADPH (Moyle and Mitchell 1973b). Thus NADPH is thought to be the effective reductant in \textbf{P. denitrificans} respiring on endogenous substrates during a short oxygen pulse after a period of anaerobiosis. However, in view of uncertainties concerning the multiple sites of action of N-ethyl maleimide (Brand 1977), this explanation must be regarded as unsatisfactory. Values obtained by various authors for the $\Delta \text{H}\,/\,O$ ratio of intact \textbf{P. denitrificans} cells are summarised in Table 1.2. It may be noted that, whatever the $\Delta \text{H}\,/\,\text{ATP}$ ratio, and whatever the basis for the changes in the $\Delta \text{H}\,/\,O$ ratio under different conditions, \textbf{P. denitrificans} is capable of altering the efficiency of free energy transduction in response to changing environmental conditions.

(b) When cells of \textbf{P. denitrificans} are allowed to become anaerobic, the cellular ATP level is low and that of AMP is high (cf. Fig. 1.1 and Atkinson 1977). On introduction of oxygen, the ATP level increases and the AMP level falls; measurement of these changes during anaerobic to aerobic transitions has yielded estimates for the $P/O$ ratio (with endogenous substrates) of 1.0 (van Verseveld and Stouthamer 1976) and 1.8 (Hanselmann 1974). However, it is generally believed that values of the $P/O$ ratio obtained with this method are underestimates of the true values, owing to the rapid rate
of utilisation of ATP by the cells (van der Beek and Stouthamer 1973; Stouthamer 1977).

(c) As noted above, in bacteria changes in the efficiency of oxidative phosphorylation are directly related to the molar growth yield of the organism (the weight in g of cells obtained per mole of substrate or terminal electron acceptor utilised), generally obtained by weighing cells grown in a chemostat (Stouthamer 1976, 1977). The early observation (Bauchop and Elsden 1960) of a constant proportionality between their growth yield and the number of moles of ATP produced via known pathways of substrate degradation in fermentative bacteria (about 10.5 g cells per mole of ATP) led to the belief that this number ($Y_{ATP}$) might be a biological constant. However, largely because of the widespread occurrence of a significant effect of growth rate on growth yield, and because of variability in measured $Y_{ATP}$ values, this belief is not now held (Stouthamer and Bettenhausen 1973; Stouthamer 1977), and it has proved necessary to calculate $Y_{ATP}$ theoretically (see Stouthamer 1973). In particular, the energy required for maintenance processes in the absence of growth must be considered. Pirt (see Pirt 1975) noted that the molar growth yield of cells was approximately proportional to their growth rate, and reasoned that this was due to the proportionately greater effect of maintenance processes at low growth rates. He developed an empirical equation in the form of a double reciprocal plot of $1/Yield$ versus $1/growth rate$, which, by extrapolation to infinite growth rate (giving $Y_{max}^{ATP}$ and $Y_{max}^{substrate}$) could be used to correct for maintenance requirements. However,
Table 1.3  P/O ratios calculated for P. denitrificans cells from molar growth yield studies

<table>
<thead>
<tr>
<th>Energy Source</th>
<th>Terminal electron acceptor</th>
<th>Limiting substrate</th>
<th>P/O</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>( O_2 )</td>
<td>Succinate</td>
<td>1.4-1.7</td>
<td>Van Verseveld and Stouthamer (1976)</td>
</tr>
<tr>
<td>Succinate</td>
<td>( O_2 )</td>
<td>Succinate</td>
<td>1.7</td>
<td>Meijer et al., (1977a)</td>
</tr>
<tr>
<td>Succinate</td>
<td>( O_2 )</td>
<td>Sulphate</td>
<td>1.0</td>
<td>Stouthamer (1977)</td>
</tr>
<tr>
<td>Glycerol )</td>
<td>( O_2 )</td>
<td>C - source</td>
<td>3</td>
<td>Edwards et al., (1977)</td>
</tr>
<tr>
<td>Glucose )</td>
<td>( O_2 )</td>
<td>C - source</td>
<td>3</td>
<td>Edwards et al., (1977)</td>
</tr>
<tr>
<td>Acetate</td>
<td>( O_2 )</td>
<td>Acetate</td>
<td>2</td>
<td>Edwards et al., (1977)</td>
</tr>
</tbody>
</table>

For methods of calculation see original texts. Note that in the case of acetate as substrate (Edwards et al., 1977) the measured \( 
\text{H}^+/\text{O} \) ratio (with endogenous substrate) was the same as that when glucose or glycerol were the substrate for growth (and the \( 
\text{H}^+/\text{O} \) ratio was measured with endogenous substrate). There is currently some controversy as to whether site 1 phosphorylation is lost under sulphate-limited condition. See Lawford (1978) for a differing view from that of Stouthamer's group (whose value is included in the Table).
recent work (Neijssel and Tempest 1976a,b) has questioned
this analysis and its assumption of a constant maintenance
energy, and in view of the well-known decrease in cell
viability at low growth rates (Postgate and Hunter 1962;
Tempest et al., 1967; Sinclair and Topiwala 1970), it is
my opinion that quantitative conclusions regarding the
P/O ratio based on molar growth yield studies of this type
should be treated with some caution. Nevertheless, if the
value of \( Y_{\text{C}_2}^{\text{max}} \) (yield in g of cells per mole \( \text{O}_2 \) utilised,
corrected for maintenance requirements) is measured, the
P/O ratio is given by

\[
\frac{Y_{\text{C}_2}^{\text{max}}}{Y_{\text{O}_2}^{\text{max}}} = 2 \frac{Y_{\text{ATP}}^{\text{max}}}{Y_{\text{O}_2}^{\text{max}}} \cdot \text{P/O} \quad \text{............ Eq 1.6}
\]

A more sophisticated theoretical analysis of the rela-
tionship between the P/O ratio and the molar growth yield
during heterotrophic growth has been presented by de Kwaad-
steniet et al., (1976), and applied to \( \text{P. denitrificans} \)
(van Verseveld and Stouthamer 1976; Meijer et al., 1977a).
It was concluded that, with succinate or gluconate as
(growth-limiting) substrate, the P/O ratio was no more than
1.7. Edwards et al., subsequently calculated the P/O ratio
using the \( Y_{\text{ATP}}^{\text{max}} / Y_{\text{O}_2}^{\text{max}} \) method, and found it equal to about
3 for cells grown with glucose, glycerol or DL-\( \text{Lactate} \), but
about 2 for cells grown with acetate. However, in the
experiments of Edwards et al., the calculated P/O ratio varied
independently with the measured \( \rightarrow \text{H}^+/\text{O} \) ratio for cells grown
on differing substrates (oxidising endogenous substrates
during \( \rightarrow \text{H}^+/\text{O} \) measurements). These and other experiments
are summarised in Table 1.3. There is still some controversy
concerning the fate of the first coupling site of oxidative phosphorylation in sulphate-limited, chemostat-grown cells (Meijer et al., 1977a,b; Lawford 1978), and such data have not been included in extenso in Table 1.3. It might be noted that experimentally determined $\Delta H^{\circ}/O$ ratios are obtained by non-equilibrium methods with high concentrations of thiocyanate present, whereas growth yield parameters are essentially thermodynamic and are obtained when the system is in a steady state, with no thiocyanate present. Therefore it may not be appropriate to compare $y_{\text{max}}^{\text{ATP}}$ values and $\Delta H^{\circ}/O$ measurements, particularly in view of the differences noted above for the value of $\Delta H^{\circ}$/ATP calculated for the mitochondrial ATPase using the two types of method (see also Chapters 4, 7).
CHAPTER 2

OXIDATIVE PHOSPHORYLATION IN P. DENITRIFICANS

MEMBRANE VESICLES: A CHEMIOSMOTIC SYSTEM?
"Models of oxidative phosphorylation can therefore no longer be based on chloroplasts and mitochondria alone, but must be compatible with the full range of coupling modes found in the bacterial world. This approach from comparative biochemistry strengthens the inference that oxidative phosphorylation is an optional accessory to the energised state which must be chemically nonspecific and involve proton translocation". 

Harold (1978)

On strategies for studying biological systems

Both the stimulus-response characteristics of living systems, and the input-output data of biochemical experimentation are described by systems analysis (e.g. Basar 1976; Calow 1976). Biochemical science is characterised by a reductive approach, presumably as a result of the complexity of living systems. Fig. 2.1 presents such a reductionist strategy of research in electron transport phosphorylation, indicating how the essence of reductionism is the isolation of a system's components. Since a full description of a biochemical mechanism includes both thermodynamic and kinetic components, research strategies may correspondingly involve thermodynamic and kinetic isolation, and tests of biochemical mechanisms based on working hypotheses may be thermodynamic or kinetic in nature. The strategy followed in the present work is thermodynamic in essence, and involves the use of a cell-free preparation of membrane vesicles to assess the thermodynamic competence of ATP synthesis of the steady-state protonmotive force predicted by the chemiosmotic hypothesis.
CHEMICAL ISOLATION

Application of pharmacological agents, electron transport inhibitors, uncouplers, ionophores, energy-transfer inhibitors, substrates.

PROBLEMS: Specificity, pathways, primary/secondary effects.

PHYSICAL ISOLATION

Removal of parts of the system; production of cell-free systems, purified enzyme, lipoprotein complexes.

PROBLEMS: Artefactual changes in organisation.

Fig. 2.1
A reductionist strategy of experimental research in electron transport phosphorylation

The figure contrasts the 2 main reductionist approaches to understanding biological electron transport phosphorylation systems, and indicates the major problems with which each is associated. In the majority of the work described in this thesis physical isolation has been followed by chemical isolation. For a discussion of the problem of artefactual changes in membrane organisation during the preparation of *P. denitrificans* membrane vesicles, see text.
Structural and functional homogeneity and the characterisation of \textit{P. denitrificans} membrane vesicles

The fourth approach, mentioned in Chapter 1, to assessing the stoichiometry of bacterial oxidative phosphorylation is to use membrane vesicles and to assay their catalysis of phosphate esterification and oxygen uptake by standard biochemical techniques. This approach was first reported for \textit{P. denitrificans} NCIB8944 by John and Whatley (1970), who found a P/O ratio, with NADH as substrate, of about 1.5. However, as indicated in Fig. 2.1, the principal problem with this approach is to assess the extent to which disruption of the bacterial plasma membrane necessary in their preparation has decreased the P/O ratio measured with the vesicles. In particular, the P/O ratio measured in this way is an average property of the vesicles in the population, and it is to this problem of vesicle inhomogeneity that I now turn.

At the vesicular level, two classes of inhomogeneity may be identified: structural inhomogeneity ('sidedness') and functional inhomogeneity (a heterogeneous distribution of energy coupling properties). Since the \textit{P. denitrificans} plasma membrane is impermeable to both pyridine nucleotides (Scholes and Smith 1968b) and adenine nucleotides (see Chapter 1), and both the NADH dehydrogenase and ATPase molecules face the inside of the intact \textit{P. denitrificans} cell, NADH oxidation and ADP phosphorylation will be catalysed only by those vesicles which have inverted during preparation such that these enzymes face the medium. Three types of vesicle may in principle be formed upon disrupting the intact cell: first, right side-out (RSO) vesicles, in which
VESICLE TYPE

Right-side-out

(ISO)

Inside-out

(IS0)

'Scrambled'

Fig 2.2
Fig. 2.2

**Topological relationships in bacterial membrane vesicles**

The figure indicates the different types of membrane vesicles which may be characterised topologically, considering *P. denitrificans* as a specific example. The blocks containing the letters N and A refer respectively to the NADH dehydrogenase and ATPase enzymes of the plasma membrane. Two types (if only 2 enzymes are considered) of 'scrambled' membrane vesicles may be identified. For methods of characterisation, see text.
all components of the vesicle membrane have the same orientation as in the plasma membrane of the intact cell; secondly inside-out vesicles, in which complete inversion has occurred during their preparation so that the orientation of the vesicle membrane is the reverse of that of the plasma membrane of the intact cell; and finally 'scrambled' vesicles, in which some components of the plasma membrane change their relative orientation during vesicle preparation so that the vesicles are not simply related, in a topological sense, to the intact cell. These relationships are indicated in Fig. 2.2.

After growth under appropriate conditions, osmotic lysis of spheroplasts derived from _P. denitrificans_ cells produces a population of membrane vesicles which is believed to consist predominantly of inside-out vesicles (Burnell _et al._, 1975). In order to characterise their topology further, an affinity chromatographic technique was employed. Separations based on biological specificity, particularly those utilising immobilised ligands, have gained wide popularity in recent years (Jakoby and Wilchek 1974), and it was reasoned that the plasma membrane ATPase, which is expected to have a high affinity for ADP, would bind strongly to an affinity column containing immobilised agarose-ADP, thus causing the column to retain only the ISO vesicles in which the ATPase faces outwards.

Table 2.1 shows that when a sample of _P. denitrificans_ vesicles was applied to the affinity column, a fraction of the preparation (fraction A) was not retained by the column. Subsequent washing of the column with buffer did not cause
Table 2.1

<table>
<thead>
<tr>
<th>Fractions</th>
<th>mg protein</th>
<th>NADH dehydrogenase activity (nmol min(^{-1}) mg protein(^{-1}))</th>
<th>No Bee Venom</th>
<th>+ Bee Venom</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&lt; 0.05</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 + 3 pooled</td>
<td>0.42</td>
<td>15</td>
<td></td>
<td>107</td>
</tr>
<tr>
<td>4 + 5 pooled</td>
<td>0.10</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>6,7,8 pooled</td>
<td>&lt; 0.05</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>9,10,11 pooled</td>
<td>1.95</td>
<td>55</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>12 + 13 pooled</td>
<td>0.15</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>14 + 15 pooled</td>
<td>&lt;0.05</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1

Separation of *P. denitrificans* membrane vesicles using immobilised ADP

0.4 ml (3.0 mg protein) of a preparation of *P. denitrificans* membrane vesicles was applied to a 5 mm x 20 mm column containing 1 ml ADP-agarose preequilibrated with 10 mM Tris acetate/5 mM magnesium acetate pH 7.3. It was eluted with 2.5 ml of the same buffer. 0.5 ml fractions were collected, using an LKB Ultrorac fraction collector, at a gravity-induced flow rate of approximately 0.4 ml min$^{-1}$. After 5 fractions had been collected a pulse (0.5 ml) of 0.1 M ADP (sodium salt, pH 7.0) was applied to the column and further fractions collected. Fractions were pooled and assayed for protein and NADH dehydrogenase activities as described in Materials and Methods. The protein recovered represented about 85% of that applied to the column. The NADH dehydrogenase activities of the initial preparation were 48 (no bee venom) and 77 (plus bee venom) nmol min$^{-1}$ mg protein$^{-1}$. All operations were carried out at room temperature, except that NADH dehydrogenase activities were measured at 30°C.
elution of the retained vesicles, but when a pulse of ADP (sodium salt) was applied to the column, the retained vesicles (fraction B) were eluted (Table 2.1).

It has been suggested that discrimination between RSO and ISO vesicles may be made on the basis of the extent to which bee venom stimulates the NADH dehydrogenase of the vesicles (Burnell et al., 1975). As noted above, the plasma membrane is effectively impermeable to 1 mM NADH (Scholes and Smith 1968b) so that RSO vesicles are not expected to oxidise added NADH unless the permeability of the membrane to added NADH is increased, thus permitting access of NADH to its inwardly-facing dehydrogenase. Bee venom contains mellitin (see Burnell et al., 1975), a haemolytic agent with mild detergent properties, and this destroys the integrity of the plasma membrane, enabling access of NADH dehydrogenase in the RSO vesicles.

Table 2.2 shows the results of experiments with four different preparations of P. denitrificans vesicles in which the stimulation of NADH dehydrogenase by bee venom was examined in (a) the original vesicle preparation, (b) the vesicles (fraction A) that were not retained by the column, and (c) the vesicles (fraction B) that were eluted from the column by ADP. The stimulation of NADH dehydrogenase activity by bee venom was always very much greater in fraction A than in either the original vesicle preparation or in fraction B (Table 2.2). This suggests that fraction A is composed predominantly of RSO vesicles, whereas the ISO vesicles are retained by the column and eluted by ADP.
<table>
<thead>
<tr>
<th>Preparation</th>
<th>Sample</th>
<th>NADH dehydrogenase activity + Bee Venom</th>
<th>NADH dehydrogenase activity - Bee Venom</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fraction A</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fraction B</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Initial</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fraction A</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fraction B</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Initial</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fraction A</td>
<td>&gt;200</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fraction B</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Initial</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fraction A</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fraction B</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2

Effect of bee venom on NADH dehydrogenase activity of unfractionated and fractionated *P. denitrificans* membrane vesicles

NADH dehydrogenase activities were measured as described in Methods. Bee Venom was added to a final concentration of 50 µg ml⁻¹. Fraction A and Fraction B have the meanings indicated in the text.
However, it is apparent from Table 2.2 that there was significant variability in the proportions of RSO and ISO vesicles, both in the original preparations and in the chromatographic fractions. Only in preparation 3 was stimulation of NADH dehydrogenase by bee venom of fraction A as large as might be expected if the vesicles were entirely RSO. Furthermore, only in preparation 2 was no stimulation by bee venom of the NADH dehydrogenase activity of fraction B observed. These observations indicate that both fractions A and B (and thus the original vesicle preparation) are usually contaminated by scrambled vesicles in which molecules of ATPase or of NADH dehydrogenase have migrated topologically during the preparation of the vesicles. Thus the RSO (fraction A) vesicles might be contaminated by vesicles in which the NADH dehydrogenase but not the ATPase is outward-facing, thus giving NADH dehydrogenase activity before the addition of bee venom.

A stimulation by bee venom of the NADH dehydrogenase activity of the fraction B vesicles could be accounted for either if some NADH dehydrogenase molecules have everted so as to face the vesicle lumen in otherwise ISO vesicles or if some ATPase molecules have migrated to the outer surface of otherwise-RSO vesicles.

There has been much controversy regarding the orientation of membrane vesicles derived from bacterial cells by osmotic lysis (reviewed by Simoni and Postma 1975; Konings 1977). However, for the present purpose, it is sufficient to note that my preparations usually consist of approximately 40% inside-out vesicles as assayed by the
increase in NADH dehydrogenase activity caused by the addition of bee venom. In the present work, the vesicles were used without further purification by the affinity chromatographic method described above. Meijer et al., (1977b) have also reported that vesicles prepared from P. denitrificans using the method described in the present work consist of 40% ISO vesicles, as judged both by the bee venom assay and by freeze-fracture analysis (Meijer et al., 1977b).

As the present work is concerned with oxidative phosphorylation, only the ISO vesicles in a given population are of importance, and the RSO vesicles may be regarded as being essentially inert. This point is discussed further in Chapter 5.

Further work by John and coworkers (reviewed by John and Whatley 1977b) established the existence of a marked respiratory control by phosphate acceptor in these vesicles. The ADP/O ratio (Chance and Williams 1956) determined (John and Hamilton 1970) was identical to the P/O ratio, indicating that the vesicles were capable of phosphorylating essentially all of the added ADP, in contrast to the topologically analogous bovine heart submitochondrial particles (Chapter 4; Ferguson and Sorgato 1977). The two experiments presented next are based on this property of respiratory control by 'tightly coupled' vesicles (current hypotheses concerning respiratory control were indicated in Chapter 1; see also Boyer et al., 1977), both of which indicate that the inside-out P. denitrificans vesicles are not a significantly heterogeneous population with respect to their coupling properties. First, the ADP/O ratio of approximately 1.5 which is observed
Fig 2.3

RESPIRATION (μatom O/min/mgprot) vs. [HQNO] / μM
Fig. 2.3

Effect of HQNO on coupled and uncoupled respiration of *P. denitrificans* vesicles

Respiration was monitored polarographically in a reaction medium containing 10 mM Pi-Tris pH 7.3, 5 mM magnesium acetate, 1% (v/v) ethanol, 50 µg alcohol dehydrogenase and membrane vesicles (1.0 mg of protein) in a final volume of 3 ml. The temperature was 30°C. The particles were preincubated with the appropriate concentration of HQNO before initiating respiration by addition of 0.6 mM NAD⁺. 0.2 mM ADP (C–C–C–C–) or 0.5 µg gramicidin D plus 30 mM ammonium acetate (◇–◇–◇–◇–) were present as indicated. (After John and Ferguson 1975).
when NADH is substrate (John and Whatley 1975) means that even if the P/O ratio of fully coupled vesicles is 3, then only half of the vesicles can be non-phosphorylating. Secondly, the profile of sensitivity of respiration to the inhibitor HQNO (Fig. 2.3) (after John and Ferguson 1975) suggest that only a negligible fraction of the vesicles can be uncoupled. The rate of NADH oxidation in the vesicles in the presence of an uncoupler was inhibited strongly by increasing concentrations of HQNO whereas the rate of respiration in the absence of an uncoupler (state 4) (Chance and Williams 1956) was not affected over the same concentration range (Fig. 2.3). If a significant fraction of vesicles were naturally uncoupled the respiration by these vesicles in state 4 would have been partially sensitive to HQNO, as the state 4 rate would reflect the controlled rate of respiration by the coupled vesicles as well as the uncontrolled rate of respiration by the uncoupled vesicles. That the thermodynamic back-pressure of the high-energy intermediate of oxidative phosphorylation is partially released by ongoing phosphorylation, is indicated by the partial sensitivity to HQNO of the state 3 (Chance and Williams 1956) respiratory rate.

Thus, having indicated the nature of the problem of vesicle heterogeneity, and of experiments which suggest that this problem is not likely to be a significant one here, I shall describe the properties of respiratory control and oxidative phosphorylation in membrane vesicles from P. denitrificans.
The kinetics of NADH dehydrogenase in \textit{P. denitrificans} membrane vesicles

Van Verseveld \textit{et al.}, (1977) proposed that metabolism in heterotrophically grown \textit{P. denitrificans} cells was regulated at the level of phosphorylation at site I of the electron transport chain. Certainly the NADH dehydrogenase occupies an important place in the metabolism of this organism, and the reaction it catalyses could justifiably be regarded as a flux-generating step (Newsholme and Start 1973). On this basis (Newsholme and Start 1973), a subtle feedback system affecting both the \( K_m \) and the \( V_{\text{max}} \) of the enzyme might be expected. For this reason, the kinetics of the enzyme were investigated. In assays of oxygen uptake (e.g. Fig. 2.3), NADH was generated \textit{in situ}, and it was first established spectrophotometrically that, under the conditions routinely employed for the assay of oxygen uptake, more than two thirds of the NAD\(^+\) added existed as NADH in the steady state.

A progress-curve method (e.g. Johnston and Diven 1969; Atkins and Nimmo 1973; Thomas and Christian 1975; Markus \textit{et al.}, 1976) was adopted for reasons of convenience and accuracy. \( K_m \) and \( V_{\text{max}} \) data could be obtained from a single sample, using the following relationship based on an integrated form of the Michaelis-Menten equation:

\[
\frac{2.303 \log \left( \frac{S_0}{S_0 - P} \right)}{t} = \frac{V_m}{K_m} - \frac{1}{K_m} \frac{P}{t} \quad \text{Eq. 2.1}
\]

where \( S_0 \) is the initial substrate concentration, \( P \) is the product concentration and \( t \) is the time after substrate addition. \( S_0 \) was calculated from the final change in the
1 min

Time

$\text{OD}_{340}$

0.1 A

$60 \, \mu\text{M NADH}$

Fig 2.4
Fig. 2.4

Progress curve for NADH oxidation by _P. denitrificans_ vesicles, obtained spectrophotometrically

The OD₃₄₀ was monitored in a Pye Unicam SP 8000 spectrophotometer. The glass cuvette contained, in a final volume of 3 ml: 10 mM Pi-Tris, pH 7.3, 5 mM magnesium acetate and membrane vesicles (64 μg protein). At the time indicated NADH was added from a stock solution (made up freshly and stored in the dark). The initial concentration of NADH was calculated from the difference between the initial and final OD₃₄₀ values after addition of NADH using a millimolar extinction coefficient of 6.23.
\[
\frac{1}{t} \ln \frac{S_0}{S_0 - P}
\]

\(\text{min}^{-1}\)

0.5

0.01  0.02  0.03

\(P/t\)

(mmol. min\(^{-1}\). l\(^{-1}\))

Fig 2.5
Fig. 2.5

Secondary plot for the calculation of $K_m$ and $V_{\text{max}}$ values from progress curve of Fig. 2.4

Data of Fig. 2.4 are plotted out using equation 2.1 to give a slope equal to $-1/K_m$ and an intercept on the abscissa of $V_{\text{max}}$. 
OD$_{340}$ nm of the reaction mixture using a molar extinction coefficient for NADH of 6.22, and P was calculated by difference. A typical progress curve of coupled NADH oxidation by \textit{P. denitrificans} membrane vesicles is shown in Fig. 2.4. The analysis of this progress curve using equation 2.1 is plotted in Fig. 2.5, where it may be seen that a plot of $\frac{1}{t} \ln \frac{S_0}{S_0 - P}$ versus P/t gives good linearity, with a slope equal to $-1/K_m$ and an intercept on the abscissa equal to $V_{max}$. In this particular experiment values for $K_m$ and $V_{max}$ of 16 $\mu$M and 0.28 $\mu$mol NADH oxidised min.$^{-1}$ mg$^{-1}$ protein were obtained. Thus it appears that the enzyme obeys Michaelis-Menten kinetics; similar experiments under phosphorylating or uncoupled conditions (data not displayed) showed that the $K_m$ (but not, of course, the $V_{max}$) was independent of the level of uncoupling.

Commercial samples of NADH contain a potent inhibitor of some NADH-requiring enzymes, possessing similar spectral properties to NADH (e.g. Gallati 1976), and although this would not interfere with the calculation of $S_0$ (see above), it was not known whether the inhibitor was effective against NADH dehydrogenase. Since the inhibitor is said to be competitive with NADH (Gallati 1976), it was reasoned that a plot of the apparent $K_m$ obtained from progress curves versus the initial NADH concentration would resolve this question, for, if the inhibitor were effective against the enzyme, the apparent $K_m$ would increase with increasing NADH (and hence inhibitor) concentration. Such a plot is shown in Fig. 2.6,
APPARENT $K_m$ (µM)

0.5 1.0

INITIAL SUBSTRATE CONCENTRATION (millimolar)

Fig 2.6
Effect of initial substrate concentration on apparent $K_m$ for NADH dehydrogenase of *P. denitrificans* vesicles

Data were obtained from experiments similar to those described in Figures 2.3 to 2.5. An absence of an effect of concentration on the observed $K_m$ obtained from progress curve measurements is consistent with the notion that the 'oxidoreductase inhibitor' that is present in commercial NADH samples does not inhibit the NADH dehydrogenase enzyme.
where it may be seen that there was no significant effect of $S_o$ on the measured $K_m$ of approximately 20-25 μM. Thus the 'oxidoreductase inhibitor' does not inhibit the NADH dehydrogenase of _P. denitrificans_. This experiment also indicated that the dehydrogenase is not inhibited by its product NAD$, although such an inhibition would not be expected on thermodynamic grounds alone (Dutton and Wilson 1974).

The progress curve method described above was also applied to the forward reaction catalysed by the pyridine nucleotide transhydrogenase of these vesicles:

\[ \text{NADPH} + \text{NAD}^+ \rightarrow \text{NADH} + \text{NADP}^+ \quad \text{Eq. 2.2} \]

which under the conditions of the assay is coupled to the NADH dehydrogenase reaction:

\[ \text{NADH} + \text{H}^+ + \frac{1}{2}\text{O}_2 \rightarrow \text{NAD}^+ + \text{H}_2\text{O} \quad \text{Eq. 2.3} \]

It was found (data not shown) that the effective reaction

\[ \text{NADPH} + \text{H}^+ + \frac{1}{2}\text{O}_2 \rightarrow \text{NADP} + \frac{1}{2}\text{H}_2\text{O} \quad \text{Eq. 2.4} \]

has an apparent $K_m$ for NADPH of 1.7(2) mM. It is concluded that this latter reaction is not of physiological significance, for the free NADPH concentration in _P. denitrificans_ cells (London and Knight 1966) is very much lower.

The effect of ionophores on respiratory control in _P. denitrificans_ membrane vesicles

John and Hamilton (1971) studied the effects on the coupled respiration of _P. denitrificans_ membrane vesicles of ion-translocating antibiotics of the valinomycin and nigericin groups (review: Pressman 1977) in the presence and absence of various salts. The effects of the antibiotics on energy-
transducing membrane systems have been widely interpreted in terms of their ability to increase the permeability of both natural and artificial membranes to specific cations (e.g. Montal et al., 1970; Harold 1972; Hamilton 1975) and their mode of action has been interpreted as indicating the importance of chemiosmotic phenomena in biological energy transduction. John and Hamilton (1971) found that valinomycin plus K⁺ ions did not stimulate particle respiration unless either nigericin or an ammonium salt was present, under incubation conditions in which all anions present (acetate) could not cross the particle membrane. They interpreted these results as consistent with the proposition that the (NADH-oxidising) vesicles were predominantly ISO. I have extended this work using the above ion-translocating antibiotics with a variety of potassium salts; the results are interpreted to indicate that the transmembrane permeability of the anion present both alters and may be predicted from the effects of these compounds on respiratory control.

Figs. 2.7 - 2.9 illustrate the release of respiratory control by these antibiotics in the presence of potassium acetate, ammonium acetate or potassium thiocyanate. It may be seen that Nigericin stimulates respiration only if a membrane permeable ion is present, e.g. potassium plus valinomycin (Fig. 2.7) or thiocyanate (Fig. 2.9). Evidence that this thiocyanate permeates the P. denitrificans plasma membrane has been given by Scholes and Mitchell (1970b). A similar series of experiments (data not displayed) using other potassium salts indicated that chloride was slightly permeable
POTASSIUM ACETATE CONCENTRATION (millimolar)

Fig 2.7
Release of respiratory control of P. denitrificans vesicles by ionophores and potassium acetate

Respiration was monitored polarographically in a 3 ml reaction mixture containing 10 mM Pi-Tris pH 7.3, 5 mM magnesium acetate, 1% v/v ethanol, 0.1 mg alcohol dehydrogenase, 1 ug valinomycin, 520 μg vesicle protein, and the appropriate concentration of potassium acetate (O, □). Respiration was initiated with 0.6 mM NAD⁺. The closed symbols indicate experiments in which nigericin was also present (1 μg). Nigericin alone (with no valinomycin present) exerted only a minimal stimulation of respiration (data not shown).
ADDITIONAL TEXT...
Fig. 2.8

Effect of ammonium acetate and ionophores on controlled respiration of G. denitrificans vesicles

Respiration was monitored polarographically in a 3 ml reaction mixture containing 10 mM Pi-Tris pH 7.3, 5 mM magnesium acetate, 1% v/v ethanol, 0.1 mg alcohol dehydrogenase, 0.39 mg vesicle protein and the appropriate concentration of ammonium acetate. Respiration was initiated by the addition of 0.6 mM NAD⁺ (O, ☯). Further additions of valinomycin (1 µg) or valinomycin plus nigericin (1 µg each) were made as indicated (☯).
Fig 2.9
Fig. 2.9

Effect of potassium thiocyanate and ionophores on controlled respiration of _P. denitrificans_ vesicles

Respiration was monitored polarographically in a 3 ml reaction mixture containing 10 mM Pi-Tris pH 7.3, 5 mM magnesium acetate, 1% v/v ethanol, 0.1 mg alcohol dehydrogenase, 0.39 mg vesicle protein and the appropriate concentration of potassium thiocyanate. Respiration was initiated by the addition of 0.6 mM NAD⁺ (○, ●). Nigericin (1 μg) was either present (●) or absent (○). Valinomycin addition (1 μg) did not further influence these activities.
Fig 2.10
Fig. 2.10

A chemiosmotic explanation of the role of solutes and ionophores in releasing the respiratory control of *P. denitrificans* vesicles

The figure represents 3 situations in which a vesicle containing a protonmotive electron transport system, $H^+$, is exposed to various types of ionophore and permeant base. According to the chemiosmotic viewpoint uncoupling results from a dissipation of both the pH gradient and transmembrane potential terms of the protonmotive force which is set up upon initiation of respiration. Thus full uncoupling requires the release of both these components of the protonmotive force.

In case A, when no permeant anions are present, nigericin (N) acts to catalyse an electroneutral exchange of $K^+$ for $H^+$ (Ashton and Steinrauf 1970). This replaces the pH gradient by an increased membrane potential. Thus in case A valinomycin (V), which catalyses an electrogenic transmembrane movement of potassium ions, is required to express the uncoupling effect of nigericin (cf. Fig. 2.7). In case B, the valinomycin is shown to be effectively replaced by a permeant anion $A^-$, which is concentrated in the vesicle lumen in the act of dissipating the membrane potential generated by electron transport. Thus nigericin plus a permeant anion cause full uncoupling (or release of respiratory control) (Fig. 2.9).

In case C, nigericin is replaced, in its ability to remove the pH gradient component of the protonmotive force, by a weak base B, which can permeate only in the uncharged form, and which removes protons. Again no uncoupling or release of respiratory control is seen unless the membrane potential component of the protonmotive force is removed, either by
Fig. 2.10 (continued)

valinomycin (Fig. 2.8) or by a permeant anion (Fig. 2.11; case C).

It should be noted that these explanations apply only to vesicles in which respiration causes the vesicle lumen to become positive and acidic relative to the external phase. Such an orientation is characteristic of P. denitrificans vesicles respiring on NADH.
RESPIRATION RATE (patm/min/mg protein)

METHYLMELINE HYDROCHLORIDE CONCENTRATION (millimolar)
Synergistic release of respiratory control of \textit{P. denitrificans} membrane vesicles by the weak base methylamine and the permeant thiocyanate ion

Respiration was monitored polarographically in a 3 ml reaction mixture containing 10 mM Pi-Tris pH 7.3, 5 mM magnesium acetate, 1% v/v ethanol, 0.1 mg alcohol dehydrogenase, the appropriate concentration of methylamine and 0.6 mM NAD$^+$ (O, \textcircled{1}). In some experiments (O) 10 mM potassium thiocyanate was also present. The rate of respiration when 0.2 mM ADP was included in the reaction mixture is also indicated (A). The slight uncoupling effect of methylamine hydrochloride alone, in the absence of thiocyanate, is ascribed to a limited, but finite, permeability of the vesicle membrane to the chloride ion.
and that the tetraphenylborate (TPB) and tetrafluoroborate ions were as permeable as thiocyanate. The chemiosmotic interpretation, that the steady-state transmembrane electrochemical proton gradient is responsible for respiratory control (Mitchell 1966a, b), is diagrammed in Fig. 2.10. Thus neither antibiotic is expected to stimulate the (already uncoupled) particle respiration in the presence of potassium acetate and ammonium thiocyanate (10 mM each), as is indeed observed. The ability of the weak base, methylamine, and the permeant thiocyanate ion to act synergistically in releasing control in the absence of membrane-active ionophores (Fig. 2.11) is similarly explained (Fig. 2.10). Thus, (Fig. 2.11) although 10 mM potassium thiocyanate has no effect on coupled respiration (Fig. 2.11), when respiration has been partially uncoupled by the weak base methylamine (as the hydrochloride) the thiocyanate does act to release the respiratory control of the P. denitrificans vesicles.

The transmembrane distribution of permeant ions and weak acids and bases (at low concentrations) may be used as a probe of the components of the protonmotive force, and such measurements have been widely reported (review: Rottenberg 1975). In my opinion, hydrophobic ion-distribution methods are the only methods currently available which may be used to give a quantitative estimate of the bulk transmembrane electrochemical proton gradient defined by Mitchell (1966a, 1968). In view of the ability of thiocyanate and methylamine to act synergistically in releasing the respiratory control of the P. denitrificans vesicles, it was decided to utilize these compounds to undertake a series of measurements of the value
of the protonmotive force across the vesicles under different conditions. The rationale behind these methods, and the way in which they may be used to give a quantitative measure of the components of the protonmotive force is described in the next chapter, in which the determination of the protonmotive force generated by *Rhodospirillum rubrum* chromatophores is also described. The methods developed were also used with bovine heart submitochondrial particles (Chapter 4) before being applied (Chapter 5) to the phosphorylating *P. denitrificans* membrane vesicles.

**Methods**

**Culture of bacteria**

*Paracoccus denitrificans* NCIB 8944 (Torry Research Station, Aberdeen) was maintained as a stab culture in a medium containing (per litre) 4 g bactopeptone (Difco), 2 g yeast extract (Difco), 10 g K$_2$HPO$_4$, 10.1 g KNO$_3$ and 13.5 g sodium succinate hexahydrate, solidified with 2% purified agar. The pH was adjusted to 6.8 with sulphuric acid prior to sterilisation by autoclaving at 120 lb in $^{-2}$. Inocula were taken from these agar slants, inoculated into and subcultured in 25 or 100 ml 'succinate' medium of the following composition (per litre). 0.68 g KH$_2$PO$_4$ adjusted to pH 6.8 with KOH; 0.60 g (NH$_4$)$_2$HPO$_4$ adjusted to pH 6.8 with H$_2$SO$_4$; 0.1 ml of 'modified Hoagland's trace element solution' (Collins 1969); 12 mg EDTA, ferric monosodium salt; 25 mg CaCl$_2$.2H$_2$O, 25 mg MgSO$_4$.7H$_2$O, 13.5 g sodium succinate hexahydrate, 10.1 g KNO$_3$. Cells for use were grown from these subcultures in 21 flasks filled with the same medium.
Single-glass-distilled water was used throughout, and all cells were grown at 30°C. Growth was monitored by measuring the optical density at 550 nm in a Beckman DB spectrophotometer. The OD\textsubscript{550} was related to bacterial dry weight by a standard gravimetric method. 3 determinations gave a mean value (±S.E.M.) of 0.50 (±0.03) mg dry weight ml\textsuperscript{-1} = OD\textsubscript{550} 1.0.

**Preparation of membrane vesicles**

The procedure used in the preparation of phosphorylating membrane vesicles is based on a method described by John and Whatley (1970). All solutions and apparatus used were maintained at 1-4°C and all operations except the lysozyme treatment were carried out at these temperatures. Stock buffer solutions at a concentration of 0.4 M were adjusted to a pH of 7.3 at room temperature, and then diluted to 10 or 100 mM as required.

Cells from 2 litres of a mid-exponential phase (OD\textsubscript{550} = 1.7-1.9) culture in 'succinate-nitrate' medium were sedimented by centrifugation at 5000 x g for 30 min and were washed in 800 ml of 150 mM NaCl containing 10 mM Tris-HCl buffer. The cells were then suspended in 400 ml of 0.5M sucrose containing 10 mM Tris-HCl buffer, so that a 0.1 ml sample of the suspension diluted to 2.5 ml with water had an absorbance at 550nm of 0.3 when read in a Beckman DB spectrophotometer. Lysozyme was added at a concentration of 250 µg ml\textsuperscript{-1} to this suspension which was then left in a water bath at 30°C for 20-30 min until the absorbance of a sample diluted 1:25 with water decreased from 0.3 to 0.05.
After treatment with lysozyme the cells were sedimented by centrifugation at 40,000 x g for 10 min and resuspended in 40 ml of 100 mM Tris-acetate buffer (containing 10 mM sodium ATP pH 7.0) by using a Potter-Elvehjem homogeniser. The suspension was then poured into 360 ml water to disrupt the cells. The suspension was left for 20 min, then a trace of deoxyribonuclease (DNase) and 2 mM magnesium acetate were added. The suspension was shaken gently to distribute the magnesium acetate. When the viscosity of the suspension was sufficiently lowered, the suspension was centrifuged at 40,000 x g for 50 min to yield a double-layered pellet and a clear supernatant, which was discarded. The upper, red, layer of the pellet was resuspended in 400 ml of 10 mM Tris-acetate buffer containing 1 mM magnesium acetate. The lower, white, layer, which probably consists of poly-β-hydroxybutyrate (Scholes and Smith 1968a), was discarded. The suspension was centrifuged at 40,000 x g for 40 min and the resulting pellet was resuspended in 10 mM Tris-acetate containing 1 mM magnesium acetate to a concentration of about 5-10 mg protein ml⁻¹ (about 5-10 ml). The suspension of membrane vesicles was stored at 1-4°C and could be used for experiments over a period of 4-5 days.

Determination of NADH dehydrogenase activity

The oxidation of NADH was linked to the reduction of 2,6-dichlorophenol indophenol and the initial rate in decrease in E₆₀₀ monitored spectrophotometrically at 30°C in a Pye Unicam SP8000. The reaction mixture contained in 3 ml: 50 mM Tris-Cl pH 8.0, 33 μM 2,6-dichlorophenolindophenol and 2 mM KCN pH 7.5. The reaction was started by the addition of 1 mM NADH, followed by membrane vesicles (approximately 0.1 mg protein) and bee venom (150 μg). The small rate of NADH-
dependent 2,6-dichlorophenylindophenol reduction sometimes observed in the absence of vesicles was subtracted from the rates observed in the presence of membrane vesicles to give a measure of the NADH dehydrogenase activity. For a population lacking 'scrambled' vesicles and consisting only of RSO and ISO vesicles, the percentage of vesicles of ISO configuration is given by the relationship.

\[
\frac{\text{% ISO vesicles}}{\text{NADH dehydrogenase activity before bee venom}} = \frac{x \times 100}{\text{NADH dehydrogenase activity after bee venom}}
\]

\text{Eq. 2.5}

The molar extinction coefficient for 2,6-dichlorophenolindophenol was taken to be 21 000 litre. mol\(^{-1}\). cm\(^{-1}\) (King 1963). Protein was determined by the Folin method of Lowry et al., (1951) with bovine serum albumin (Cohn fraction V) as standard. Determinations were carried out on 30- or 50-fold dilutions of the membrane vesicle suspension in water to minimise possible interference by Tris and to bring the protein concentration into the range required for this assay.

\(N^\delta(6\text{-aminohecanoyl})\ ADP\text{-agarase} \) was obtained from Sigma (Cat. No. A1642).
CHAPTER 3

MEASUREMENT BY A FLOW DIALYSIS TECHNIQUE OF THE STEADY-STATE PROTONMOTIVE FORCE IN CHROMATOPHORES FROM RHODOSPIRILLUM RUBRUM; COMPARISON WITH THE PHOSPHORYLATION POTENTIAL
'There is little doubt that in chromatophores sufficient energy can be preserved in an electrochemical hydrogen gradient to drive the synthesis of ATP'.

Jones (1977)

INTRODUCTION

Chromatophores derived from photosynthetic bacteria, which are topologically analogous to the *P. denitrificans* membrane vesicles described in the previous chapter, are a useful system for studying the mechanism of coupling between electron flow and ATP synthesis in biological membranes. Much experimental data has accrued to indicate that this coupling involves a transmembrane electrical (Jackson and Crofts 1969) potential difference ($\Delta \Psi$), and a transmembrane pH gradient ($\Delta \text{pH}$) (Casadio et al., 1974a; Jones 1977) as the two components of the protonmotive force that is defined in the chemiosmotic hypothesis (Mitchell 1966a). Indeed, chromatophores from *Rhodopseudomonas capsulata* are the only energy transducing system for which evidence has been obtained (Casadio et al., 1974a) for a sufficiently large protonmotive force to account for the extent of ATP synthesis on the basis that two $\text{H}^+$ are translocated across the membrane for each ATP molecule synthesised, as originally suggested by Mitchell (1966a). However, the methods used for measuring the membrane potential and the pH gradient, respectively the extent of the carotenoid band shift and the quenching of 9-amino acridine fluorescence, are not universally accepted as quantitative indicators of
these two parameters (Piolet et al., 1974; Rottenberg 1975; Baltscheffsky 1976; Kraayenhof et al., 1976; Searle et al., 1977).

The more direct, and more generally acceptable, method for determining the membrane potential across energy transducing membranes is to measure the distribution of an appropriately charged permeant ion between the lumen of the membrane vesicle and the suspending medium, while the pH gradient is determined from the distribution of a weak acid or base across the membrane (Rottenberg 1975). Schuldiner et al., (1974) have already employed these methods for illuminated chromatophores from Rhodospirillum rubrum, using thiocyanate as permeant ion and methylamine as a weak base, but were hampered by the difficulty of separating chromatophores rapidly from the suspending medium, for which precipitation of the chromatophores with protamine sulphate was necessary. In the present work this difficulty was overcome by using a flow dialysis technique to monitor the concentration of either thiocyanate ions or methylamine in reaction mixtures containing Rhodospirillum rubrum chromatophores. This technique allows the extent of uptake of these two species into the chromatophores to be followed without separating the chromatophores from the reaction medium. It was introduced by Colowick and Womack (1969) and applied more recently to studies of bacterial membrane vesicles (Ramos et al., 1976; Ramos and Kaback 1977). The main aim was to compare the size of the protonmotive force measured using the flow dialysis technique with the magnitude of the phosphorylation potential that the chromatophores could generate. A diagram of the flow dialysis cell used
FLOW DIALYSIS

- radioactive species
- membrane vesicles
- dialysis membrane
- pump
- buffer
- fraction collector
- magnetic stirrer
A flow dialysis cell

The figure illustrates schematically the design of the flow dialysis cell used in the present work. The principle of the method is that the rate of flow of a (radioactive) species from the upper to the lower chamber, and hence its concentration in the outflow from the lower chamber, is proportional to the free solute concentration in the upper chamber. Thus, if membrane vesicles are also present, and energisation causes them to take up a certain amount of the radioactive species present, the concentration of free solute will fall, and thus the concentration in the outflow from the lower chamber will faithfully reflect this, without the need to separate the vesicles from the incubation medium.
Calculation of $\Delta \psi$ and $\Delta \text{pH}$

$\Delta \psi$ was measured assuming that thiocyanate ions passively equilibrate across the membrane so that there is no electrochemical potential difference for the thiocyanate ion across the chromatophore membrane. In this case, from the Nernst equation,

$$\Delta \psi = \frac{RT}{zF} \ln \frac{[\text{thiocyanate}]_{\text{in}}}{[\text{thiocyanate}]_{\text{out}}} \quad \text{Eq. 3.1}$$

The concentration terms, $[\text{thiocyanate}]_{\text{in}}$ and $[\text{thiocyanate}]_{\text{out}}$ are obtained from the amount of thiocyanate uptake and an estimate of the internal volume of the chromatophores.

$\Delta \text{pH}$ was evaluated from the extent of methylamine uptake. As the $\text{pK}_a$ of methylamine ($10.47$) is substantially greater than the pH on either side of the membrane then (Rottenberg 1975)

$$\Delta \text{pH} = \log \frac{[\text{methylamine}]_{\text{in}}}{[\text{methylamine}]_{\text{out}}} \quad \text{Eq. 3.2}$$

Again the internal volume must be known. Details of this determination are given in Table 3.1. Use of equations 3.1 and 3.2 assumes equal activity coefficients for both methylamine and thiocyanate ions on each side of the membrane.

Determination of the internal volume of the chromatophores

The internal volume of the chromatophores was estimated by a modification of the sucrose impermeable space method.
Table 3.1

Determination of the internal volume of R. rubrum chromatophores

<table>
<thead>
<tr>
<th></th>
<th>cpm 1st supernatant</th>
<th>cpm 2nd supernatant</th>
<th>( V_1 ) (( \mu l ) mg(^{-1}) Bchl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td>( ^{14}C )</td>
<td>50 469</td>
<td>1 713</td>
</tr>
<tr>
<td></td>
<td>( ^3H )</td>
<td>270 215</td>
<td>11 388</td>
</tr>
<tr>
<td></td>
<td>( ^{14}C )</td>
<td>57 637</td>
<td>5 983</td>
</tr>
<tr>
<td>Expt 2</td>
<td>( ^3H )</td>
<td>354 097</td>
<td>46 053</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>49.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>52.2</td>
</tr>
</tbody>
</table>
Table 3.1 Determination of the internal volume of *R. rubrum* chromatophores

Chromatophores were suspended in the reaction medium given in Fig. 3.2 with approximately 0.15 μCi 2-¹⁴C·³H₂O to a final volume of 5 ml containing 0.82 (expt 1) or 2.52 (Expt 2) mg bacteriochlorophyll. The mixtures were centrifuged at 100 000 x g in a Spinco 50 rotor at 4°C for one hour. One ml aliquots of the supernatant were counted for radioactivity in 9 ml of triton-toluene scintillant (Turner 1969). The pellets of the chromatophores were dried by removing as much as possible of the remaining supernatant by suction. Then the pellets were resuspended to a volume of 5 ml with homogenisation in the original medium lacking the radioactive components, and centrifuged as above. One ml aliquots of the supernatant were again counted for radioactivity. This protocol avoids counting radioactivity in the pellet from the first spin, and thus no correction for differential quenching of ³H and ¹⁴C counts is required.

The channels on the liquid scintillation counter were set such that ³H registered only in channel A in which ¹⁴C was counted with an efficiency of about 93%. The efficiency of counting of ¹⁴C in channel B was approximately 42%. A standard containing only ¹⁴C was run so as to obtain the factor by which the ¹⁴C counts in channel B had to be multiplied to obtain the ¹⁴C counts in channel A. The ³H counts in channel A were thus calculated by subtracting the ¹⁴C counts in channel A (calculated from the ¹⁴C counts in channel B) from the total counts in channel A.

\[ V_1 \text{, the specific internal volume of the chromatophores in } \mu\text{l} \cdot \text{mg}^{-1} \text{ bacteriochlorophyll, is given by:} \]
\[ V_i = \frac{5000}{x} \left[ \frac{\text{cpm } ^3\text{H in 2nd supernatant}}{- \text{cpm } ^{14}\text{C in 2nd supernatant}} - \frac{\text{cpm } ^3\text{H in 1st supernatant}}{\text{cpm } ^{14}\text{C in 1st supernatant}} \right] \]

where \( x \) - mg bacteriochlorophyll in the 5 ml starting reaction mixture. The results from the 2 experiments given are typical. From a series of determinations the mean and S.D. for \( V_i \) were 49.8 and 4.3 \( \mu \)l mg\(^{-1}\) bacteriochlorophyll.
INTERNAL VOLUME OF VESICLES (μl/ml reaction mixture)

Fig 3.2
Fig. 3.2  **Nomograph for calculating transmembrane ion gradients from flow dialysis measurements**

The ordinate represents the external ligand concentration as a percentage of the concentration before membrane energisation. The abscissa represents the enclosed volume of the membrane vesicles in $\mu\text{l} \text{ml}^{-1}$ reaction mixture.

The values of the lines of isopotential are in mV at 30°. The equation from which the nomograph is derived may be found in Kamo et al., (1976).
(e.g. Papa et al., 1973a), which gave a mean value \textpm S.E.M. of $50 \pm 1.6 \mu l$ per mg bacteriochlorophyll (Bchl) at the osmolarity of 65 milliosmolar that was used in the present work (Table 3.1). This volume is close to that obtained by others for chromatophores from \textit{R. rubrum} (Jackson et al., 1968; Schuldiner et al., 1974; Leiser and Gromet-Ethanan 1977).

It is evident from equations 3.1 and 3.2 that an accurate determination of the internal volume of the chromatophores is critical for the quantitative estimation of $\Delta \psi$ and $\Delta \text{pH}$ across the membrane. For this reason it is instructive to compare the experimentally determined value with the internal volume obtained by direct calculation. If it is assumed that there are between 2000 and 4000 bacteriochlorophyll molecules per chromatophore (Nishimura 1970; Jackson and Crofts 1971; Saphon et al., 1975b) and that the average chromatophore radius is 30 nm an estimate of between 40 and 20 $\mu l$ per mg bacteriochlorophyll is obtained. Such calculated values are in good agreement with our experimentally determined value.

A more recent value (Jackson, J.B. personal communication; Packham et al., 1978) of 1400 Bchl per chromatophore is in exact agreement on the above basis with the value of 50 $\mu l$ mg$^{-1}$ Bchl determined by the sucrose-impermeable-space method. In the present work I have assumed that the chromatophore volume remains constant upon illumination as there are no changes in light-scattering by chromatophores under these conditions (A.R. Crofts, unpublished, cited in Casadio et al., 1974b). Fig. 3.2 shows how the potential calculated from equation 3.1 depends upon the internal volume of the chromatophores at a given decrease in the external concentration of thiocyanate ions. Underestimation by a factor of two of the
internal volume will increase the value of $\Delta \psi$ calculated from equation 3.1 by only 18 mV. Similar considerations apply to the determination of $\Delta \mathrm{pH}$ from the uptake of methylamine into chromatophores whose inside is acidic relative to the outside.

**Determination of $\Delta \psi$**

Fig. 3.3 shows the thiocyanate concentration, monitored by counting radioactivity, in the outflow from the flow dialysis cell. Thiocyanate was added to the upper compartment of the cell either in the presence or in the absence of chromatophores. Before the cell was illuminated the amount of thiocyanate equilibrating across the dialysis membrane was similar whether or not chromatophores were present. Thus, in the dark, there appears to be neither a significant binding of thiocyanate to chromatophores, nor a detectable Donnan potential across the chromatophore membranes. The flow rate through the lower chamber of the flow dialysis cell was 2 ml min$^{-1}$ and thus it can be seen from Fig. 3.3 that equilibrium across the dialysis membrane was attained after about 3 min.

Illumination of the chromatophores resulted in a marked decrease in the thiocyanate concentration in the outflow from the cell, and a new equilibrium of thiocyanate ions across the dialysis membrane was reached (Fig. 3.3). This result indicates that thiocyanate ions were taken up by the illuminated chromatophores, thus lowering the free external thiocyanate concentration in the upper chamber of the cell. The thiocyanate ions were released from the chromatophores when FCCP was added (Fig. 3.3). From a series of eleven experiments an
Fig 3.3

\[10^{-3} \times \text{cpm}\]

\textit{Fraction No.}
Flow dialysis was performed as described in Methods. The radioactivity measured in the outflow of the flow dialysis cell is plotted against the fraction number. Reaction mixtures contained in a final volume of 1 ml: 10 mM P-i-Tris (pH 8.0), 5 mM magnesium acetate, 20 mM sucrose, 0.2 mM sodium succinate. At time zero, 10 μl of 2.08 mM \(^{14}C\) KSCN (60 mCi mmol\(^{-1}\)) were added to the upper chamber and the flow started. After collecting fraction 10 of the outflow from the lower chamber, the light was turned on (open arrow), and after fraction 20 had been collected, 1 μl of 2.5 mM FCCP was added to the upper chamber (second arrow).

- No chromatophores
- Plus chromatophores (0.18 mg bacteriochlorophyll)
average light-dependent $\Delta \Psi$ of 100 mV (S.D. = ± 9 mV) was obtained. After addition of FCCP the ratioactivity in the outflow corresponded to an extrapolation of the radioactivity found in the fractions of the outflow that were collected before the chromatophores were illuminated (Fig. 3.3). Hence the uptake of thiocyanate ions into chromatophores is fully reversible.

Fig. 3.3 also shows that uptake of thiocyanate into the chromatophores was complete within five minutes of illumination. It is noteworthy that very little thiocyanate was lost from the upper chamber during this period so that the rate of thiocyanate uptake by the chromatophores is faster than the rate of dialysis (cf. Chapter 6).

The light-dependent $\Delta \Psi$ was reduced by between 15 and 20 mV (data not shown) when ADP and $P_i$ were present so as to permit ATP synthesis. This observation indicates that thiocyanate uptake is responsive to the magnitude of $\Delta \Psi$, since ATP synthesis is known to lower the steady state proton-motive force in several systems including chloroplasts (Pick et al., 1973) and mitochondria (Nicholls 1974; Rottenberg 1975) as well as in R. rubrum chromatophores (Leiser and Gromet-Elhanan, 1977).

The extent of thiocyanate uptake was not increased by addition of oligomycin to the chromatophores, in harmony with the results of Leiser and Gromet-Elhanan (1977) who found that oligomycin did not cause any additional energy-linked enhancement of anilinonaphthalene sulphonate fluorescence with R. rubrum chromatophores.
Fig. 3.4  Effect of varying the $^{14}C$ thiocyanate concentration on the proportion of thiocyanate taken up by illuminated chromatophores.

The reaction conditions were as in the legend to Fig. 3.3, except that the concentration of radioactive thiocyanate used to start the reaction was varied.

(A) The initial thiocyanate concentrations in the upper chamber (constant specific activity) were 5.2 μM (○—○), 10.4 μM (▼—▼), 20.8 μM (▽—▽), 31.2 μM (□—□), 41.6 μM (△—△) and 52.0 μM (▲—▲). Open arrow: light on. Closed arrow: light off.

For clarity, the chromatophore-free controls have been omitted.

(B) Data of (A) replotted to show that a constant fraction of the total thiocyanate added is taken up by the chromatophores, over the whole range of thiocyanate concentrations tested. (○—○) represents the radioactivity in fraction 16 as a function of the total added thiocyanate concentration. (●—●) represents the radioactivity that was found in fraction 16 of the chromatophore-free controls, or calculated by interpolation of the counts found in fractions 8 and 28 so as to obtain the value which would be expected for fraction 16 in the dark. As may be seen in Fig. 3.2, the constancy of the fraction taken up indicates that a constant potential is formed across the chromatophore membrane.
Fig. 3.5  **Effect of antimycin on $\text{C}^{14}$ thiocyanate uptake by chromatophores.**

The reaction mixture was as in the legend to Fig. except that the chromatophore concentration was 0.15 mg bacteriochlorophyll ml$^{-1}$ and antimycin (0.37 mg µg$^{-1}$ bacteriochlorophyll) was present ($\circ-\circ-\circ$). ($\bigstar-\bigstar-\bigstar$) shows a similar experiment in which antimycin was present at the same concentration and 0.1 mM phenazine methosulphate was also added.
Fig. 3.4 shows some of the results from an experiment in which the uptake of thiocyanate ions into chromatophores was monitored over a ten-fold range of initial thiocyanate concentration. The percentage of thiocyanate taken up in the light was similar at all thiocyanate concentrations tested. Therefore it appears that thiocyanate is not binding to the membrane, which would be a saturable effect, but rather that it is taken up into the chromatophore lumen. Further evidence (not shown) that thiocyanate taken up is not bound was obtained by determining that the measured $\Delta \Psi$ was independent of the chromatophore concentration over the range 0.05 to 0.25 mg bacteriochlorophyll ml$^{-1}$ at a constant thiocyanate concentration. Whole cells of *R. rubrum* would presumably contain the same putative membrane binding sites for the permeant thiocyanate ion as chromatophores. Illumination of cells of *R. rubrum* did not drive an uptake of thiocyanate ions (data not shown), thus confirming that thiocyanate uptake occurs only into membrane preparations (chromatophores) that have an opposite polarity to whole cells, and that light-induced binding of thiocyanate to the membrane is significant (cf. Scholes *et al.*, 1969).

When antimycin (0.37 µg, µg$^{-1}$ bacteriochlorophyll) was present, no light-dependent uptake of thiocyanate into chromatophores was observed unless the site of antimycin inhibition was bypassed through addition of phenazine methosulphate (0.1 mM) (Fig. 3.5).

Hydrolysis of ATP by the chromatophores in the dark caused an uptake of thiocyanate that corresponded to a $\Delta \Psi$ of 80mV.
Flow dialysis was performed as described in Methods except that light was excluded throughout. The reaction mixture in a final volume of 1 ml: 10 mM Tris-acetate (pH 8.0), 10 mM magnesium acetate and 10 mM ATP (sodium salt, pH 8.0). The flow rate was 1 ml min\(^{-1}\), and the reaction was initiated by adding thiocyanate as described in the legend to Fig. 2. Two µl of 2.5 mM FCCP were added where indicated by the arrow.

- O O No chromatophores
- • O Plus chromatophores (0.12 mg bacteriochlorophyll)
Fig. 3.7  

Uptake of $\lambda$-methylamine by R. rubrum chromatophores

Conditions as in Fig. 2 except that the radioactive thiocyanate was replaced by 10 ul of 2.25 mM $\lambda$-methylamine hydrochloride (55.5 mCi mmol$^{-1}$).

•—• No chromatophores

○—○ Plus chromatophores (0.12 mg bacteriochlorophyll)

▲—▲ Plus chromatophores (0.12 mg bacteriochlorophyll) plus 10 mM unlabelled KSCN

Open arrow: light on.  Closed arrow: light off.
As shown in Fig. 3.6 this determination was made by adding ATP at the beginning of the experiment, and subsequently adding an uncoupler so that $\Delta \psi$ was determined from the thiocyanate released. It was found that the addition of ATP (as its sodium salt) to the upper chamber of the flow dialysis cell altered the rate of flow of thiocyanate across the dialysis membrane irrespective of whether chromatophores were also present.

The addition of nitrate ions (as 10 mM KNO$_3$) decreased the light-dependent $\Delta \psi$ from 103 mV to 83 mV, estimated from the decrease in thiocyanate uptake into chromatophores, a result consistent with previous work which has shown that the nitrate ion is permeable to chromatophores and thus reduces the membrane potential (Jackson and Crofts 1971; Pick and Avron 1976).

**Determination of $\Delta \text{pH}$**

Under those conditions that were routinely employed to measure $\Delta \psi$ (20 $\mu$M thiocyanate present), there was no observable light-dependent methylamine uptake (Fig. 3.7). However, on addition of 10 mM KSCN (unlabelled), methylamine uptake into the chromatophores corresponding to a $\Delta \text{pH}$ of 1.6 units (acid inside) was observed (Fig. 3.7). Addition of thiocyanate ions to chromatophores from *R. rubrum* is known to stimulate light-induced proton uptake (Gromet-Elhanan and Leiger 1973), but the data of Fig. 3.7 indicate that only at relatively high initial concentrations of thiocyanate is the proton uptake sufficient to produce a significant $\Delta \text{pH}$.
The appearance of this $\Delta$ pH of 1.6, which is energetically equivalent to a $\Delta\psi$ of 100 mV, coincided with a decrease in $\Delta\psi$ to practically zero, in agreement with previous evidence that the $\Delta\psi$ and $\Delta$ pH components of the proton-motive force are interchangeable in chromatophores (Gromet-Elhanan and Leiser 1973). Further evidence for this interpretation of the methylamine uptake at high concentrations of thiocyanate was that addition of nigericin (5 $\mu$g mg$^{-1}$ bacteriochlorophyll) in the presence of 10mM KSCN completely blocked methylamine uptake. Nigericin catalyses an electrically neutral $K^+/H^+$ exchange (Ashton and Steinrauf 1970; Chapter 2) and so should decrease any $\Delta$ pH that can be formed in the presence of 10 mM KSCN.

Fig. 3.2 shows that a small pH gradient will be reflected by only slight changes in the concentration of methylamine in the suspending medium in the upper chamber of the flow-dialysis cell. Therefore the question arises as to the magnitude of the smallest $\Delta$ pH that would have been detected in my experiments. In an experiment in which sufficient chromatophores were used to give 16 $\mu$l of internal volume per ml of reaction mixture, no methylamine uptake was detected upon illuminating the chromatophores. The experimental conditions were such that uptake of 3% of the methylamine would have been detected. Fig. 3.2 shows that uptake of 3% of the methylamine corresponds to a pH gradient of less than 0.3 units (20 mV) with 16 $\mu$l internal volume per ml reaction mixture.

As a further check against the possibility that the flow dialysis method was failing to detect a small but significant
Fig 3.8
Fig. 3.8  Effect of nigericin and valinomycin on the uptake of $[^{14}C]_{\text{thiocyanate}}$ by R. rubrum chromatophores

Reaction conditions as in the legend to Fig. 3.3 except that 10 mM potassium acetate was also present and the chromatophore concentration was 0.22 mg bacteriochlorophyll ml$^{-1}$. One µg nigericin was either present (○—○) or absent (●—●). At the first (open) arrow, the light was switched on, and at the second arrow 1 µg valinomycin was added to the upper chamber.
ΔpH when Δψ was large, or a small Δψ when ΔpH was large, both Δψ and ΔpH were estimated at concentrations of KSCN at which Δψ and ΔpH were expected to be of comparable magnitude. These reaction conditions should allow uptake of both thiocyanate and methylamine to be detected by the flow dialysis method. It was found that, with a preparation of chromatophores that generated a Δψ of 100 mV and an insignificant ΔpH under standard conditions, Δψ was decreased to 50 mV and 59 ΔpH increased to 40 mV in the presence of 2 mM KSCN, and with 5 mM KSCN Δψ was 35 mV and 59 ΔpH was 60 mV (data not shown).

The conclusion from these experiments is that a Δψ of 100 mV and a ΔpH of virtually zero does reflect the proton-motive force under my reaction conditions. If the method of measurement had been failing to detect a ΔpH of as much as 30 mV, then at intermediate concentrations of KSCN, where both components of the protonmotive force are detectable, it might have been anticipated that the total protonmotive force would have been greater than the value obtained under standard conditions when only Δψ could be detected.

The quantitative conversion of Δψ to ΔpH upon addition of 10 mM KSCN indicates that the uptake of \(^{14}C\) thiocyanate or \(^{14}C\) methylamine does reflect an equilibrium distribution of these solutes with Δψ and ΔpH respectively.

Addition of potassium acetate (10 mM) and nigericin (5 μg mg\(^{-1}\) bacteriochlorophyll) to illuminated chromatophores did not stimulate an extra uptake of thiocyanate ions (Fig. 3.8). The ΔpH component of a protonmotive force should, in the presence of K\(^+\) and nigericin, be replaced by an energetically equivalent Δψ as a result of nigericin exchanging
any accumulated H⁺ for added K⁺. Hence failure to observe an increased uptake of thiocyanate ions under these conditions suggests that a significant ∆pH is not normally formed. A light-dependent ∆pH was absent not only in the usual Tris-acetate or Tris-phosphate reaction media but also when the chromatophores were suspended in similar concentrations of Hepes-KOH (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) or Tris-chloride; my failure to observe a light-dependent ∆pH is not then a consequence of using an unusual suspending medium for the chromatophores.

Finally it is of interest that while K⁺ and nigericin had no effect on the extent of thiocyanate ion uptake, the combination nigericin, valinomycin and K⁺ was effective in releasing all the thiocyanate taken up in the light (Fig. 3.8). This result is consistent with previous work on the synergistic uncoupling effect of these two ionophores (Jackson et al., 1968; cf. Chapter 2).

**Determination of the phosphorylation potential (∆Gᵖ)**

According to the chemiosmotic hypothesis of energy coupling (Mitchell 1966a), the sum of ∆Ψ and ∆pH is termed the protonmotive force, and can be equated with the free energy stored in ATP if it is assumed that the ATPase reaction is poised against the protonmotive force. Equation 3.3 (cf. Eq. 1.4) expresses the relationship between the protonmotive force and the phosphorylation potential (∆Gᵖ):

\[
ΔΨ - 2.3RTΔpH = -\frac{ΔG_p}{zF} \quad \text{................. Eq. 3.3}
\]
### Table 3.2 The phosphorylation potential and protonmotive force in *R. rubrum* chromatophores

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Other Additions</th>
<th>Initial Substrate Concentrations (mM)</th>
<th>Final Substrate Concentrations (mM)</th>
<th>$\Delta G_p$ (kcal mol$^{-1}$)</th>
<th>p.m.f. (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>ATP 1.7      ADP 0.25   P$\text{$_i$}$ 1.7</td>
<td>ATP 1.95  ADP 0.044   P$\text{$_i$}$ 1.5</td>
<td>14.1</td>
<td>105</td>
</tr>
<tr>
<td>2</td>
<td>10 mM KSCN</td>
<td>ATP 2.5      ADP 0.25   P$\text{$_i$}$ 1.7</td>
<td>ATP 2.75  ADP 0.058   P$\text{$_i$}$ 1.5</td>
<td>14.1</td>
<td>105</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>ATP 0        ADP 1.4   P$\text{$_i$}$ 2.5</td>
<td>ATP 1.35  ADP 0.952   P$\text{$_i$}$ 1.0</td>
<td>14.0</td>
<td>99</td>
</tr>
</tbody>
</table>
Table 3.2

For determination of the phosphorylation potential, the reaction mixture contained in a final volume of 2 ml:
20 mM sucrose, 5 mM magnesium acetate, 1.7 mM or 2.5 mM phosphate-Tris, 0.2 mM sodium succinate and approximately 0.1 mg bacteriochlorophyll at pH 8.0, plus the other additions shown in the Table. The samples were illuminated for 20 min with the same light intensity and at the same temperature (24°C) as used in the flow dialysis experiments. The rate of ATP synthesis catalysed by the chromatophores, approximately 1.5 μmol min⁻¹ mg⁻¹ bacteriochlorophyll, was such that a 20 min incubation was sufficient to allow a steady state extent of phosphorylation to be reached. At the end of the incubation, 0.2 ml of 40% perchloric acid was added to the reaction mixture and after standing this acid extract on ice for 10 min, the precipitated protein was removed by centrifugation. The supernatant was neutralised by addition of a predetermined amount of 0.25M Tris in 10% KOH, and EDTA was also added to a final concentration of 2 mM. ATP in the neutralised extracts was determined with hexokinase and glucose-6-phosphate dehydrogenase, and ADP was assayed with pyruvate kinase and lactate dehydrogenase (Bergmeyer 1970). Pi was measured by the method of Hurst (1964) in a Technicon Autoanalyser. In calculating ΔGᵰ a value for ΔGᵰ of 7.8 kcal mol⁻¹ was used (Rosing and Slater 1972). The protonmotive force was determined in experiments 1 and 3 from the extent of thiocyanate uptake (ΔΨ as sole component) in parallel experiments using the reaction conditions given in the legend to Fig. 3.3. In experiment 2 the protonmotive force was determined from the extent of methylamine
uptake (Δ pH sole component), using the reaction conditions given in the legend to Fig. 3.7 with 10 mM KSCN present.
where \( F \) is the Faraday constant and \( z \) the number of protons that are translocated across the chromatophore membrane for each ATP synthesised (cf. Chapter 1). \( z \) is postulated to have a value of 2 (Mitchell 1966a), although to my knowledge a direct measurement of the \( \rightarrow H^+/ATP \) ratio for chromatophores has not yet been reported. Data in Table 3.2 show that the chromatophores were capable of maintaining a phosphorylation potential of approximately 14 kcal mol\(^{-1}\).

The phosphorylation potential was determined routinely in the absence of 20 \( \mu \)M thiocyanate as higher concentrations of thiocyanate (10 mM) were found not to affect the phosphorylation potential (Table 3.2).

In the determination of the protonmotive force (Table 3.2), neither ADP nor ATP was added to the reaction mixture. ADP was not added because phosphorylation lowered the steady-state protonmotive force generated by illuminated chromatophores (see above). ATP was not added because it was observed that it had no effect on the value of the light-dependent protonmotive force. The latter result is explicable on the basis that when there is no ATP synthesis there is no energetic demand on the protonmotive force.

The results described in this chapter indicated that, under the standard reaction conditions, the protonmotive force in \( R. \ rubrum \) chromatophores is 100 mV, composed solely of a membrane potential. From equation 3.3 it may be calculated that for the phosphorylation potential to be in equilibrium with a protonmotive force of this magnitude, at least five or six protons must be translocated for each ATP molecule synthesised if the ATPase is operating by a chemiosmotic mechanism.
DISCUSSION

The protonmotive forces reported in this chapter are apparently low when compared with the phosphorylation potential that can be generated by the *R. rubrum* chromatophores. Determinations of both $\Delta \text{pH}$ and $\Delta \psi$ rely upon the estimate of the internal volume of the chromatophores (equations 3.1 and 3.2), and therefore possible sources of error in the estimate of this volume must be considered. First, I assume that all the internal volume is enclosed by coupled chromatophores that are capable of photophosphorylation. If the preparations of chromatophores contain substantial amounts of 'uncoupled' chromatophores, or membrane preparations that are incapable of photophosphorylation because the orientation of the membrane is the same as in the whole cell (i.e. of opposite polarity to the phosphorylating chromatophores), then the value taken for the internal volume in equations 3.1 and 3.2 would need to be reduced. However, to produce a significant increase in $\Delta \psi$ or $\Delta \text{pH}$, a large reduction in the internal volume is required, since a two-fold decrease raises $\Delta \psi$ or $\Delta \text{pH}$ by only the equivalent of 18 mV. I have no direct evidence as to the extent of contamination of my chromatophores by non-phosphorylating membranes, but two lines of evidence suggest that these preparations are not unduly so contaminated. The fact that the chromatophores maintained very high phosphorylation potentials (Table 3.2) suggests that only a small proportion of uncoupled chromatophores are likely to be present. The rate of ATP hydrolysis catalysed by these preparations was typically 1.5 $\mu$mol ATP mg$^{-1}$ bacteriochlorophyll min$^{-1}$ (assayed in 50 mM Tris-chloride, pH 7.5, at 30°C with 5 mM MgCl$_2$).
and 5 mM ATP), while the rate of ATP synthesis was between 1 and 2 μmol ATP mg⁻¹ bacteriochlorophyll min⁻¹ (assayed under the same conditions as employed for determination of the phosphorylation potential (Table 3.2)). These values are similar to those reported by others (e.g. Edwards and Jackson 1976). If the ATP hydrolysis activity were mainly associated with the putative uncoupled chromatophores, then it is difficult to see how the high phosphorylation potentials of Table 3.2 could be attained. Presumably upon illumination the ATP hydrolysis activity of the chromatophores become ATP-synthesising activity; thus either any 'uncoupled' chromatophores lack ATPase activity or they are present in relatively small quantities. There is no evidence as to the possibility that there are a substantial fraction of non-phosphorylating membranes orientated with an opposite polarity to phosphorylating chromatophores. However, the similarity of the phosphorylation rate catalysed by these chromatophores and those of others (Edwards and Jackson 1976) at least suggests that the present preparations are not grossly unusual in composition. It is also noted that there is immunological data and evidence from the electron microscope that chromatophore membranes are generally orientated in the sense that the ATPase is facing the suspending medium (Baltscheffsky 1976).

A second problem in estimating an internal volume could be that in chromatophores a significant fraction of the internal volume is occupied by the electrical double layer on the inner surface of the chromatophore membrane.
However, this effect appears to be small, as, if representative value of 0.8 nm for the thickness of this layer (Mitchell 1968) is taken, then its volume is an insignificant fraction of the total internal volume of a chromatophore with a radius of 30 nm.

In contrast to the results in the present chapter, there have been several previous reports of significant light-dependent pH gradients in chromatophores from *R. rubrum* (Jackson *et al.*, 1968; Schuldiner *et al.*, 1974; Leiser and Gromet-Elhanan 1977). Schuldiner *et al.*, (1974) found a light-dependent $\Delta$ pH of 1.8 units from the distribution of methylamine across the chromatophore membranes, which was measured using a precipitation method to separate the chromatophores from the suspending medium. The precipitation step may have resulted in an overestimation of the methylamine uptake, especially as a $\Delta$ pH of 1.1 units was measured even when the chromatophores were in the dark (Schuldiner *et al.*, 1974).

Jackson *et al.*, (1968) estimated a $\Delta$ pH of 1 unit from the difference between the concentrations of external KCl at which added nigericin caused no pH change in the medium when added to *R. rubrum* chromatophores in the dark and light. Comparison of their result with the present data is difficult as Jackson *et al.*, (1968) used rather different conditions of ionic strength and pH than those employed in the present experiments.

Recently Leiser and Gromet-Elhanan (1977) obtained evidence for a $\Delta$ pH of 2.6 units from the extent of quenching of 9-amino-acridine fluorescence using reaction conditions not very different from those used in the present work.
The present failure to observe a significant pH gradient may thus add weight to the previous criticisms that the fluorescence of 9-amino-acridine is not a reliable indicator of pH gradients (Piolet et al., 1974; Kraayenhof et al., 1976; Kraayenhof 1977; Searle et al., 1977) (but see Casadio and Melandri 1977).

As previous work has suggested that a substantial $\Delta$ pH is formed when chromatophores are illuminated (see above) I now consider some other possibilities as to why I failed to observe a $\Delta$ pH. The evidence for the permeability of the uncharged form of methylamine is that the chromatophores did accumulate a significant amount of methylamine when a permeant ion was present (Fig. 3.7). It is improbable therefore that methylamine was unable to equilibrate with any transmembrane pH gradient that was formed in the absence of a permeant ion. A lack of methylamine uptake in response to a pH gradient would be explicable if the chromatophore membrane is permeable to the charged form of methylamine. The charged form might, for instance, act as a $K^+$ analogue and thus be pumped out of the chromatophore lumen via a $K^+$ transport system (see e.g. Stevenson and Silver 1977). A consequence of such behaviour would be that methylamine, at low concentrations, should be an uncoupler which it is not known to be. Furthermore, as methylamine uptake can be observed in the presence of high concentrations of a permeant ion, a rapid transport of the charged form of methylamine from the chromatophore lumen seems unlikely.
Alternatively it may be that the chromatophores used in the present work have a higher internal buffering capacity than the chromatophores used by other works; this would account in a simple way for my failure to observe a pH gradient. The number of protons that must be translocated to produce a membrane potential of 100 mV can be estimated at between 18 and 90 if a membrane capacitance of between 0.3 and 1.5 μfarad cm$^2$ and a chromatophore radius of 30 nm are assumed (Jackson and Crofts 1971). It is plausible that this number of protons can be buffered within the lumen of the chromatophores thus giving no significant pH gradient (see Chapter 7).

The estimate described in this chapter of the light-dependent $\Delta \Psi$ in *R. rubrum* chromatophores is similar to the values obtained by (1) Schuldiner *et al.*, (1974) from the distribution of thiocyanate; (2) Pick and Avron (1975) from the fluorescence of an oxacarboxyamine dye; (3) Leiser and Gromet-Elhanan (1977) from the fluorescence of 1-anilino-naphthalene-8-sulphonate. The agreement of the data from thiocyanate distributions with methods (2) and (3) may, however, be fortuitous as reservations have been expressed about the calibration procedures for these dyes (Ferguson *et al.*, 1976a; Pick and Avron 1976). It is also of interest that membrane potentials in the range 100 to 120 mV have been reported for plasma membrane preparations from a number of bacteria (Hamilton 1977).

In chromatophores from other photosynthetic bacteria such as *Rhodopseudomonas sphaeroides* or *Rhodopseudomonas capsulata* light-dependent membrane potentials of between
200 and 250 mV have been estimated in the steady state from the extent of the carotenoid band shift (Jackson and Crofts 1971; Casadio et al., 1974a). In view of the low value of $\Delta \Psi$, and of the protonmotive force, reported in the present chapter some discussion of the thiocyanate distribution method is warranted, especially as Tedeschi (1975; Tedeschi and Maloff 1976) has suggested that the basis of the method is unsound.

The use of thiocyanate distribution to estimate membrane potentials depends on a number of factors. (a) Accurate estimation of the internal concentration (activity) of thiocyanate. (b) There should be insignificant binding of thiocyanate to both the energised and unenergised membrane. (c) The free acid HJCN is not present in significant amounts. (d) Thiocyanate is a permeant ion which can equilibrate with the potential across the membrane, is not actively accumulated, and does not perturb the membrane potential.

Point (a) requires estimation of the internal volume of the chromatophores and determination of the thiocyanate uptake; as discussed earlier I believe that these quantities have been realistically measured in the work described here. In using equation 3.1 I have assumed activity coefficients of unity; in practice lower values should be used as it is likely that the ionic strength inside the chromatophores is higher than outside. In this case the activity coefficient inside the chromatophores will be lower than that outside so that the use of concentration terms in equation 3.1 will tend to slightly over-estimate $\Delta \Psi$. 
Point (b) is satisfied by the observations that similar membrane potentials are obtained when either the thiocyanate or the chromatophore concentration is varied, and that illuminated cells of *R. rubrum* do not take up thiocyanate (see above).

As the pKₐ of thiocyanate is very low (−1.8) (Morgan *et al.*, 1965), HSCN will be present in very small quantities, thus satisfying point (c).

Evidence that chromatophores are freely permeable to thiocyanate has been obtained by previous workers (Gromet-Elhanan 1972; Gromet-Elhanan and Leiser 1973; Pick and Avron 1976), and is supported by the observations that thiocyanate is rapidly lost from the chromatophores either upon addition of an uncoupler or in a subsequent dark period after illumination. Moreover, after illumination of chromatophores, thiocyanate in the outflow from the dialysis cell reaches a new equilibrium as rapidly as may theoretically be expected (Colowick and Womack 1969). This result is also indicative of a passive equilibration of thiocyanate into the chromatophores rather than an active uptake, the rate of which might have been expected to be slower and possibly dependent on the thiocyanate concentration.

It has been suggested (Tedeschi 1975; Tedeschi and Maloff 1976) that an ion like thiocyanate will be taken by a direct coupling involving a co-transport of one or more protons with the thiocyanate ion, and that there is not necessarily a steady state membrane potential, generated by electron flow, which drives the uptake. The argument, by analogy with valinomycin treated-mitochondria, is that upon addition
of thiocyanate to illuminated chromatophores, extra protons will be taken up stoichiometrically with the thiocyanate until a Donnan equilibrium for thiocyanate is reached. It seems improbable that there should be a direct coupling of protons to thiocyanate uptake, although extra proton uptake even in the presence of low thiocyanate concentrations is expected on the following basis. Assuming that light-driven electron flow generates a membrane potential, presumably as a result of proton movement, then the potential-driven uptake of negatively charged thiocyanate will tend to lower the potential. This in turn can cause an extra proton uptake so as to re-establish the original potential. The extent of this proton uptake can be calculated as follows. For an experiment (Fig. 3.3) in which 20 μM thiocyanate is added to chromatophores, illumination results in the uptake of approximately half the thiocyanate, and the concentration of thiocyanate inside the chromatophores will be approximately 1 mM. If the radius of a chromatophore is 30 nm then 60 negative thiocyanate ions have been taken up by each chromatophore. As noted earlier a membrane potential of 100 mV is established by the net uptake of between 18 and 90 protons, so that if the uptake of 60 thiocyanate ions is not compensated by an extra and equivalent uptake of 60 protons, the membrane potential would be collapsed or reduced. Thus it appears that the chromatophore membrane tends to maintain its potential by compensating thiocyanate uptake by an additional uptake of protons.

It may be argued that the uptake of either K⁺ into valinomycin-treated mitochondria, or of synthetic ions into
chromatophores, is not irrefutable evidence for a membrane potential. However, for chromatophores there is evidence independent of ion-uptake studies for a light-induced membrane potential. The light causes changes in the absorption spectrum of the membrane carotenoids, which can be mimicked by imposing $K^+$ diffusion potentials across the chromatophore membranes (Jackson and Crofts 1969). Further, Barsky et al., (1976) fused chromatophores from *R. rubrum* onto a planar phospholipid membrane separating two aqueous phases and measured the light-induced potential with a voltmeter. Hence there is good evidence that illumination of chromatophores produces a membrane potential and the permeant thiocyanate ion may be expected to equilibrate with it across the membrane according to equation 3.1. In this respect I would emphasise that the same value for the membrane potential was determined over a range of thiocyanate concentrations (Fig. 3.4), which can be taken as further evidence that thiocyanate uptake, except at much higher concentrations of added thiocyanate, does not perturb the potential. I feel that requirement (d) (above) is satisfied.

It is noteworthy that addition of antimycin to *R. rubrum* chromatophores abolished the light-dependent thiocyanate uptake (Fig. 3.5). The carotenoid band shift, which is generally taken as an indicator of membrane potential, is only partially inhibited by antimycin (Baltscheffsky 1976). Hence I am inclined to support the view of Baltscheffsky (1976) that only the antimycin-sensitive component of the carotenoid shift should be used as an indicator of the steady-state membrane potential in the light, at least for *R. rubrum*. The $\Delta \Psi$ is...
chromatophores from other bacteria, which were estimated from the full extent of the carotenoid shift, would be much lower if only the antimycin-sensitive component of the band shift were used (Jackson and Crofts 1971; Casadio et al., 1974a; Jones 1977).

From a total protonmotive force of 100 mV and a $\Delta G_p$ of approximately 14 kcal mol$^{-1}$ it was calculated from equation 3.3 that at least six protons must be translocated for each ATP molecule synthesised. The chemiosmotic hypothesis envisages that two protons are translocated for each ATP made (Mitchell 1966a, 1977b), and some evidence has been obtained with chromatophores that agrees with this view (Casadio et al., 1974a; Jackson et al., 1975). Casadio et al., (1974a) reached this conclusion from a comparison of the protonmotive force with $\Delta G_p$, but as discussed above they may have overestimated the protonmotive force by using the total extent of the carotenoid shift and relying on the quenching of 9-amino-acridine fluorescence to determine $\Delta \text{pH}$.

Studies on the movement of protons during ATP synthesis following single flash excitation of chromatophores from Rps. sphaeroides have led Jackson et al., (1975) to conclude that approximately two protons are translocated per ATP synthesised. The discrepancy between the kinetic work of Jackson et al., (1975) and the present thermodynamic measurements cannot be explained at present (cf. Chapter 1), but it is noteworthy that Gruber and Witt (1976) have found recently in chloroplasts that the $\rightarrow H^+/ATP$ ratio may not be the same with single turnover flashes as during continuous illumination.
METHODS

Growth of organism and preparation of chromatophores

*R. rubrum* (strain S1, a gift from Dr O.T.G. Jones, University of Bristol, U.K.) was grown anaerobically in the medium of Ormerod et al., (1961) under continuous illumination from a 100 watt tungsten bulb at about 23°C. Cells were harvested in late logarithmic or early stationary phase, washed once in cold 0.2M glycylglycine pH 7.4, resuspended in the same buffer and stored at -20°C. Chromatophores were prepared by exposing cells (about 0.2 mM in bacteriochlorophyll) suspended in 0.2M glycylglycine and 5 mM MgCl₂ at pH 7.4 to sonic oscillation at the maximum output of a Dawe Soniprobe for two periods of one min. The temperature of the cell suspension did not rise above 10°C during this treatment. Cell debris was removed by centrifugation at 17 000 r.p.m. (28 000 x g) for 15 min in the SS 34 rotor of a Sorvall RC2B centrifuge. Chromatophores were sedimented at 110 000 x g by centrifugation at 44 000 r.p.m. for 50 min in a Spinco rotor, and then resuspended in 10 mM Tris-acetate pH 8.0 containing 5 mM magnesium acetate. They were either used immediately or stored overnight under nitrogen at 4°C.

Flow dialysis

The uptake of \(^{14}C\) thiocyanate and \(^{14}C\) methylamine was measured in a cylindrical flow dialysis cell (Fig. 3.1) (constructed in the workshop of the Botany School) which essentially followed the design of Colowick and Womack (1969).
The volume of the lower chamber was 1.2 ml and the upper chamber had a maximum capacity of 2.5 ml. Visking dialysis tubing (Gallenkamp) of average pore diameter 24 Å was boiled for one hour in 5 mM EDTA (sodium salt) and stored in distilled water at 4°C before being inserted between the two chambers with Parafilm gaskets to ensure water tightness. Water was pumped through the lower chamber at 2 ml min⁻¹ by means of a Watson-Marlow MHRE peristaltic pump. 1 ml fractions of the outflow were collected in scintillation vials inserts containing 2 ml Triton-toluene scintillant (Turner 1969) held in an LKB Ultrorac fraction collector. The dead volume between the flow dialysis cell and the fraction collector was 0.3 ml. Radioactivity was counted in a Tracerlab Corumatic 200 liquid scintillation counter. Chromatophores in the flow dialysis cell were illuminated by a 500 watt tungsten bulb from which the light was filtered through a Kodak Cinemoid 5A deep orange filter and 5 cm of water. The light intensity behind the cell was approximately 2.8 x 10⁵ erg cm⁻² sec⁻¹ as measured with an EEL Lightmaster photometer. For dark controls, the cell was covered with aluminium foil. Details of the reaction mixtures are given in the legends to the figures. The flow dialysis experiments were done at room temperature (23 - 24°C).

**Bacteriochlorophyll and protein determinations**

Bacteriochlorophyll concentrations were estimated from the absorbance at 880 nm using the in vivo millimolar extinction coefficient (\( \frac{1\text{cm}}{880} = 140 \)) given by Clayton (1963). Protein was determined by the Folin method (Lowry et al., 1951) using bovine serum albumin (Cohn fraction V) as standard. The
bacteriochlorophyll: protein ratio (mean ± S.D.) for three different preparations of chromatophores was 37 ± 3.7 µg bacteriochlorophyll mg⁻¹ protein.
CHAPTER 4

THE PROTONMOTIVE FORCE IN BOVINE HEART SUBMITOCHONDRIAL PARTICLES: MAGNITUDE, SITES OF GENERATION AND COMPARISON WITH THE PHOSPHORYLATION POTENTIAL
"Despite a popular belief to the contrary, thermodynamic energetic methods are useful, not only for describing initial and final states, but also for describing intermediary states, and thus for investigating biochemical mechanisms"

Mitchell (1977b)

INTRODUCTION

As noted in previous chapters, the chemiosmotic hypothesis (Mitchell 1966a,b) originally envisaged that either the passage of a pair of electrons through one proton-translocating segment of the mitochondrial respiratory chain, or the hydrolysis of one molecule of ATP, was linked to the translocation of 2 protons across the inner mitochondrial membrane. Although a good deal of evidence has been obtained that is consistent with this view (Mitchell 1976a), many recent experimental findings indicate that the stoichiometry of proton translocation may be higher than two.

Re-examination of the number of protons ejected by mitochondria after an oxygen or substrate pulse, has shown that the stoichiometry of proton-translocation is three or even four (for a review see Brand 1977). A recent study of the number of protons ejected in electroneutral exchange for Ca$^{2+}$ is consistent with the translocation of four protons as a pair of electrons pass through a proton-translocating segment of the respiratory chain (Reynafarje & Lehninger 1977). A stoichiometry of 2.8 protons has been estimated by Nicholls (1977a), on the basis of the number of Ca$^{2+}$ ions taken up by mitochondria respiring on succinate.
Hydrolysis of a pulse of ATP by the ATPase in mitochondria is still thought to be coupled to the translocation of two protons across the membrane, although the measurements are complicated by the need to allow for, or eliminate, any proton movements that may be associated with the transport of ATP, ADP or Pi across the membrane (Brand & Lehninger 1977).

Thermodynamic measurements also suggest that more than two protons must be translocated per two electrons passing through a proton-translocating segment of the respiratory chain. Comparison of the extramitochondrial phosphorylation potential \( \Delta G^\circ + RT \ln \frac{[ATP]}{[ADP][Pi]} \) with the electrochemical gradient of protons (the protonmotive force of Mitchell (1966)) has indicated that the overall process of producing extramitochondrial ATP from ADP and Pi involves the translocation of three protons per molecule of ATP synthesised (Nicholls 1974; Rottenberg 1975; Wiechmann et al., 1975). Thus if the P/O ratio for succinate is two, these observations mean that the oxidation of a molecule of succinate must be associated with the translocation of six rather than four protons as originally proposed (Mitchell 1966a, b).

With intact mitochondria the study of respiration-or ATP-driven proton ejection, or the analysis of the thermodynamic relationship between the protonmotive force and the extramitochondrial phosphorylation potential, is complicated by proton movements associated with the transport systems of the inner membrane. Comparison of the phosphorylation potential with the protonmotive force is most probably complicated by the electrogenic nature of the adenine nucleotide translocator (Klingenberg & Rottenberg 1977), and by proton movements
connected with the movement of $P_i$ into or out of mitochondria (Bhand 1977). In view of the inherent uncertainties, and possible experimental discrepancies (Mitchell 1977c), there is a need for additional approaches to the problem of the stoichiometry of proton translocation.

Submitochondrial particles, which are inside out relative to mitochondria (Racker 1970), have an ATP-synthesising apparatus directly available to added substrates. Thus they provide a system free of the complicating factors that are experienced with intact mitochondria. In particular the adenine nucleotide translocator is not involved in oxidative phosphorylation in submitochondrial particles. The advantages of working with submitochondrial particles have so far been exploited to only a limited extent in the determination of the $\rightarrow H^+/ATP$ ratio. For example the number of protons taken up by submitochondrial particles during the passage of a pair of electrons through a proton-translocating segment of the respiratory chain has been shown to be approximately two at pH 6.0 to 6.5 (Hinkle & Horstman 1971). An uptake of around two protons has also been found to be linked to the hydrolysis of one molecule of ATP by the particles (Moyle & Mitchell 1973a; Thayer and Hinkle 1973). However, advantage has not been taken of the properties of submitochondrial particles for comparison of the phosphorylation potential with the protonmotive force. The value of such measurements would be that the number of protons translocated by the ATPase alone for each molecule of ATP synthesised could be determined, and compared with the values obtained by other methods.
This chapter describes measurements of both the phosphorylation potential and protonmotive force in submitochondrial particles. Although the phosphorylation potential in submitochondrial particles is distinctly lower than its extra-mitochondrial counterpart, it has been argued (Ferguson & Sorgato 1977) that the phosphorylation potential is not restricted to a low value by the poor coupling properties of the particles. Thus the work described in this chapter had two main objectives: to determine whether, despite the low phosphorylation potential, submitochondrial particles were able to maintain a protonmotive force of similar magnitude to those found in other energy-coupling membranes, and to estimate the number of protons translocated per ATP molecule synthesised, by comparing the protonmotive force with the phosphorylation potential.

If the stoichiometry of proton translocation is higher than was suggested in the chemiosmotic hypothesis, then the original concept of a looped respiratory chain (Mitchell 1966a, b; see Chapter 7) cannot account for the full extent of proton translocation. Wikström (1977) has presented evidence that cytochrome oxidase acts as an intrinsic proton pump, a property that could account for some extra proton translocation. In contrast Hauska et al., (1977) have suggested that electron flow through cytochrome oxidase is not coupled to proton translocation, as judged by the apparent absence of ATP synthesis during oxidation of ascorbate plus N,N,N',N'-tetramethyl-p-phenylene diamine (TMPD) by submitochondrial particles. A further purpose of the work described in this chapter was therefore to examine whether
electron flow through cytochrome oxidase was coupled to the generation of a protonmotive force.

As in the previous chapter, the protonmotive force was determined by a flow dialysis assay of the uptake of $^{14}$C methylamine hydrochloride and K$^{14}$CN. Use of the method requires that the membranes of submitochondrial particles, which are derived from the inner mitochondrial membrane, are permeable to SCN$^{-}$. Evidence that SCN$^{-}$ can permeate the inner mitochondrial membrane (or submitochondrial particles) has been given by Mitchell & Moyle (1969b), Papa et al., (1973a, b) and Lehninger (1974). In support of this evidence it was found (data not shown) that addition of 50 mM KSCN to submitochondrial particles in the presence of valinomycin does not cause an increase in the fluorescence of added 8-anilinonaphthalene-1-sulphonate. A similar experiment using KCl instead of KSCN did produce a fluorescence increase, which is regarded as being diagnostic of the generation of a K$^{+}$ diffusion potential (Azzi et al., 1971; Jasaitis et al., 1971). It seems that SCN$^{-}$, unlike Cl$^{-}$, can rapidly permeate the membrane, thus preventing the formation of a K$^{+}$ diffusion potential.

**Determination of the internal volume of the submitochondrial particles**

The internal volume of the particles was estimated from the sucrose-impermeable space (as in Chapter 3). An average value of 1.3 µl per mg of protein was obtained in the Pi/Tris magnesium acetate reaction medium that was routinely used for measurements of the protonmotive force (Table 4.1). Papa et al., (1973a) reported a value of 2.5 µl per mg of protein
Table 4.1

<table>
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<th>cpm 2nd supernatant</th>
<th>$V_i$ (µl mg protein $^{-1}$)</th>
</tr>
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<td>1 772</td>
<td>1.0</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>$^3H$</td>
<td>409 365</td>
<td>16 041</td>
<td>2.1</td>
</tr>
<tr>
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<td>76 252</td>
<td>2 192</td>
<td></td>
</tr>
<tr>
<td>Expt 2</td>
<td></td>
<td></td>
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<tr>
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<td>723 922</td>
<td>26 287</td>
<td></td>
</tr>
<tr>
<td>$^{14}C$</td>
<td>51 949</td>
<td>1 314</td>
<td></td>
</tr>
<tr>
<td>Expt 3</td>
<td></td>
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<td>1.0</td>
</tr>
<tr>
<td>$^3H$</td>
<td>478 545</td>
<td>14 957</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.1

Submitochondrial particles were suspended (final concentrations were: Expt 1, 7.54 mg ml⁻¹; Expt 2, 3.63 mg ml⁻¹; Expt 3, 5.14 mg ml⁻¹) in 10 mM Pi/Tris pH 7.3, 5 mM magnesium acetate with approximately 0.15 µCi U-14C sucrose and 2.5 µCi 3H₂O to a final volume of 5 ml. The mixtures were centrifuged and further analysed as described in the legend to Table 3.1.
for the internal volume of Mg-ATP particles using the dextran-impermeable space as a marker for the internal volume. A possible explanation for the smaller internal volume determined by me is that dextran, being a considerably larger molecule than sucrose, is excluded from the interface between the particles and the suspending medium, thus giving a larger apparent impermeable space than sources. For EDTA-particles an internal volume of 1.4 μl mg of protein has been reported (Papa et al., 1973b), using the dextran-impermeable space. However, this value cannot strictly be related to the internal volume of Mg-ATP-particles as the different preparative procedures for the two types of particle may well produce particles of differing sizes.

The internal volume of the submitochondrial particles is determined under conditions where the particles are not energised, but the internal volume under energised conditions is needed for measurement of Δψ and ΔpH. Energisation of submitochondrial particles may cause swelling of the particles owing to the inward movement of H⁺ plus any migrating anions into the particles. However, the magnitude of this effect appears to be small, except where relatively high concentrations of permeant ions are present (Papa et al., 1973b). Even when significant swelling of submitochondrial particles was demonstrated (Papa et al., 1973b), the maximum effect was less than a twofold increase in internal volume. As noted in Chapter 3, twofold errors in the estimate of internal volume will be reflected by errors of only 18 mV in the estimation of Δψ or 59 ΔpH. In the present chapter
Fig. 4.1  Respiration-driven uptake of the thiocyanate ion by submicrochondrial particles as determined by flow dialysis.

The upper chamber of the flow dialysis cell contained in a final volume of 1 ml: 20 μM K$^{14}$CN (60 μCi μmol⁻¹), 0.6 mM NAD⁺, 1% v/v ethanol, 0.05 mg alcohol dehydrogenase, 10 mM P/Tris, 5 mM magnesium acetate. Submitochondrial particles (6.25 mg protein) were either included in (●—●) or omitted from (●—○) this reaction mixture as indicated. The temperature was 23°C and the pH was 7.3. FCCP (5 μM) and Triton X-100 (0.1% w/v) were added as shown.
an internal volume of 1.3 µl per mg of protein has been used throughout unless otherwise indicated. Use of this estimate of the volume means that, if there is a significant energy-linked swelling of the particles under the present reaction conditions, the results will err in the direction of over-estimating $\Delta \psi$ and $\Delta \text{pH}$.

**Determination of the protonmotive force with NADH as substrate**

Fig. 4.1 shows two plots of the radioactivity ($S^{14}\text{CN}^-$) in sequentially collected fractions of the outflow from the lower chamber of the flow-dialysis cell. The open symbols represent an experiment in which no submitochondrial particles were added to the upper chamber. The experiment was started by adding 20 µM KS$^{14}\text{CN}$ to the upper chamber of the dialysis cell; simultaneously the collection of the outflow from the lower chamber was begun. After 2.5 minutes (collection of 5 fractions) a steady-state distribution of $S^{14}\text{CN}^-$ across the dialysis membrane was attained (Fig. 4.1). At this point the rate of entry of $S^{14}\text{CN}^-$ ions into the lower chamber almost equalled the rate of loss of $S^{14}\text{CN}^-$ from the lower chamber to the fraction collector. In subsequent fractions the level of radioactivity in the outflow declined slightly owing to the slow (approximately 3% depletion in 20 minutes) decrease of the KS$^{14}\text{CN}$ concentration in the upper chamber. When the $S^{14}\text{CN}^-$ concentration in the upper chamber was varied (no submithochondrial particles present) there was a directly proportional variation in the level of radioactivity in the outflow from the dialysis cell. Thus (cf. Chapter 3) the concentration of unbound $S^{14}\text{CN}^-$ in the upper chamber of the dialysis cell could be reliably determined from the
concentration of $S^{14}$CN$^-$ in the outflow from the lower chamber.

The enclosed symbols (Fig. 4.1) show an experiment in which the uptake of $S^{14}$CN$^-$ by respiring particles was measured. 20 μM $K{}^{14}$CN was added to the upper chamber of the dialysis cell, which also contained submitochondrial particles oxidising NADH, and the collection of fractions of the outflow was started simultaneously.

The concentration of $S^{14}$CN$^-$ in the outflow first rose, and then fell before reaching a concentration at which there was an essentially steady-state distribution of $S^{14}$CN$^-$ across the dialysis membrane. Thereafter the concentration of $S^{14}$CN$^-$ in the outflow decreased very slowly (Fig. 4.1). The rise in $S^{14}$CN$^-$ concentration in the outflow before the fall to the steady-state concentration can be attributed to a relatively slow uptake of $S^{14}$CN$^-$ into the respiring particles. Mitchell and Moyle (1969b) and Lehninger (1974) have shown that SCN$^-$ crosses the inner mitochondrial membrane with a $t_\frac{1}{2}$ between 30 and 60 seconds (for a direct estimate, see Chapter 6). Thus in these flow dialysis experiments (Fig. 4.1) the concentration of free $S^{14}$CN$^-$ decreased slightly during the approach to a steady-state distribution of $S^{14}$CN$^-$ across the dialysis membrane, and consequently the concentration of $S^{14}$CN$^-$ in the outflow 'overshot' (Fig. 4.1, closed symbols) (cf. Fig. 3.3).

The response time of the flow dialysis apparatus to a change in the $S^{14}$CN$^-$ concentration in the upper chamber was approximately 2.5 minutes, as judged by the time taken for an approximately steady-state concentration of $S^{14}$CN$^-$ to be
reached in the outflow following addition of 20 μM K$^{14}$CN to the upper chamber (open symbols, Fig. 4.1). The final extent of S$^{14}$CN$^{-}$ uptake by the respiring particles must be reached within this period as it can be seen from Fig. 1 (closed symbols) that a steady-state distribution of S$^{14}$CN$^{-}$ was reached within 2.5 to 3 minutes of adding K$^{14}$CN to respiring particles. The rate of S$^{14}$CN$^{-}$ uptake by respiring particles was very much faster than the rate of S$^{14}$CN$^{-}$ dialysis (cf. Chapter 6). This is evident from Fig. 4.1 in which it can be seen that the particles took up half the total added SCN$^{-}$ during a period that virtually no S$^{14}$CN$^{-}$ was lost from the upper chamber of the cell by dialysis.

Addition of an uncoupler of oxidative phosphorylation carbonyl cyanide-p-trifluoromethoxy phenylhydrazone (FCCP) caused efflux of the S$^{14}$CN$^{-}$ accumulated by respiring submitochondrial particles (Fig. 4.1, closed symbols). The subsequent addition of a lytic amount of Triton X-100 did not cause any extra efflux of S$^{14}$CN$^{-}$ (Fig. 4.1), which indicated that addition of FCCP had completely dissipated the membrane potential and thus caused complete efflux of the accumulated S$^{14}$CN$^{-}$. Fig. 4.1 (open symbols) shows that addition of either FCCP or Triton X-100 had no effect on the rate of S$^{14}$CN$^{-}$ transfer across the dialysis membrane when no submitochondrial particles were present in the upper chamber.

$\Delta \psi$ was routinely calculated using the nomograph (Fig. 3.2) from the extent of S$^{14}$CN$^{-}$ uptake before uncoupling; the difference between the level of radioactivity in a given fraction of the outflow during the steady state before un-
coupling, and, by extrapolation, the level of radioactivity that would have been in the same fraction had FCCP been present throughout was taken as the measure of $^{14}{\text{CN}}^-$ uptake (Fig. 4.1). This procedure was adopted (cf. Fig. 3.6) rather than the alternative of measuring the decrease in radioactivity in the outflow after addition of respiratory substrate, for the following reason. It was found that the introduction into the upper chamber of substrates such as NADH, succinate or ascorbate perturbed the rate of $^{14}{\text{CN}}^-$ transfer across the dialysis membrane, so that the extent of $^{14}{\text{CN}}^-$ uptake following energisation of the particles could not be readily calculated. A plausible explanation of this effect is that diffusion potentials were set up across the dialysis membrane owing to the different diffusion rates of the cation-anion pair added as substrate. Similar effects were observed on adding ATP to Rhodospirillum rubrum chromatophores in the upper chamber of the flow dialysis cell (Chapter 3), and Van Dam et al., (1977) have also reported similar phenomena.

After addition of FCCP to the respiring particles the concentration of $^{14}{\text{CN}}^-$ (closed symbols, Fig. 4.1) in the outflow from the dialysis cell was slightly higher than in the experiment in which no particles were present (open symbols, Fig. 4.1). This effect is interpreted as follows. First, the uptake of $^{14}{\text{CN}}^-$ by the respiring particles resulted in a substantially decreased concentration of free $^{14}{\text{CN}}^-$ relative to that in the experiment in which no particles were present. Consequently, over a given period, slightly more $^{14}{\text{CN}}^-$ was lost from the upper chamber in the
experiment without particles. Therefore after uncoupling
the concentration of $^{14}\text{CN}^-$ in a given fraction of the
outflow is expected to be slightly higher compared with the
corresponding fraction of the outflow from the experiment
without particles.

The data in Fig. 4.1 indicate that the extent of binding
of $^{14}\text{CN}^-$ to the non-energised particles is relatively small.
The binding of a significant fraction of the total $^{14}\text{CN}^-$
to the particles would have been reflected by a markedly
lower level of radioactivity in the outflow from the dialysis
cell when particles plus uncoupler (closed symbols Fig. 4.1)
were present, compared with the experiment without particles
(open symbols, Fig. 4.1). In other runs of the same
experiment, the counts both after uncoupling the particles
and in the particle-free control were more nearly identical
(cf. Chapters 3, 5), indicating that the slight difference
is partially due either to differing rates of $^{14}\text{CN}^-$ diff-
uslon across the dialysis membrane depending on the presence
of particles, or to slight changes in the volume of $\text{K}^{14}\text{CN}$
added to the upper chamber.

If equation 3.1 is to be used to calculate the membrane
potential, it is essential that, the energy-linked uptake
of $^{14}\text{CN}^-$ represents an accumulation of the ion into the
lumen of the particles, rather than an energy-dependent bind-
ing of $^{14}\text{CN}^-$ to the particles. Discrimination between
binding and accumulation can be made by varying the concen-
tration of $^{14}\text{CN}^-$. Binding of $^{14}\text{CN}^-$ should be a saturable
process, whereas accumulation of $^{14}\text{CN}^-$ inside submitochon-
drial particles until the electrochemical equilibrium for
$^{14}\text{CN}^-$ is reached requires that the ratio $(\text{SCN}^-)$ in/$(\text{SCN}^-)$
out (equation 3.1) be independent of the total concentration of $S^{14}\text{CN}^-$. When the $S^{14}\text{CN}^-$ concentration was varied from 5 to 50 μM the $S^{14}\text{CN}^-$ accumulation ratio was constant (data not displayed; cf. Chapter 3), consistent with the notion that $S^{14}\text{CN}^-$ uptake does represent an accumulation of $S^{14}\text{CN}^-$ to electrochemical equilibrium. The $S^{14}\text{CN}^-$ accumulation ratio was also unchanged when, at a fixed added $S^{14}\text{CN}^-$ concentration (20 μM), the particle concentration was varied from 1 to 8 mg of protein per ml. This result was again consistent with equilibrium accumulation of $S^{14}\text{CN}^-$ inside the particles rather than an energy-linked binding (data not displayed).

When a solute is extensively accumulated within sub-mitochondrial particles, the concentration in the suspending medium in the upper chamber will greatly decrease. In these circumstances the rate of dialysis of the solute across the dialysis membrane will also decrease, but van Dam et al., (1977) have suggested that the accumulated solute can act as a buffer of solute so that the concentration of solute in the external medium, and hence the rate of solute dialysis, may not decrease to the 'true' extent. An effect of this kind would mean that, at a fixed total concentration of $S^{14}\text{CN}^-$, a higher accumulation ratio, $\frac{[\text{SCN}^-]_{\text{in}}}{[\text{SCN}^-]_{\text{out}}}$, would be observed at lower particle concentrations when the proportion of the total $S^{14}\text{CN}^-$ taken up would be less. However, as already noted, the accumulation ratio was constant over an eight-fold range of particle concentration, so that it appears that the effect by van Dam et al., (1977) was not a source of error in the experiments described here.
Fig 4-2

\(10^{-3} \text{ counts/min}\)

5 µM FCCP
5 µM rotenone
0.1% Triton X-100

Fraction Number
Absence of detectable respiration-driven uptake of methylamine into submitochondrial particles as determined by flow dialysis

The upper chamber of the flow dialysis cell contained in a final volume of 1 ml: 20 μM \(^{14}C\) methylamine hydrochloride (55.5 μCi μmol\(^{-1}\)), 0.6 mM NAD\(^+\), 1% v/v ethanol, 0.05 mg alcohol dehydrogenase, 10 mM Pi/Tris, 5mM magnesium acetate. Submitochondrial particles (12.5 mg protein) were either included in (▪▪▪) or omitted from (〇〇〇) this reaction mixture as indicated. The temperature was 23°C and the pH was 7.3 5 μM FCCP together with rotenone (5 μM), and Triton X-100 (0.1% w/v) were added as shown.
From a series of experiments with ten different particle preparations, a value for the membrane potential of 145 mV (± 5 mV) was obtained with NADH as substrate in the standard Pi/Tris reaction mixture. The mean value of 145 mV is included in Table I.2 (experiment 1).

The open symbols in Fig. 4.2 are a plot of the radioactivity (\(^{14}C\) methylamine) in sequentially collected fractions of the outflow from the lower chamber of the flow-dialysis cell, in an experiment where the upper chamber contained \(^{14}C\) methylamine plus the standard Pi/Tris reaction mixture without submitochondrial particles. The rise in radioactivity over the first five fractions, and the ensuing slow decay in the level of radioactivity, is similar to, and has the same basis as, that seen with \(^{14}CN^-\) in the upper chamber (Fig. 4.1).

The closed symbols in Fig. 4.2 show an experiment in which 20 µM \(^{14}C\) methylamine was added to the upper chamber of the dialysis cell which also contained submitochondrial particles oxidising NADH. Subsequent addition of FCCP, together with rotenone, did not cause any efflux of \(^{14}C\) methylamine from the particles, as judged by the absence of a detectable increase in the level of radioactivity in the outflow from the lower chamber. The addition of FCCP alone cannot necessarily be guaranteed to reduce \(\Delta \psi\) or \(\Delta pH\) (or their sum) to zero in submitochondrial particles. Therefore rotenone was added with FCCP (Fig. 4.2) to inhibit respiration and thus slow the rate of proton translocation across the membrane of the particles. At low rates of proton translocation (rotenone present), FCCP, by carrying protons back across the membrane would be expected to reduce the steady-state \(\Delta pH\) or \(\Delta \psi\).
to a lower level than in the absence of a respiratory inhibitor. The precaution of adding rotenone was taken as the logarithmic nature of equation 3.2 means that the magnitude of $\Delta pH$ could be underestimated if FCCP were only partially to dissipate a small $\Delta pH$. If $\Delta pH$ (or $\Delta \psi$) is large, a small residual gradient left after addition of FCCP will not cause a significant error, as the amount of $\zeta^{-14}C_7$ methylamine or $S^{14}CN^-$ retained in the particles by any residual gradient will be very small relative to the amount of $\zeta^{-14}C_7$ methylamine or $S^{14}CN^-$ released upon adding FCCP. In practice, no extra efflux of either $\zeta^{-14}C_7$ methylamine of $S^{14}CN^-$ was observed upon adding rotenone in addition to FCCP. The conclusion that FCCP effectively dissipated $\Delta pH$ or $\Delta \psi$ is supported by the finding that no additional efflux of $\zeta^{-14}C_7$ methylamine or of $S^{14}CN^-$ was seen on adding a lytic amount of Triton X-100 (Figs. 4.1 and 4.2).

The open symbols (Fig. 4.2) show that addition of FCCP, rotenone of Triton X-100 had no effect on the rate of $\zeta^{-14}C_7$ methylamine transfer across the dialysis membrane when no submitochondrial particles were present in the upper chamber.

It is concluded from the data in Fig. 4.2 that respiration generated an essentially insignificant $\Delta pH$. The experiment shown in Fig. 4.2 was done at a high particle concentration so as to increase the extent of any $\zeta^{-14}C_7$ methylamine uptake (see Fig. 3.2). The reaction conditions were such that an uptake of 3% of the total added $\zeta^{-14}C_7$ methylamine would have been detected. The particle concentration was 12.5 mg of protein per ml (giving 16 µl of internal volume per ml), and from Fig. 3.2 it may be calculated that uptake of less
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<th>Substrate</th>
<th>Additions</th>
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<th>$-59 \Delta \mathrm{pH}$ (mV)</th>
<th>$\Delta \rho$ (mV)</th>
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<td>150</td>
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<td>125</td>
<td>n.d</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Succinate</td>
<td>0.3 mM ADP, 10 mM glucose, 20 units hexokinase</td>
<td>130</td>
<td>n.d</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4.2  The magnitude of the components of the protonmotive force measured under various conditions

$\Delta \psi$ and $\Delta$ pH were measured from the extent of $S^{14}CN^-$ or $\gamma^{14}C$ methylamine uptake using the flow dialysis procedure. The upper chamber of the flow dialysis cell contained in a volume of 1 ml: 10 mM P$_i$/Tris, 5 mM magnesium acetate and submitochondrial particles, plus other components as detailed below or in the Table. The pH was 7.3 and the temperature was 23°C. In experiments 1 and 8, 12 mg submitochondrial particle protein was present; in other experiments approximately 4 mg particle protein was used. For measurements of $\Delta \psi$ 20 μM K$^{14}$CN (60 μCi μmol$^{-1}$) was added to the upper chamber, while for measurements of $\Delta$ pH 20 μM $\gamma^{14}$C methylamine hydrochloride (55.5 μCi μmol$^{-1}$) was added). When NADH was the substrate, 0.6 mM NAD$^+$, 1% v/v ethanol and 0.05 mg alcohol dehydrogenase were added. When succinate was the substrate, 10 mM sodium succinate was added. For experiments in which either $\Delta \psi$ or $\Delta$ pH was not observed the lower limit of detection is signified by $<$. The values of $\Delta$ pH given in parentheses represent upper limits on $\Delta$ pH, obtained by adding the lower limit of detection for $\Delta$ pH or $\Delta \psi$ to the observed $\Delta$ pH or $\Delta \psi$. n.d. = not determined. (* 1 unit of hexokinase catalyses the phosphorylation of 1.0 umol glucose min$^{-1}$ at 25°C, pH 8.5).
than 3% of the methylamine sets an upper limit of $\Delta pH$ of approximately 0.5 units (±30 mV). At an equally high concentration of particles the $S^{14}CN^-$ accumulation ratio was similar to that observed with lower concentrations, which therefore indicated that anaerobiosis of such concentrated suspensions of particles was not occurring.

The addition of permeant ions to respiring submitochondrial particles is expected to cause a decrease in $\Delta \Psi$ and a compensating increase in $\Delta pH$ (Rottenberg & Lee 1975). Table 4.2 (experiments 2 and 3) shows that indeed the presence of either 10 mM KSCN or 10 mM KNO$_3$ resulted in the appearance of a substantial $\Delta pH$. (Evidence that nitrate can permeate the inner mitochondrial membrane has been given by Montal et al., (1970) and by Lehninger (1974)). When 10 mM KSCN was present no accumulation of $S^{14}CN^-$ inside the particles was detected in an experiment with sufficient particles to allow a $\Delta \Psi$ of 40 mV to have been detected. Experiment 4 (Table 4.2) shows that with 2 mM KNO$_3$ added to the standard reaction mixture (Pi/Tris) both $\Delta \Psi$ and $\Delta pH$ were detectable. The magnitude of the protonmotive force was virtually the same as the value of $\Delta \Psi$ measured in the absence of permeant ions (experiment 1). This suggests that the flow dialysis method was not failing to detect a small but significant $\Delta pH$ when only $\Delta \Psi$ could be measured (as in experiment 1, Table 2). The logarithmic nature of equations 3.1 and 3.2 means that the flow dialysis method is relatively insensitive to a very small $\Delta \Psi$ or $\Delta pH$, but if a $\Delta pH$ of as much as 0.5 units (±30 mV) had escaped detection in experiment 1, it might have been anticipated that the total protonmotive force would have been greater with 2 mM KNO$_3$ present (experiment 4) than
under standard conditions (experiment 1). The basis of this argument would be undermined if 2 mM KNO₃ had an uncoupling effect on the particles, but as shown later (Table 4.5), this concentration of KNO₃ did not reduce the magnitude of the phosphorylation potential generated by the submitochondrial particles and hence did not cause any uncoupling.

Δψ was not increased when K⁺ plus nigericin were present (Table 4.2, experiment 5) (cf. Chapter 3). Nigericin catalyses an electroneutral exchange of accumulated protons inside the particles for externally added K⁺, and so the effect of adding nigericin plus K⁺ should be to increase Δψ at the expense of ΔpH. (Ashton & Steinrauf, 1970; Montal et al., 1970; see also Chapter 3). The failure of K⁺ plus nigericin to increase is thus consistent with the absence of a significant ΔpH across respiring submitochondrial particles in the Pi/Tris reaction medium.

Experiment 6, Table 4.2, showed that inclusion of ADP in the standard reaction mixture did not change the magnitude of the respiration-dependent Δψ. Submitochondrial particles do not phosphorylate all the added ADP (Ferguson & Sorgato, 1977; Table 4.5), and the conditions in experiment 6 were such that the final extent of conversion of added to ATP would have been reached in less than one minute. Experiment 6 shows, therefore, that there is no energetic demand on the proton-motive force when there is no net ATP synthesis.

Oligomycin improves, probably by blocking a proton channel (Mitchell & Moyle 1974), the coupling properties of submitochondrial particles (e.g. EDTA-particles) that are deficient in ATPase molecules. Addition of oligomycin (experiment 7, Table 4.2) to the Mg-ATP particles used in the present work
resulted in only a slight increase in $\Delta \psi$, consistent with the view that Mg-ATP particles do not lose ATPase molecules during preparation.

**The effect of osmolarity and ionic strength on the magnitude of the components of the protonmotive force**

Most of the experiments described in this chapter were performed in reaction media of both low osmolarity and low ionic strength. However, reaction media of high osmolarity and/or high ionic strength are often used for work with submitochondrial particles, and it was therefore of interest to make some determinations of the protonmotive force with reaction conditions similar to those employed by other workers. Table 4.3 shows that in a Hepes/sucrose/KCl medium similar to that used by Thayer and Hinkle (1973) and by C.L. Bashford and W.S. Thayer (personal communication), the total protonmotive force was approximately 40 mV higher than in the Pi/Tris medium (Table 4.2). It is noteworthy that in medium A (Table 4.3) both $\Delta \psi$ and $\Delta \mathrm{pH}$ are of comparable magnitude. The relatively large $\Delta \mathrm{pH}$ (compare Table 4.2, experiment 1) can be attributed to the use of a relatively impermeable buffer, Hepes, and the inclusion of a high Cl$^-$ concentration which will presumably result in some accumulation of chloride ions at the expense of $\Delta \psi$. In calculating $\Delta \psi$ and $\Delta \mathrm{pH}$ in medium A, a particle internal volume of 1 μl per mg of protein was used. This value was obtained from an experiment similar to those detailed in Table 4.1 except that medium A (Table 4.3) was substituted for the Pi/Tris suspending medium.

Inclusion of nigericin in reaction medium A (Table 4.3) resulted in an increased $\Delta \psi$ at the expense of a decreased $\Delta \mathrm{pH}$, as expected in view of the mode of action of nigericin out-
<table>
<thead>
<tr>
<th>Reaction Medium</th>
<th>Addition</th>
<th>$\Delta \Psi$ (mV)</th>
<th>$-59 \Delta pH$ (mV)</th>
<th>$\Delta p$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A.</strong> 200 mM sucrose</td>
<td>None</td>
<td>90</td>
<td>95</td>
<td>185</td>
</tr>
<tr>
<td>10 mM Hepes-NaOH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM KCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mM KP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM$\text{MCl}_2$; pH 7.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nigericin ($0.5 \mu g$ per mg protein)</td>
<td>135</td>
<td>$&lt; 40$</td>
<td>135 (175)</td>
</tr>
<tr>
<td><strong>B.</strong> 180 mM sucrose</td>
<td>None</td>
<td>135</td>
<td>$&lt; 40$</td>
<td>135 (175)</td>
</tr>
<tr>
<td>30 mM Tris/acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mM magnesium acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM $P_1$-Tris pH 7.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C.</strong> 100 mM choline chloride</td>
<td>None</td>
<td>$&lt; 50$</td>
<td>100</td>
<td>100 (150)</td>
</tr>
<tr>
<td>1.5 mM Tris/chloride; pH 7.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.3  The magnitude of the components of the protormotive force measured in reaction media of differing osmolarity and ionic strength

Submitochondrial particles (approximately 4 mg protein) were incubated in one of the reaction mixtures listed, together with 0.6 mM NAL\(^+\), 1% v/v ethanol, and 0.05 mg alcohol dehydrogenase. The total volume was 1 ml and the temperature was 23°C. For measurement of $\Delta \Psi$ 20 $\mu$M K\(^{14}\)CN (60 $\mu$Ci $\mu$mol\(^{-1}\)) was added to the upper chamber, while for measurement of $\Delta \text{pH}$ 20 $\mu$M $\text{N}\text{N}^{-14}\text{C}$ methylamine hydrochloride (55.5 $\mu$Ci $\mu$mol\(^{-1}\)) was added. $\Delta \Psi$ and $\Delta \text{pH}$ were calculated from the extent of thiocyanate and methylamine uptake determined from flow dialysis measurements. The symbol $<$ and the values of \(\Delta \text{p} \) in parentheses have the significance in the legend to Table 4.2. Volatile amines were removed from medium C by evaporation under reduced pressure.
lined earlier.

Reaction B (Table 4.3) is similar to that used by Rottenberg and Lee (1975) except that Mg$^{2+}$ and Pi were included. It differs from medium A in that the ionic strength is lower, Cl$^-$ is replaced by the less permeant acetate ion, and Hepes is replaced by Tris as buffer. The protonmotive force generated in reaction medium B is similar to that developed in the Pi/Tris medium (Table 4.2, experiment 1) which was of comparable ionic strength but lower osmolarity.

Rottenberg and Lee (1975) reported that in reaction medium C (Table 4.3), $\Delta\psi$ was probably very small, but $\Delta$ pH was very substantial. I could not detect $\Delta\psi$ in this medium but the extent of respiration-dependent $^{14}$C methylamine uptake indicated a $\Delta$ pH of 1.6 units (≈ 100 mV). The appearance of $\Delta$ pH was attributed (Rottenberg & Lee, 1975) to the presence of a high concentration of Cl$^-$ and the virtual absence of a buffer.

Rottenberg & Lee (1975) determined $\Delta$ pH from the quenching of 9-amino acridine fluorescence, obtaining values of 2.2 units under conditions similar to those of medium B (Table 4.3) and 3.6 units in medium C (Table 4.3). These values for $\Delta$ pH are much larger than those obtained with the flow dialysis method (Table 4.3). Although part of this discrepancy may be because Rottenberg and Lee (1975) used a different type of particle preparation from that used in the present work, it seems that the 9-amino acridine fluorescence quenching method gives larger values for $\Delta$ pH than the methylamine uptake procedure. When measurements were made using the 9-amino acridine method, $\Delta$ pH appeared to be 2.8 units in medium A,
2.4 units in medium B and 3.0 units in the standard Pi/Tris reaction mixture plus 10 mM KNO₃. Comparison with results obtained from the extent of \( ^{-14}C_7 \) methylamine uptake (Tables 4.2 and 4.3) shows that the 9-amino acridine method gives values which are larger by about 1.5 units. In the standard Pi/Tris reaction mixture the extent of respiration-linked 9-amino acridine fluorescence quenching was very small (less than 1% of the total fluorescence) and so comparison of the two procedures for measuring \( \Delta \)pH was not possible.

The estimation of \( \Delta \)pH in the present work relies on methylamine uptake rather than on 9-amino acridine fluorescence quenching because doubts have been raised about the validity of the fluorescence method (Fiolet et al., 1974, 1975; Kraayenhof et al., 1976; Kraayenhof 1977) although there is evidence that in sonicated phospholipid vesicles the procedure is reliable (Deamer et al., 1972; Casadio & Melandri 1977). The recent report of Searle et al. (1977) that 9-amino acridine binds, with fluorescence quenching, as a cation to the surface of chloroplast thylakoid membranes may add weight to the reservations noted above (see also Chapter 7).

**The protonmotive force with succinate as substrate**

Submitochondrial particles generate the same phosphorylation potential with either NADH or succinate as substrate (Ferguson & Sorgato 1977), and Table 4.2 (experiments 1 and 8) shows that both substrates also established a \( \Delta \psi \) of similar magnitude. A figure of 0.5 units (\( \approx 30 \) mV) for \( \Delta \)pH has been given as an upper limit as no \( ^{-14}C_7 \) methylamine uptake was detected under conditions where a \( \Delta \)pH as low as 0.5 units would have been detected. The protonmotive force with succinate as substrate was measured only in the standard Pi/Tris reaction medium.
The protonmotive force with ascorbate plus an electron mediator as substrate

The purpose of the experiments in Table 4.4 was to determine the magnitude of the membrane potential that could be generated by electron flow through the cytochrome c/cytochrome aa₃ segment of the respiratory chain. In general, oxidation of ascorbate plus either TMPD or DAD generated a lower membrane potential (Table 4.4) than was observed with NADH or succinate as substrate. However, there was a greater variability in the size of the membrane potential linked to ascorbate oxidation. In two sets of experiments ascorbate oxidation generated a potential as high as 130 mV with either TMPD or 2,3,5,6-tetramethyl-p-phenylene diamine (DAD) as electron donor, and in later work membrane potentials of 140-150 mV were commonly obtained (data not shown).

Uptake of SCN⁻ driven by oxidation of ascorbate plus TMPD or DAD was measured in the presence of either antimycin or HQNO, which were added to inhibit any electron flow through the cytochrome b/cytochrome c₁ section of the electron transport chain. This ensured that electron flow was restricted to the cytochrome c/cytochrome aa₃ region of the chain. The titres of antimycin and 2-n-haptyl-4-hydroxyquinoline N-oxide (HQNO) necessary to inhibit electron flow between cytochromes b and c₁ were determined by titrating the rate of succinate oxidation with the two inhibitors polarographically. It is important to use the minimum titre of these two inhibitors as when present in significant excess they can act as uncouplers (see e.g. Wikström 1978). Measurements of Δρ were made with
<table>
<thead>
<tr>
<th>Electron carrier</th>
<th>Respiratory chain inhibitor</th>
<th>Concentration (μg per mg protein)</th>
<th>Δψ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMPD</td>
<td>Antimycin</td>
<td>0.2</td>
<td>90</td>
</tr>
<tr>
<td>TMPD</td>
<td>HQNO</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>TMPD</td>
<td>Antimycin plus HQNO</td>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td>DAD</td>
<td>Antimycin</td>
<td>1</td>
<td>95</td>
</tr>
</tbody>
</table>
Membrane potentials generated by ascorbate oxidation in submitochondrial particles

Submitochondrial particles were incubated (approximately 6 mg protein) in a reaction mixture that contained the following components in the upper chamber of the flow dialysis cell: 10 mM P$_i$/Tris, 5 mM magnesium acetate, 10 mM sodium D-isoascorbate and 20 μM KS$_{14}$CN (60 μCi μmol$^{-1}$). Respiratory chain inhibitors were added as indicated. The reaction was started by adding either TMPD (0.1 mM) or DAD (0.1 mM). The pH, measured potentiometrically, remained at 7.3 throughout the experiments.
either antimycin or HQNO present as it has been suggested (Eisenbach & Gutman 1975; Mitchell 1976b; Papa et al., 1977, 1978) that these two inhibitors may have different modes of action, despite the indications that they share a common binding site (Brandon et al., 1972; van Ark and Berden 1977). The combined effect of both antimycin and HQNO was also studied, since Papa et al., (1977, 1978) have suggested that only when these two inhibitors are added together is electron flow through the cytochrome b/cytochrome c\(_1\) region of the respiratory chain completely inhibited. Table 4.4 shows that with TMPD as electron donor similar values of $\Delta \psi$ were found with either or both of the inhibitors present.

Oxidation of ascorbate plus either TMPD or DAD did not generate a detectable $\Delta \text{pH}$ when the submitochondrial particles were suspended in the standard Pi-Tris reaction mixture. Moreover, whereas with NADH or succinate as substrate $\Delta \text{pH}$ was formed when the permeant nitrate ion was added to the standard reaction mixture (Table 4.2), no $\Delta \text{pH}$ linked to oxidation of ascorbate plus TMPD was detected even when 5 mM KNO\(_3\) was included in the same reaction mixture. The latter experiment was done under conditions where a $\Delta \text{pH}$ of 0.5 units could have been detected, and in a separate experiment it was shown that 5 mM KNO\(_3\) did not inhibit the oxidation of ascorbate plus TMPD. Attempts to detect a $\Delta \text{pH}$ linked to ascorbate plus TMPD oxidation with 50 mM KCl and valinomycin (0.5 $\mu$g/mg particle protein) added to the standard Pi/Tris reaction mixture were also unsuccessful under conditions where a pH gradient of 0.5 units would have been detected. Valinomycin and KCl were added to allow full expression of the
The protonmotive force is expressed as $\Delta pH$ (Thayer & Hinkle 1973). Oxidation of ascorbate (a 2 electron plus 1 proton donor at pH 7.0) at neutral pH can result in an alkalinisation of the reaction medium, followed by an acidification due to hydrolysis of dehydroascorbate. In these experiments, a well-buffered reaction medium was used, and no pH changes were detected during ascorbate oxidation. Thus the failure to detect a $\Delta pH$ (expected to be more acid inside the particles) was not due to a decrease in the external pH.

Comparison of the phosphorylation potential with the proton-motive force

Data from experiments in which $\Delta G_p$ (see Chapter 3) was compared with $\Delta p$ under several different sets of reaction conditions are given in Table 4.5. In the standard Pi/Tris reaction medium oxidation of either HADH or succinate generated similar values for both $\Delta G_p$ and $\Delta p$. The magnitude of $\Delta G_p$ generated by the particles used for experiments shown in Table 4.5 was slightly lower than was found, under similar conditions, in earlier work (Ferguson & Sorgato 1977). A lower $\Delta G_p$ was not due to the presence of either 20 $\mu$M KSCN or 20 $\mu$M methylamine both of which had no effect on $\Delta G_p$. Some preparations of particles gave higher values of $\Delta G_p$ (up to 44.3 kJ mol$^{-1}$ / 10.6 kcal mol$^{-1}$) but an unchanged $\Delta p$, so that there appeared to be some variation in the exact value of $\Delta G_p$ between different preparations of particles.

Substitution of the values of $\Delta p$ and $\Delta G_p$ into equation 4 gives a value of 3.1 for the $\rightarrow H^+$/ATP ratio with either NADH or succinate as substrate in the Pi/Tris reaction medium. No $\Delta pH$ was detected under these conditions (Table 4.2) when
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction medium</th>
<th>$\Delta p$ (mV)</th>
<th>$\Delta G_p$/kJ mol$^{-1}$ (kcal mol$^{-1}$)</th>
<th>$\Delta H^+/\Delta ATP$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>10 mM Pi/Tris, 5 mM magnesium acetate, pH 7.3</td>
<td>145 (175)</td>
<td>43.1</td>
<td>(10.3) 3.1 (2.6)</td>
</tr>
<tr>
<td>NADH</td>
<td>10 mM Pi/Tris, 5 mM magnesium acetate, 2 mM KNO$_3$, pH 7.3</td>
<td>150</td>
<td>43.1</td>
<td>(10.3) 3.0</td>
</tr>
<tr>
<td>Succinate</td>
<td>10 mM Pi/Tris, 5 mM magnesium acetate, pH 7.3</td>
<td>140 (170)</td>
<td>42.2</td>
<td>(10.1) 3.1 (2.6)</td>
</tr>
<tr>
<td>NADH</td>
<td>200 mM sucrose, 10 mM Hepes/NaOH, 50 mM KCl, 5 mM KPi, 2 mM MgCl$_2$, pH 7.5</td>
<td>185</td>
<td>43.1</td>
<td>(10.3) 2.4</td>
</tr>
<tr>
<td>NADH</td>
<td>180 mM sucrose, 30 mM Tris/acetate, 5 mM Mg acetate, 10 mM, Pi/Tris, pH 7.3</td>
<td>135 (175)</td>
<td>43.5</td>
<td>(10.4) 3.3 (2.6)</td>
</tr>
</tbody>
</table>
Parallel experiments were done with the same preparation of submitochondrial particles to determine both the protonmotive force and the phosphorylation potential under the same set of conditions. For determination of the protonmotive force, submitochondrial particles (approx. 4 to 8 mg of protein) were incubated in one of the reaction mixtures listed, together with 0.2 mM ADP. The total volume was 1 ml, and the temperature was 23°C. For measurement of \( \Delta \psi \), 20 \( \mu \)M KSCN (60 \( \mu \)Ci/\( \mu \)mol) was added; for measurements of \( \Delta p \), 20 \( \mu \)M methylamine hydrochloride (55.5 \( \mu \)Ci/\( \mu \)mol) was added. When NADH was the substrate, 0.6 mM NAD, 1% v/v ethanol and 0.05 mg alcohol dehydrogenase were added; when succinate was the substrate, 10 mM sodium succinate was added. The phosphorylation potential was determined by incubating submitochondrial particles (approx. 1.5 mg of protein) in the appropriate reaction mixture to which were added 0.2 mM ADP and 20 \( \mu \)M KSCN. The presence of 20 \( \mu \)M KSCN did not reduce the phosphorylation potential but was routinely omitted. The temperature was 23°C, and the total volume 3 ml. The experiments were done in a cell which was fitted with a Clark-type oxygen electrode. The reaction mixtures
Table 4.5 continued

were open to the atmosphere and the response of the electrode showed that they did not become anaerobic during the five-minute period in which the particles were allowed to phosphorylate added ADP. At the end of this period (which has been previously shown to be sufficient to allow the maximum extent of ADP phosphorylation to be reached (Ferguson & Sorgato 1977)), 2 ml of the reaction mixture were added to 0.2 ml of ice-cold 40% HClO₄. The acid extracts were left on ice for 10 min., and then the precipitated protein was removed by centrifugation at 2000g. The supernatants were neutralised by addition of the predetermined amount of 0.25M Tris/10% (w/v) KOH, and EDTA was also added to a final concentration of 2 mM. ATP in the neutralised extracts was determined with hexokinase and glucose-6-phosphate dehydrogenase as described by Bergmeyer (1970). In calculating \( \Delta G_p \) a value for \( \Delta G^0 \) of 30.1 kJ mol\(^{-1}\) (7.2 kcal mol\(^{-1}\)) was used (Rosing & Slater 1972). Under conditions in which a pH gradient could not be detected the value of \( \Delta p \) given comprises \( \Delta \psi \) alone. For these experiments a value of \( \Delta p \) assuming \( \Delta \) pH equivalent to 30 mV (or in one case 40 mV), which we regard as upper limits, is also shown in parentheses. Similarly the values for \( \Delta H^+/ATP \) shown in parentheses also include an estimate of 30 mV (or 40 mV) for \( \Delta \) pH in the total protonmotive force.
the lower limit of detection for \( \Delta \) pH was around 0.5 pH units which is equivalent to 30 mV. The values shown in parentheses for \( \Delta p \) and the \( \rightarrow H^+/ATP \) ratio (Table 4.5) thus represent respectively upper limits on \( \Delta p \) and lower limits for \( \rightarrow H^+/ATP \).

When the particles were suspended in a sucrose/Tris-acetate reaction medium, the values for \( \Delta p \) and \( \Delta G_p \) were very similar to those obtained in the Pi/Tris reaction medium (Table 4.5). The lower limit of detection for \( \Delta \) pH in the experiment with a sucrose/Tris-acetate reaction medium was judged to be 0.65 pH units, and so the values of \( \Delta p \) and \( \rightarrow H^+/ATP \) in parentheses were obtained by including a contribution of 40 mV for the \( \Delta \) pH.

It was shown in Table 4.2 that addition of 2 mM KNO\(_3\) to the Pi/Tris reaction medium resulted in a decrease in the value of \( \Delta \psi \) with a compensating increase in \( \Delta \) pH. Table 4.5 shows that particles respiring with NADH as substrate in the presence of 2 mM KNO\(_3\) generated a \( \Delta G_p \) of the same magnitude as in the absence of 2 mM KNO\(_3\). The \( \rightarrow H^+/ATP \) ratio in the presence of 2 mM KNO\(_3\) was thus 3.0, and under those reaction conditions there was no uncertainty about a failure to detect a very small \( \Delta \psi \) or \( \Delta \) pH as both these variables have values (Table 4.2) that are easily detected by the flow dialysis method. As \( \Delta G_p \) was not lowered by the addition of 2 mM KNO\(_3\), and as \( \Delta p \) (\( \Delta \psi - 59 \Delta \) pH) in the presence of KNO\(_3\) was very similar to the \( \Delta p \) (\( \Delta \psi \) only) in the absence of KNO\(_3\), it seems probable that, as suggested earlier, \( \Delta \psi \) is the sole component of \( \Delta p \) in the Pi/Tris reaction medium.
Although $\Delta p$ was found to be as much as 35 mV larger in the Hepes-sucrose-KCl reaction medium (Tables 4.2 and 4.5), the magnitude of $\Delta G_p$ generated under these conditions was no larger than in the Pi/Tris reaction medium. Hence the $\rightarrow H^+/ATP$ ratio was lower in the Hepes-sucrose-KCl medium.

Ascorbate oxidation with either TMPD or DAD as mediator did not generate a significant $\Delta G_p$. It was found that virtually all the added ADP was recovered as AMP. A rather low value of $\Delta G_p$ might be expected with ascorbate as substrate since a $\Delta p$ of 80 to 90 mV means that, in comparison with NADH or succinate as substrate, $\Delta G_p$ should be reduced by approximately one half to $5.1 \text{ kcal mol}^{-1}$. Presumably when ascorbate is being oxidised the ATPase is able to hydrolyse the ATP that is produced, together with AMP, by the adenylate kinase reaction (Fig. 1.1). Hence the final product is AMP. However, TMPD-mediated ascorbate oxidation did support the synthesis of ATP with a glucose and hexokinase trap present (the rate was 50 nmol min$^{-1}$ mg$^{-1}$ protein), and so it appears that the protonmotive force on the ATPase is sufficient to drive ATP synthesis at only very low prevailing phosphorylation potentials. This contrasts with the situation in intact rat liver mitochondria where ascorbate plus TMPD has been shown to generate an extra-mitochondrial phosphorylation potential of very similar magnitude to that generated by either NADH or succinate oxidation (van Dam et al., 1977). van de Stadt et al., (1973) indirectly showed that the oxidation of ascorbate plus TMPD in submitochondrial particles maintained a lower protonmotive force than either NADH or succinate oxidation, as
they found that activation of the ATPase by the protonmotive force ('energy pressure' in their terminology) was markedly less with ascorbate plus TMPD as substrate.

An alternative explanation for the inability of ascorbate/TMPD to generate a high phosphorylation potential even when it generated a high protonmotive force (145-150 mV) is offered in Chapter 7.

Rate of ATP synthesis and magnitude of the protonmotive force

Submitochondrial particles catalyse NADH-driven ATP synthesis at about twice the rate of succinate-driven ATP synthesis (see e.g. Thayer & Hinkle 1975; Ferguson & Sorgato 1977). As a possible relationship between the protonmotive force and the rate of ATP synthesis is of interest (Nicholls 1974; Portis & McCarty 1974, 1976; Baccarini-Melandri et al., 1977; Ferguson 1977; Schönfeld & Neumann 1977), the magnitude of the protonmotive force was determined during ATP synthesis driven by either NADH or succinate. Table 4.2 (experiments 9 and 10) shows that Δp was similar with both substrates, but was around 15 mV lower than the value when no ATP synthesis was occurring. Thus in submitochondrial particles ongoing ATP synthesis puts an energetic demand on the protonmotive force just as in other systems such as mitochondria (Nicholls, 1974; Rottenberg 1975) and chloroplasts (Pick et al., 1973) and chromatophores (Chapter 3). Assuming that only a very small or zero ΔpH is present during ATP synthesis driven by either NADH or succinate oxidation, it is concluded that although the rate of ATP synthesis is lower with succinate than with NADH as substrate, the protonmotive force does not decrease upon replacing NADH by succinate (see also Chapter 5).
DISCUSSION

Respiring submitochondrial particles have been shown to generate a phosphorylation potential of around 14.3 kJ mol\(^{-1}\) (10.6 kcal mol\(^{-1}\)). It has been argued that this relatively low value is not because these submitochondrial particles are poorly coupled relative to systems capable of generating a higher phosphorylation potential (Ferguson & Sorgato 1977). The phosphorylation potential generated by submitochondrial particles is of similar magnitude to the intramitochondrial phosphorylation potential, and thus it has been suggested that the maximum phosphorylation potential observed with particles reflects the limit to which the mitochondrial ATP-synthesising apparatus can drive the formation of ATP (Ferguson & Sorgato 1977) and that the difference between the intra- and extramitochondrial phosphorylation potentials is due to the energy expended in adenine nucleotide (and perhaps phosphate) translocation (Brand 1977; Brand & Lehninger 1977; Ferguson & Sorgato 1977; Klingenberg & Rottenberg 1977). This suggestion requires that the protonmotive force (which, according to the chemiosmotic hypothesis, drives ATP synthesis) in submitochondrial particles is not markedly lower than in other energy coupling systems, as the relationship between the protonmotive force and phosphorylation potential (equation 3.3) shows that a low protonmotive force alone would account for a low phosphorylation potential.

The experiments described in this chapter show that the protonmotive force generated by submitochondrial particles is in the range 145-185 mV, the exact value depending on the osmolarity and ionic composition of the medium in which the
particles are suspended. A proton motive force in this range is comparable with some of the estimates that have been made for other systems. For example Padan and Rottenberg (1973) and Wiechmann et al., (1975) have obtained values of 160 mV and 174 mV respectively for respiring rat liver mitochondria. Furthermore, chromatophores from Rhodospirillum rubrum and phosphorylating vesicles from Paracoccus denitrificans generate proton motive forces of 100 mV and 145 mV respectively (Chapters 3, 5). Yet although the proton motive force in sub-mitochondrial particles is similar to its counterpart in other systems, the phosphorylation potential is distinctly lower than is found with either the chromatophores or the bacterial vesicles for which \( \Delta G_p \) values of between 54 and 59 kJ mol\(^{-1}\) (13 and 14 kcal mol\(^{-1}\)) have been found. Hence it appears that the phosphorylation potential generated by sub-mitochondrial particles is not restricted to a low value by an unusually low proton motive force, thus supporting the arguments of Ferguson and Sorgato (1977).

Comparison of the proton motive force with the phosphorylation potential generated by sub-mitochondrial particles enables a value to be estimated for the number of protons (\( \rightarrow H^+/ATP \) ratio) that would be translocated across the mitochondrial membrane via the ATPase enzyme for each molecule of ATP synthesised, provided that a chemiosmotic mechanism is operating, and assuming that equilibrium is reached between the proton motive force and the phosphorylation potential. Values for the \( \rightarrow H^+/ATP \) ratio in the range 2.4 and 3.1 are obtained in this way (Table 4.5). Thus the present data indicate that the value of this ratio is three, whereas some previous work is consistent with a value of two. The evidence for an \( \rightarrow H^+/ATP \)
ratio of two is, briefly, as follows.

Thayer and Hinkle (1973) measured the \( \text{H}^+ \) uptake into submitochondrial particles which followed the addition of a pulse of ATP to the particles at a pH (6.2) at which no \( \text{H}^+ \) release was associated with the ATP hydrolysis reaction. It was found that the \( \rightarrow \text{H}^+/\text{ATP} \) ratio approached two, and so the results of kinetic experiments would seem to be at variance with the present data, which were thermodynamic measurements (see Chapters 1, 7). A possible explanation for this discrepancy is that the \( \rightarrow \text{H}^+/\text{ATP} \) ratio is lower at the pH of 6.3 employed by Thayer and Hinkle (1973) than at pH 7.3 which was used in our work. Studies on active transport by bacterial membrane vesicles have indicated a dependence on pH of the \( \rightarrow \text{H}^+/\text{substrate} \) ratio (Ramos & Kaback 1977b). However, Moyle and Mitchell (1973a) also obtained an \( \rightarrow \text{H}^+/\text{ATP} \) ratio of two using a similar experimental approach to that of Thayer and Hinkle except that the measurements were made with rat liver submitochondrial particles and the pH was 7.3, which meant that pH changes due to the scalar hydrolysis of ATP had to be corrected for.

An \( \rightarrow \text{H}^+/\text{ATP} \) ratio of two for the mitochondrial ATPase is also indicated by the recent work of Brand and Lehninger (1977) who carefully determined the value of this ratio in experiments in which pulses of ATP were added to rat liver mitochondria under conditions where any complicating transmembrane movements of phosphate were inhibited. Nevertheless, the determination of the \( \rightarrow \text{H}^+/\text{ATP} \) ratio from experiments with whole mitochondria is complicated by the necessary involvement of the adenine nucleotide carrier in the overall process of hydrolysing added ATP, and thus there may be proton movements
associated with ATP hydrolysis by mitochondria which arise from processes other than the simple hydrolysis of ATP.

Agreement between the values of the $\frac{H^+}{ATP}$ ratio estimated from kinetics and thermodynamic experiments would be expected if energy coupling proceeds via a purely chemiosmotic mechanism. However, if membrane-bound protons (e.g. Gould and Cramer 1977; Williams 1977, 1978a, b; Chapter 7) were also to participate in coupling, measurements of the bulk protonmotive force might underestimate the true thermodynamic potential of the energised state, and so lead to an overestimate of the $\frac{H^+}{ATP}$ ratio. Kinetic measurements of $H^+$ uptake, on the other hand, would presumably measure the total number of protons moved into or across the membrane, and thus give the number of protons that participate in the ATPase reaction (see also Chapter 7).

Returning to the widely held view that coupling does occur via a chemiosmotic mechanism (Mitchell 1977a), one must consider that an $\frac{H^+}{ATP}$ ratio of two for the ATPase would become consistent with the results presented in this chapter if either the protonmotive force had been underestimated or the value for the phosphorylation potential is an overestimate. The latter is unlikely as the value of the phosphorylation potential depends on measuring concentrations of ATP, ADP and Pi, all of which can be done with relative accuracy. In addition a value for $\Delta G^\circ$ must be known. I have used the data of Rosing and Slater (1972) which is one of the lowest estimates in the literature; another recent determination (Guynn & Veech 1973) gave a value for $\Delta G^\circ$ that was approximately $2.5 \text{ kJ mol}^{-1}$ (0.6 kcal mol$^{-1}$) higher than the value
of Rosing and Slater (1972) (see also Table 1.1). Thus it is possible that the values of the phosphorylation potential reported in Table 4.5 should be increased by this amount (but see discussion of Slater (1976)), which would result in a small increase in the $\text{H}^+/\text{ATP}$ ratio.

Underestimation of the protonmotive force could arise from use of a value for the internal volume that is too high (see figure 3.2 and equations 3.1 & 3.2). For instance, if the value of 1.3 μl per mg of protein were a two-fold overestimate, then the values for both $\Delta \Psi$ and $\Delta \text{pH}$ (Tables 4.2-4.5) would have to be increased by 18 mV. The addition of an extra 36 mV to the protonmotive force measured in the sucrose/Hepes/KCl reaction medium (Table 4.5) would raise the protonmotive force to 220 mV and lower the $\text{H}^+/\text{ATP}$ ratio to approximately two. The $\text{H}^+/\text{ATP}$ ratio with 2 mM KNO$_3$ present would also be reduced, from 3.0 to 2.4. Thus for all the data presented in this chapter to become consistent with an $\text{H}^+/\text{ATP}$ ratio of two, the error in overestimating the internal volume would have to be of the order of three-fold. Failure of the flow dialysis technique to measure the full extent of $\text{S}^{14}\text{CN}^-$ or $\text{C}^{14}_{7}$ methylamine uptake accurately would also lead to an underestimation of the protonmotive force, but I have argued earlier why I consider that the full extent of uptake was measured in these experiments.

An additional source of error in this work would arise if the assumptions made in using equations 3.1 and 3.2 were invalid. Portis and McCarty (1973) have pointed out that at pH 7.3 the concentration of the unprotonated form of methylamine may be so low that the assumption of a much higher membrane permeability towards the uncharged rather than the charged may
no longer be justified. However, Casey et al., (1977) have recently shown that the uptake of $^{14}C_7$ methylamine into non-energised chromaffin granules leads to an estimate of the intragranular pH that is very close to the value obtained from observation of pH-dependent chemical shifts of the $^{31}P$ nuclear magnetic resonance signals from ATP molecules inside the granules (Ritchie 1976). As the external pH at which the granules were suspended was 6.5, it may be concluded that the presence of only a very small fraction of the methylamine as the free base does not introduce significant errors into this method for determining pH gradients. Further, I have determined the pH gradient across the membranes of the chromaffin granules used by Casey et al., (1977) (kindly provided by Dr D. Njus) and obtained a similar value by flow dialysis of $^{14}C_7$ methylamine uptake to that reported by Casey et al., (1977) (data not shown).

Evidence that $^{14}CN^-$ reaches an equilibrium distribution with the membrane potential is that the accumulation ratio $[^{14}CN^-]/[^{14}CN^-]$ in/[^{14}CN^-] out is independent of the total $^{14}CN^-$ concentration. It is also significant in view of the different assumptions which underlie the use of equations 3.1 and 3.2 that almost quantitative conversion of $^{14}CN^-$ into $^{14}$pH can be achieved by increasing the concentration of KNO$_3$ or KSCN (Table 4.2). Failure to demonstrate complete quantitative interconversion was probably due to the chaotropic SCN$^-$ and NO$_3^-$ ions damaging the submitochondrial particles at the concentrations required ( $\geq$ 10 mM) (see Chapter 7) as judged by the slightly lowered phosphorylation potential that was observed with either 10 mM KSCN or KNO$_3$. However, with Rhodospirillum rubrum chromatophores, addition of 10 mM KSCN results in a quantitative conversion of
ΔΨ to ΔpH (Chapter 3), an observation that provides support for the contention that $^{14}S$ and $^{14}C$ methylamine uptake are reliable indicators of ΔΨ and ΔpH in membrane vesicles.

A final reason why I might have underestimated the protonmotive force is that the preparations of submitochondrial particles might contain a significant proportion of uncoupled and therefore non-phosphorylating, particles. Ferguson and Sorgato (1977) have discussed previously the abundant evidence that Mg-ATP particles appear to be relatively homogeneous with respect to their coupling properties.

Thus the present data indicate that the $\rightarrow H^+/ATP$ ratio for the mitochondrial ATPase is three, and indeed there are some cogent reasons for supposing that the ratio is three. Rottenberg and Gutman (1977) have concluded that an $\rightarrow H^+/ATP$ ratio of three together with an $\rightarrow H^+/2e^-$/site ratio of four would best explain their data on the energetics of ATP-driven reversed electron flow from succinate to NAD in submitochondrial particles. Indications that the $\rightarrow H^+/2e^-$/site ratio could be as high as four have been obtained (Brand 1977; Brand et al., 1976a, b; Reynafarje et al., 1976; Reynafarje and Lehninger 1977), and the possibility has been recognised that the $\rightarrow H^+/ATP$ ratio for the mitochondrial ATPase in vivo might be three, with one proton being used to drive energy-linked transport of substrates across the mitochondrial membrane (Brand 1977; Brand & Lehninger 1977) (see also Chapter 7).

The ATPase of the thylakoid membrane in chloroplasts is structurally closely related to the mitochondrial ATPase (Postwa and van Dam 1976) and considerable evidence has accrued that the $\rightarrow H^+/ATP$ ratio for the thylakoid ATPase is three, as judged by several independent methods (Avron et al., 1976; McCarty & Portio 1976; Junge 1977; Hasiska and Trebst 1977).
McCarty and Portis (1976) have previously suggested that the discrepancy between an $\text{H}^+/\text{ATP}$ ratio of three for thylakoid ATPase and two for mitochondrial ATPase is disquieting. An $\text{H}^+/\text{ATP}$ ratio of three for the mitochondrial ATPase seems attractive in view of the foregoing considerations, although if this is the case and if the overall $\text{H}^+/\text{ATP}$ ratio for synthesis of extramitochondrial ATP is also three (Nicholls 1974; Wiechmann et al., 1975), then accommodating the charge movement that is associated with the adenine nucleotide translocator (Klingenberg & Rottenberg 1977) poses a problem.

A very similar protonmotive force is generated when either NADH or succinate is oxidised by submitochondrial particles (Table 4.2). As the rate of succinate oxidation is only approximately 66% of the NADH oxidation rate in these particles (Ferguson & Sorgato 1977), and allowing for the smaller number of protons that are translocated for each molecule of succinate oxidised, it is evident that the same protonmotive force can be maintained over a 2.5-fold range of proton-pumping rates. This is consistent with the idea that the proton conductance of the membrane in submitochondrial particles is non-ohmic at higher respiratory rates, just as has been shown for the inner membrane of mitochondria from rat liver and adipose tissue (Nicholls 1974, 1977b; see Chapter 6). The rate of ATP synthesis by submitochondrial particles depends on whether NADH or succinate is substrate, and typically the rate with NADH is twice that found with succinate (Thayer & Hinkle 1975; Ferguson & Sorgato 1977). As the size of the protonmotive force does not depend on whether NADH or succinate
is driving ATP synthesis (Table 4.2), there does not seem to be a simple relationship between the size of the protonmotive force and the rate of ATP synthesis (cf. Chapter 5). This result contrasts with the reports of a close relationship between the protonmotive force and the rate of ATP synthesis in thylakoids (Graber & Witt, 1976; McCarty & Portis 1976; Schonfeld & Neumann 1977).

Hauska et al., (1977) have pointed out that as TMPD is an electron carrier, oxidation of ascorbate plus TMPD should not be able to drive energy-linked reactions via a protonmotive force in submitochondrial particles, provided that the terminal segment of the respiratory chain is organised according to the generally held view (Scheme 7.1a). However, earlier work has indicated that oxidation of ascorbate plus TMPD can be coupled both to the generation of a membrane potential (Grinius et al., 1970), and to the synthesis of ATP (Tyler et al., 1966). It has been suggested that, in the experiments of Grinius et al., (1970) oxidation of ascorbate was not responsible for the membrane potential, as the measurements were made in the presence of succinate and antimycin, so that TMPD might have been acting as an internal bypass of the antimycin binding site and thus the membrane potential could have been linked to a TMPD-mediated oxidation of succinate (Hauska et al., 1977). From a re-examination of the antimycin sensitivity of ascorbate plus TMPD-driven ATP synthesis (Hauska et al., 1977) it has also been suggested that the entry of electrons into the respiratory chain at the level of cytochrome b is responsible for the observed ATP synthesis, and that electron flow from cytochrome c to oxygen is not coupled to ATP synthesis (Hauska et al., 1977).
Scheme 1a

Oxidation of ascorbate mediated by TMPD in subcellular particles, assuming no proton translocation linked to electron flow between cytochrome c and cytochrome oxidase.

\[
\begin{align*}
\text{粒子} & \\
\text{lumen} & \\
\end{align*}
\]

\[
\begin{align*}
\text{中性} & \\
\text{质子} & \\
\end{align*}
\]

\[
\begin{align*}
\text{中性} & \\
\text{氧化} & \\
\end{align*}
\]

\[
\begin{align*}
\text{粒子} & \\
\text{lumen} & \\
\end{align*}
\]

All⁺ is ascorbate, A is dehydroascorbate, WB⁺ is Wurster’s Blue.

Scheme 1b

Oxidation of ascorbate mediated by TMPD in subcellular particles, assuming a proton pump activity associated with cytochrome oxidase.

\[
\begin{align*}
\text{粒子} & \\
\text{lumen} & \\
\end{align*}
\]

\[
\begin{align*}
\text{中性} & \\
\text{质子} & \\
\end{align*}
\]

\[
\begin{align*}
\text{中性} & \\
\text{氧化} & \\
\end{align*}
\]

\[
\begin{align*}
\text{粒子} & \\
\text{lumen} & \\
\end{align*}
\]

All⁺ is ascorbate, A is dehydroascorbate, WB⁺ is Wurster’s Blue.
Doubt has been cast on the conclusions of Hauska et al. (1977), by Wikstrom (1978b) who has pointed out that the levels of antimycin used to inhibit ascorbate driven ATP synthesis were in excess of those needed to inhibit electron flow between cytochrome b and cytochrome c₁, and were likely to inhibit ATP synthesis by a secondary uncoupling effect. The data presented in Table 4.4 also suggest that the conclusion of Hauska et al., is not correct, as a significant membrane potential linked to the oxidation of ascorbate plus TMPD is observed in the presence of sufficient HQNO or antimycin to block electron flow between cytochrome b and c₁. Thus there is good reason to believe that electron flow from cytochrome c to oxygen is intrinsically coupled to the production of a protonmotive force, and that Scheme 1a must be modified.

Wikstrom (1977, 1978; Wikstrom and Saari 1977) has presented evidence that cytochrome oxidase has a proton-pumping activity, and incorporation of this feature into the terminal segment of the respiratory chain is shown in Scheme 1b. It can be seen that the oxidation of ascorbate plus TMPD is expected to generate a protonmotive force according to Scheme 1b, and so the data presented in this chapter would appear to support the concept of proton pumping activity associated with cytochrome oxidase. Scheme 1b predicts that the oxidation of ascorbate plus TMPD should be linked to the production of Δψ and/or ΔpH, the relative magnitudes of these two components depending on the reaction conditions. However, an enigmatic feature of our experiments was that we were unable to observe a pH gradient linked to ascorbate plus TMPD oxidation even under conditions (presence of 5 mM KNO₃ or 50 mM KCl plus...
valinomycin) which should have increased $\Delta$ pH at the expense of $\Delta \Psi$. Hence although Wikstrom & Saari (1977) have found evidence for proton pumping associated with oxidation of ascorbate plus TMPD in submitochondrial particles, the present experiments do not directly confirm this proposal, although it is possible that a small $\Delta$ pH escaped detection in these experiments.

The generation of a membrane potential linked to ascorbate plus TMPD oxidation could become consistent with Scheme 1a if preparations of TMPD were contaminated with a demethylated derivative (P.C. Hinkle, personal communication). The data given in Table 4 were obtained using recrystallised TMPD, although no difference in the magnitude of the membrane potential was noted when unpurified TMPD was used. Thus I do not think that my results can be explained on the basis that the TMPD contained substantial amounts of demethylated TMPD that could act as a proton-plus-electron carrier in an analogous manner to DAD (Hauska et al., 1977). A membrane potential could also be generated if the positively charged Wurster's Blue were accumulated inside the particles, but this seems unlikely as the potential was dissipated by an uncoupler, which is expected to reverse a proton gradient.

Papa et al., (1977, 1978) have suggested that the proton pumping activity assigned to cytochrome oxidase (Wikstrom 1977, 1978; Wikstrom and Saari 1977) is really associated with the cytochrome $b_{6}$ region of the respiratory chain. This proposal was prompted by the finding that HQNO, but not antimycin, inhibited proton movements during oxidation of ferrocyanide by mitochondria, and thus it was proposed that ferrocyanide donated electrons not only to cytochrome $c$, as assumed by
Wikstrom (1977), but also to redox carriers in the cytochrome bc₁ region of the respiratory chain. Our experiments showed that addition of sufficient HQNO to inhibit electron flow from cytochrome b to cytochrome c₁, in either the presence or absence of antimycin, did not inhibit the generation of a membrane potential linked to ascorbate-plus-TMPD oxidation. Thus it is unlikely that TMPD was donating electrons to a putative proton-pumping redox carrier in the cytochrome bc₁ region of the respiratory chain. This conclusion is supported by the work of Jasaitis et al., (1972), who showed that, in reconstituted cytochrome oxidase vesicles with internal cytochrome c, TMPD-mediated ascorbate oxidation was linked to generation of a membrane potential.

After completion of most of the experimental work described in the present chapter it was learned that van Dam et al., (1977) were making some very similar measurements of the protonmotive force in submitochondrial particles. With NADH as substrate they report a ΔV of 179 mV with no detectable ΔpH. A comparison of their data with those of the present chapter will be of value later when full details of their experiments are available.
METHODS

Submitochondrial particles. Mg-ATP bovine heart submitochondrial particles (Low & Vallin 1963) were prepared by the method of Ferguson et al., (1977) for making ATPase-inhibitor-depleted particles (Type II particles in the nomenclature of Ferguson et al., 1977). The particles were stored at 0°C as a concentrated suspension in the reaction medium that was to be used in the experiments. (Occasionally the particles were suspended in a medium containing 20 mM-Tris/HCl buffer, pH 7.4, 225 mM-mannitol and 75 mM-sucrose. No experimental difference was noted between particles which were suspended in the other reaction media). The experiments were usually completed within six hours of preparing the particles, although very little change in the membrane potential generated by the particles was seen over a period of 18 hours. Protein was determined by the Biuret method (Gornall et al., 1949).

Determination of the protonmotive force

A flow dialysis assay of K\textsubscript{14}CN and \textsuperscript{14}C\textsubscript{7} methylene uptake was carried out in the apparatus described in Chapter 3, in reaction media which are given in the legends to the Figures and Tables.

MATERIALS

All radioisotopes were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. K\textsubscript{14}CN and \textsuperscript{14}C\textsubscript{7} methylene hydrochloride were made up carrier-free to give stock solutions of 2.08 mM and 2.25 mM (60 and 55.5 mCi mmol\textsuperscript{-1}) respectively. \textsuperscript{14}C\textsubscript{7} sucrose and \textsuperscript{3}H\textsubscript{2}O were respectively diluted to specific
activities of 5 mCi mmol⁻¹ and 400 mCi ml⁻¹. FCCP, nigericin and DAD were the respective gifts of Dr P.G. Heytler (E.I. Du Pont de Nemours and Co., Wilmington, Delaware, U.S.A.), Dr R.L. Hammill (Lilly Research Laboratories, Indianapolis, U.S.A.) and Prof. A. Trebst (Ruhr-Universitat Bochum, 463 Bochum, West Germany). Sodium D-isoascorbate and TMPD were obtained from B.D.H. Ltd., Poole, Dorset, U.K. The TMPD was recrystallised from ethanol (Sanadi & Jacobs, 1967). The following reagents and enzymes were obtained from the Sigma (London) Chemical Co., Kingston-upon-Thames, U.K. Lyophilised, ammonium ion-free yeast hexokinase and yeast alcohol dehydrogenase, antimycin, oligomycin, valinomycin, HQNO, ATP, ADP, NAD, Tris base and Triton X-100. The hexokinase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase and myokinase that were used in the assay for adenine nucleotides were all purchased as suspensions in ammonium sulphate from the Boehringer Corp. (London), Lewes, Sussex, U.K.
CHAPTER 5

THE PROTONMOTIVE FORCE IN PHOSPHORYLATING MEMBRANE VESICLES FROM P. DENITRIFICANS: MAGNITUDE, SITES OF GENERATION, INHIBITOR SENSITIVITY AND COMPARISON WITH THE PHOSPHORYLATION POTENTIAL
INTRODUCTION

In the previous chapters, I have described measurements of the magnitude of the protonmotive force (Δp) (Mitchell 1966a) generated by chromatophores and by beef heart submitochondrial particles and compared this value with the phosphorylation potential (ΔGₚ) attained. The principal aim of the work described in this chapter was to make similar determinations with the phosphorylating vesicles from \textit{P. denitrificans} (Chapter 2) to ascertain whether the tight respiratory control of these vesicles is associated with a significantly higher Δp.

In the absence of previous direct determinations of the \( \rightarrow \text{H}^+/\text{ATP} \) ratio for respiratory bacteria, an \( \rightarrow \text{H}^+/\text{ATP} \) ratio of 2 has been widely adopted (see e.g. Haddock and Jones 1977) to convert experimentally determined \( \rightarrow \text{H}^+/\text{O} \) ratios into P/O ratios. In chromatophores from \textit{Rhodopseudomonas capsulata} the relatively high Δp values estimated (250-400 mV), by comparison with ΔGₚ, have been shown to be compatible with an \( \rightarrow \text{H}^+/\text{ATP} \) ratio of 2 (Casadio et al., 1974a), while from similar experiments with chloroplasts (Hauska and Trebst 1977; Junge 1977), submitochondrial particles (Chapter 4) and chromatophores from \textit{Rhodospirillum rubrum} (Leiser and Gromet-Elhanan 1977; Chapter 3) there is evidence that the \( \rightarrow \text{H}^+/\text{ATP} \) ratio is at least 3.

When \textit{P. denitrificans} is grown heterotrophically in the absence of oxygen, with nitrate as the added terminal electron acceptor, electron flow through the terminal oxidase region has been reported not to be coupled to ATP synthesis (John
and Whatley 1970). There is conflicting evidence (see Chapter 1) as to whether the terminal oxidase reaction in aerobically, heterotrophically grown _P. denitrificans_ is a site of free energy conservation (van Verseveld and Stouthamer 1976; Edwards _et al._, 1977; John and Whatley 1977b; Meijer _et al._ 1977a; Stouthamer 1977). A further purpose of the experiment described in this chapter was to investigate whether electron flow through the terminal oxidase in membrane vesicles derived from cells grown with nitrate as added terminal electron acceptor was linked to the generation of a protonmotive force. Determination of the internal volume of the vesicles

The internal volume of the vesicles was estimated by the sucrose-impermeable space method using the procedure described previously (Chapter 3). With 3 preparations, values of 5.9, 4.1 and 4.0 µl per mg protein were obtained, yielding an average of 4.7 µl per mg protein. This method estimates the total volume enclosed by the vesicles, while the present experiments are concerned only with those vesicles which have a membrane polarity opposite to that of the intact cell (cf. Chapter 2). Evidence has been presented (Burnell _et al._, 1975) to show that vesicle preparations from _P. denitrificans_ made in a similar way to those used in the present work (Chapter 2) contain two types of vesicle: right-side-out vesicles, in which the vesicle membrane has the same orientation as the plasma membrane of the intact cell; and inside-out vesicles in which the orientation of the vesicle membrane is the reverse of that of the plasma membrane of the intact cell. I estimated, from the degree of latency of the NADH dehydrogenase (Burnell _et al._, 1975), that on average about 40% of the vesicles in the preparations used here are of an
inside-out configuration (Chapter 2). I determined the percentage of the vesicles which are inside-out for each preparation used, and this percentage was used to estimate the volume of the inside-out vesicles in each preparation, for the determination of the $\Delta \psi$ and $\Delta \text{pH}$ values. The estimation of the internal volume of the inside-out vesicles will be in error if the right-side out vesicles are larger (or smaller) than the inside-out vesicles, and hence enclose a larger (or smaller) volume per mg protein. However, I found no significant correlation (in 5 different preparations) between the proportion of inside-out vesicles in a given preparation and the specific internal volume enclosed by that preparation (data not displayed). A value of 4.7 $\mu$l per mg protein is higher than the internal volumes of 0.5 - 4.0 $\mu$l per mg protein reported for membrane vesicles isolated from *Escherichia coli* (Kaback and Barnes 1971) and *Bacillus subtilis* (see Konings 1977). However, as noted before, these determinations are not very sensitive to errors in the estimation of the internal volume; for example, a 2-fold overestimation of the internal volume would lead to an underestimation of only 18 mV in either $\Delta \psi$ or $\Delta \text{pH}$.

If some of the vesicles could generate a high $\Delta p$ and were thus capable of synthesising ATP while others could generate only a low $\Delta p$ which was less than that required to drive ATP synthesis, the apparent $\Delta p$ value determined would be an average value, while the $\Delta G_p$ would be due only to the more tightly coupled vesicles. Comparison of the $\Delta p$ and $\Delta G_p$
Fig 5.1

Fraction Number

$10^{-3} \times \text{counts/min}$
Fig. 5.1. **Uptake of S$^{14}$CN$^{-}$ by membrane vesicles from *P. denitrificans***

The radioactivity measured in the outflow from the flow dialysis cell is plotted against the fraction number.

Reaction mixtures contained in a final volume of 1 ml: 10mM Pi-Tris (pH 7.3), 5 mM magnesium acetate, 50μg yeast alcohol dehydrogenase, 1% (v/v) ethanol and 0.6 mM NAD$^+$. Membrane vesicles (0.94 mg of protein) were included in (•••) or omitted from (○○○) the reaction mixture as indicated. The temperature was 23°C. At time zero, 10 μl of 2.08 mM KS$^{14}$CN (60 mCi mmol$^{-1}$) were added to the upper chamber and the flow started. After fraction 20 had been collected 2 μl of 2.5 mM FCCP were added to the upper chamber as indicated by the arrow in the Figure.
values obtained under these circumstances would be invalid. However, evidence that the inside-out _P. denitrificans_ vesicles are not a significantly heterogeneous population with respect to their energy-coupling characteristics has been given in Chapter 2.

**Determination of Δψ with NADH as substrate**

Uptake of $S^{14}$CN$^-$ into vesicles respiring with NADH is shown in Fig. 5.1, in which is plotted the radioactivity in sequentially collected fractions of the outflow from the lower chamber of the flow dialysis cell. The experiment was started by adding KS$^{14}$CN to the upper chamber of the dialysis cell, at which time collection of the outflow from the lower chamber was begun. The presence of respiring vesicles in the upper chamber resulted in decreased steady-state levels of radioactivity in the outflow. This is attributed to a decreased steady-state concentration of $S^{14}$CN$^-$ in the upper chamber due to the accumulation of $S^{14}$CN$^-$ by the vesicles. An addition of FCCP resulted in an efflux of $S^{14}$CN$^-$ from the vesicles, which was reflected in the increased levels of radioactivity in the outflow (Fig. 5.1). Δψ was calculated from the extent of $S^{14}$CN$^-$ uptake, which was, for the reasons described in Chapter 4, obtained from the difference between the level of radioactivity in a given fraction after FCCP addition and the level of radioactivity that would have been in that fraction had FCCP not been added. This latter value is obtained by extrapolation. From a series of experiments performed under the conditions of Fig. 5.1 with 22 different vesicle preparations (0.6 - 2.7 mg protein per ml reaction medium) an average value
Fig. 5.2. **Effect of varying the K$_3$CN concentration on the uptake of S$^{14}$CN$^{-}$ by *P. denitrificans* vesicles**

A. The reaction conditions were as described in the legend to Fig. 5.1 except that the concentration of radioactive S$^{14}$CN$^{-}$ used to start the reaction was varied at constant specific activity. Membrane vesicles (0.94 mg of protein) were either present ( ——— ) or absent ( — — — — — ). The initials K$S^{14}$CN concentrations (μM) in the upper chamber of the flow dialysis cells were: (a) 5.2; (b) 10.4; (c) 20.8; (d) 31; (e) 42; (f) 52. FCCP (5 μM) was added to the upper chamber after 10 fractions had been collected as indicated by the arrow. The radioactivity in individual fractions is not given, for reasons of clarity.

B. Data of (A) replotted to show the effect of K$_3$CN concentration on the value of Δψ.
Fig. 5.3.  Effect on vesicle concentration on the uptake of $S^{14}\text{CN}^-$ by *P. denitrificans* vesicles

A.  Flow dialysis was performed as described in the legend to Fig. 5.2 except that the KS$^{14}\text{CN}$ concentration was 20.8 uM, and the vesicle concentrations were (mg of protein per ml): (a) 0; (b) 0.43; (c) 0.85; (d) 1.70; (e) 2.13; (f) 2.55; (g) 2.98.

B.  The effect of particle concentration on $\Delta \psi$, calculated from the data plotted in (A).
for $\Delta \psi$ of 145 mV (S.E.M. ± 3 mV) was obtained.

When the initial concentration of $K_{\text{S}^{14}\text{CN}}$ added to the reaction mixture in the upper chamber was increased there was a slight decrease in $\Delta \psi$ (Fig. 5.2). When the initial concentration of $K_{\text{S}^{14}\text{CN}}$ was maintained at 20 $\mu$M and the vesicle concentration increased there was a slight increase in $\Delta \psi$ (Fig. 5.3). This apparent variation of $\Delta \psi$ with varying concentrations of $K_{\text{S}^{14}\text{CN}}$ and of vesicles may be due to some binding of $S_{14}^{14}\text{CN}^-$ to the vesicles, or it may be due to a slight uncoupling effect of $S_{14}^{14}\text{CN}^-$ at the higher $S_{14}^{14}\text{CN}^- :$ vesicle ratios. These effects can be seen to be of little significance, however, since extrapolation of the measured $\Delta \psi$ values back to zero KSCN concentration (Fig. 5.2B) or to an infinite vesicle concentration (Fig. 5.3B) increases $\Delta \psi$ by only 5-10 mV (Figs. 5.2 and 5.3) compared to the values measured with the 20 $\mu$M $K_{\text{S}^{14}\text{CN}}$ and ca. 2 mg vesicle protein per ml routinely present.

When ADP, glucose and hexokinase were included in the reaction medium, the extent of $S_{14}^{14}\text{CN}^-$ uptake was decreased by an amount which was equivalent to a decrease in $\Delta \psi$ of about 15 mV (Fig. 5.4). A subsequent addition of venturicidin, which inhibits the ATPase of $P.$ denitrificans (Ferguson and John 1977), caused $\Delta \psi$ to return to the value observed in the absence of ADP (Fig. 5.4). That the vesicles are indeed tightly coupled is indicated by the observation (Fig. 5.4) that venturicidin did not increase the $\Delta \psi$ beyond that in the absence of phosphate acceptor.
Fig 5.4
Flow dialysis was carried out as described in legend to Fig. 5.1. The reaction mixtures contained in a final volume of 1 ml: 10 mM Pi-Tris (pH 7.3), 5 mM magnesium acetate, 50 μg alcohol dehydrogenase, 10 μl ethanol, and 0.6 mM NAD⁺. At zero time 20.8 μM KS¹⁴CN (60 mCi mmol⁻¹) was added. Additional components were: (□-□-□) none; (□-□-□) 10 mM glucose, 5 units hexokinase and 0.5 mM ADP; (•-•-•) membrane vesicles (2.67 mg of protein); (□-□-□) 10 mM glucose, 5 units hexokinase, 0.5 mM ADP and membrane vesicles (2.67 mg of protein). The arrows indicate the successive additions of 2μg venturicidin (after collection of fraction 10), and 5 μM FCCP (after collection of fraction 20).
Determination of $\Delta \text{pH}$ with NADH as substrate

Fig. 5.5 shows that vesicles respiring with NADH as substrate did not take up a detectable amount of $\text{^{14}C}_7$ methylamine; thus, the addition of 5 $\mu$M FCCP, which caused efflux of $\text{^{14}CN}^-$ under the same conditions (Figs. 5.1 - 5.4), had no effect upon the external $\text{^{14}C}_7$ methylamine levels in the reaction mixture, even in the presence of 5 $\mu$M rotenone to restrict electron transport and thus enhance the effectiveness of FCCP as an uncoupler (cf. Chapter 4). Similarly, an addition of 0.1% triton X-100 instead of FCCP under the conditions of Fig. 5.5 did not result in any efflux of $\text{^{14}C}_7$ methylamine from the vesicles. An uptake of 3% of the methylamine present would have been detectable. This would have been equivalent to a $\Delta \text{pH}$ of 0.5 units. Thus I conclude that $\Delta \text{pH}$ under these conditions is $< 0.5$ units.

Further evidence that a significant $\Delta \text{pH}$ was absent during NADH oxidation by the *P. denitrificans* vesicles was that the addition of potassium acetate (10 mM) and nigericin (1 $\mu$g per mg protein) under the conditions of Fig. 5.1 (see Chapter 2) did not result in a measurable increase in the uptake of $\text{^{14}CN}^-$. Addition of $K^+$ and nigericin has been shown previously with chromatophores to replace the $\Delta \text{pH}$ with an energetically equivalent $\Delta \psi$ as a result of the electro-neutral exchange of internally accumulated $H^+$ for external $K^+$ (Gromet-Elhanan 1977) (see also Chapters 3 and 4).

A $\Delta \text{pH}$ was detected when KCl and valinomycin were included in the reaction mixture (Table 5.1). The appearance of a $\Delta \text{pH}$ under these conditions was accompanied by the disappearance of $\Delta \psi$. When nigericin was present in addition to KCl and valinomycin both $\Delta \psi$ and $\Delta \text{pH}$ were no longer detected (Table 5.1). Presumably the replacement of $\Delta \psi$ by
10^{-3} \times \text{counts/min}
Flow dialysis was performed with the reaction mixture and by the method described in the legends to Fig. 5.2, except that the $^3$CN$^-$ was replaced by 10 μl of 2.25 mM $^1^4$C$^7$ methylamine hydrochloride (55.5 mCi mmol$^{-1}$), and 1.82 mg of vesicle protein were present. After 20 fractions had been collected (as arrowed), 2 μl of 2.5 mM FCCP were added to the upper chamber. The addition of FCCP did not affect the rate of flow of methylamine through the dialysis membrane as was shown in a control experiment in which vesicles were omitted from the upper chamber (not shown for reasons of clarity).
<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Additions</th>
<th>pH</th>
<th>p (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>$\leq 0.5$</td>
<td>150 (180)</td>
</tr>
<tr>
<td></td>
<td>50 mM KCl</td>
<td>$\leq 0.5$</td>
<td>115 (185)</td>
</tr>
<tr>
<td></td>
<td>50 mM KCl + valinomycin (1 μg)</td>
<td>$\leq 0.5$</td>
<td>90 (120)</td>
</tr>
<tr>
<td></td>
<td>50 mM KCl + valinomycin (1 μg) + nigericin (1 μg)</td>
<td>$\leq 0.5$</td>
<td>0 (60)</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>$\leq 0.5$</td>
<td>165 (195)</td>
</tr>
<tr>
<td></td>
<td>10 mM KSCN</td>
<td>$\leq 0.5$</td>
<td>0 (60)</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>$\leq 0.5$</td>
<td>150 (180)</td>
</tr>
<tr>
<td></td>
<td>10 mM KNO$_3$</td>
<td>$\leq 0.5$</td>
<td>30 (60)</td>
</tr>
</tbody>
</table>
\[ \Delta \psi \] and \[ \Delta \text{pH} \] were measured from the extent of \(^{14}\text{CN}^-\) or \(^{14}\text{C}\text{C7}\) methylamine uptake using the flow dialysis procedure. The upper chamber of the flow dialysis cell contained in a volume of 1 ml: 10 mM Pi-Tris 5 mM magnesium acetate and membrane vesicles (Expt. 1, 1.8 mg of protein; Expt. 2, 2.9 mg of protein; Expt. 3, 2.0 mg of protein), plus other components as detailed below or in the Table. The pH was 7.3 and the temperature was 23°C. For measurements of \[ \Delta \psi \] 20 \(\mu\)M \(^{14}\text{CN}\) (60 mCi mmol\(^{-1}\)) was added to the upper chamber, while for measurements of \[ \Delta \text{pH} \] 20 \(\mu\)M \(^{14}\text{C}\text{C7}\) methylamine hydrochloride (55.5 mCi mmol\(^{-1}\)) was added.

NADH was the substrate and 0.6 mM NAD\(^+\), 1\% (v/v) ethanol and 50 \(\mu\)g alcohol dehydrogenase were added. For experiments in which either \[ \Delta \psi \] or \[ \Delta \text{pH} \] was not observed the lower limit of detection is signified by \(<\). The values of \(\Delta p\) given in parentheses represent upper limits on \(\Delta p\), obtained by adding the lower limit of detection for \(\Delta \text{pH}\) or \(\Delta \psi\) to the observed \(\Delta \text{pH}\) or \(\Delta \psi\).
ΔpH is due to the efflux of K⁺ via valinomycin neutralising Δψ and thereby allowing a greatly enhanced H⁺ uptake (Jackson et al., 1968). A similar replacement of Δψ by ΔpH should occur when Δψ is neutralised by the electrophoretic uptake of high concentrations of permeant ions. Table 5.1 shows, however, that the addition of 10 mM KNO₃ or 10 mM KSCN resulted in the loss of a measurable Δψ, but not in the appearance of an energetically equivalent ΔpH. In the case of KNO₃, the same result was found when nitrate reduction was inhibited by 0.1 mM azide (John 1977), suggesting that the effect is unrelated to the ability of nitrate to act as a terminal electron acceptor in these vesicles.

The cells from which the vesicles were isolated showed a significant rate of ¹⁴C methylamine uptake and of methylamine-dependent oxygen uptake (about 10 μmoles/min/g dry weight) (cf. Cox and Quayle 1975), which was inhibited by 0.5 mM hydrazine (data not shown). However, the vesicles showed no detectable methylamine-dependent respiration (< 3 nmol/min/mg protein) when incubated with 0.1 mM methylamine hydrochloride, either in the presence or absence of NAD⁺ (0.6 mM) under reaction conditions similar to those described for Fig. 2.3. This indicates that oxidation of methylamine by the vesicles could not account for the failure to observe respiration-dependent ¹⁴C methylamine uptake. Such oxidation would also have affected the flow rate of methylamine across the dialysis membrane, with a resultant change in radioactivity in the outflow, which was not observed.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Additions</th>
<th>$\Delta \psi$ (mV)</th>
<th>$\Delta \mathrm{pH}$</th>
<th>$-Z \Delta \mathrm{pH}$</th>
<th>$\Delta \rho$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td></td>
<td>145</td>
<td>&lt; 0.5</td>
<td>&lt; 30</td>
<td>145 (175)</td>
</tr>
<tr>
<td>Succinate</td>
<td></td>
<td>125</td>
<td>&lt; 0.5</td>
<td>&lt; 30</td>
<td>125 (155)</td>
</tr>
<tr>
<td>Ascorbate + DAD</td>
<td></td>
<td>120</td>
<td>&lt; 0.8</td>
<td>&lt; 50</td>
<td>120 (170)</td>
</tr>
<tr>
<td>Ascorbate + DAD</td>
<td>antimycin</td>
<td>85</td>
<td>n.d.</td>
<td>n.d.</td>
<td>85</td>
</tr>
<tr>
<td>Ascorbate + TMPD</td>
<td></td>
<td>90</td>
<td>n.d.</td>
<td>n.d.</td>
<td>90</td>
</tr>
<tr>
<td>Ascorbate + TMPD</td>
<td>antimycin</td>
<td>70</td>
<td>n.d.</td>
<td>n.d.</td>
<td>70</td>
</tr>
</tbody>
</table>
Table 5.2. The protonmotive force measured with vesicles from _P. denitrificans_ oxidising various substrates

$\Delta \psi$ and $\Delta \text{pH}$ were measured from the extent of $^{3}\text{H} \text{CN}^-$ or $^{14}\text{C} \text{methylamine}$ uptake using the flow dialysis procedure. The upper chamber of the flow dialysis cell contained in a volume of 1 ml: 10 mM Pi-Tris, 5 mM magnesium acetate and membrane vesicles (between 1.0 and 2.6 mg of protein), plus other components as detailed below or in the Table. The pH was 7.3 and the temperature was 23°C. For measurements of $\Delta \psi$ 20 $\mu\text{M} \text{K}^+ ^{14}\text{CN}$ (60 mCi mmol$^{-1}$) were added to the upper chamber, while for measurements of $\Delta \text{pH}$ 20 $\mu\text{M} \text{L}^- ^{14}\text{C} \text{methylamine hydrochloride}$ (55.5 mCi mmol$^{-1}$) was added. When NADH was the substrate, 0.6 mM NAD$^+$, 1% (v/v) ethanol and 50 $\mu\text{g}$ alcohol dehydrogenase were added. When succinate was the substrate 10 mM sodium succinate was added. When ascorbate was the substrate 10 mM sodium D-isoascorbate plus either 0.1 mM DAD or 0.1 mM TMPD were added. When present the concentration of antimycin was 1 $\mu\text{g}$ per mg of protein. For experiments in which either $\Delta \psi$ or $\Delta \text{pH}$ was not observed the lower limit of detection is signified by $<$. The values of $\Delta \text{p}$ given in parentheses represent upper limits on $\Delta \text{p}$, obtained by adding the lower limit of detection for $\Delta \text{pH}$ or $\Delta \psi$ to the observed $\Delta \text{pH}$ or $\Delta \psi$. n.d. = not determined.
Fig. 5.6. **Energy-linked enhancement of 1-anilinophthalene-8-sulphonate fluorescence**

5 µM anilino-naphthalene-sulphonate (ANS) was added to 3 ml of 10 mM Pi-Tris, pH 7.3, containing 5 mM magnesium acetate and *P. denitrificans* vesicles (0.37 mg of protein). The temperature was 30°C. In Experiment A sodium succinate (10 mM) and FCCP (3 µM) were subsequently added as indicated. In Experiment B antimycin (0.3 µg) and sodium D-isoascorbate (10 mM) were present initially; DAD (0.1 mM) and FCCP (3 µM) were added as shown.
Determination of $\Delta p$ generated by succinate or ascorbate oxidation, or by ATP hydrolysis

The oxidation of succinate, and the oxidation of ascorbate (mediated by either TMPD or DAD), generated a consistently lower $\Delta \psi$ than did the oxidation of NADH (Table 5.2), but in no case was there a measurable $\Delta \text{pH}$. An addition of antimycin at a concentration just sufficient to inhibit maximally the oxidation of NADH, had no significant effect upon the $\Delta \psi$ generated by the oxidation of ascorbate (Table 5.2). The presence of energy coupling associated with the oxidation of ascorbate, in the presence of DAD, was confirmed by the observation of an FCCP-sensitive enhanced fluorescence of 1-anilino naphthalene-8-sulphonate, although the magnitude of both the rate and extent of fluorescence enhancement was less than that induced by succinate (Fig. 5.6). On occasion, ascorbate oxidation mediated by TMPD or DAD gave no detectable SCN$^-$ uptake nor a significant FCCP-sensitive enhancement of 1-anilino naphthalene-8-sulphonate fluorescence, even though the same preparations gave the usual $\Delta \psi$ value and enhancement of 1-anilino naphthalene-8-sulphonate fluorescence with NADH or succinate as substrate.

When the vesicles were incubated with ATP instead of a respiratory substrate no detectable $\Delta \psi$ nor $\Delta \text{pH}$ was observed (data not shown). The absence of a detectable $\Delta p$ associated with the slow rate of ATP hydrolysis observed with the P. denitrificans vesicles is compatible with the previously noted effective irreversibility of this ATPase (Ferguson et al., 1976b).
<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Substrate</th>
<th>Addition</th>
<th>Final concentrations</th>
<th>$\Delta g_p$</th>
<th>$\Delta G_p$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NADH</td>
<td>-</td>
<td>$0.013$, $9.8$</td>
<td>53.9</td>
<td>(12.9)</td>
</tr>
<tr>
<td>2</td>
<td>Succinate</td>
<td>-</td>
<td>$0.017$, $9.8$</td>
<td>53.2</td>
<td>(12.74)</td>
</tr>
<tr>
<td>3</td>
<td>NADH</td>
<td>-</td>
<td>$0.003$, $9.8$</td>
<td>52.2</td>
<td>(12.5)</td>
</tr>
<tr>
<td>4</td>
<td>NADH</td>
<td>10 mM KNO$_3$</td>
<td>0.2, $0.003$, $9.8$</td>
<td>52.2</td>
<td>(12.5)</td>
</tr>
</tbody>
</table>

Table 5.3
Table 5.3. Phosphorylation potentials generated by P. denitrificans vesicles

In experiments 1 and 2 P. denitrificans vesicles (2 mg of protein) from the same preparation were incubated in 1 ml of a reaction mixture which contained 10 mM Pi-Tris, pH 7.3, 5 mM magnesium acetate, 20 μM KSCN and, initially, 1.7 mM ATP plus 0.2 mM ADP. When NADH was substrate (Expt. 1) 0.6 mM NAD⁺, 50 μg of yeast alcohol dehydrogenase and 1% (v/v) ethanol were also present. When succinate was the substrate (Expt. 2) 5 mM sodium succinate was also added. The incubations were carried out at 23°C in the flow dialysis cell over which water saturated oxygen was blown. After 8 min (Expt 1) or 10 min (Expt 2) the reaction mixture was withdrawn from the flow dialysis cell and added to 0.1 ml of ice-cold 40% HClO₄. In experiments 3 and 4 P. denitrificans vesicles (0.05 mg of protein) from the same preparation (which was a different preparation from that used for Expts. 1 and 2) were incubated in 3 ml of a reaction mixture which contained 10 mM Pi-Tris, pH 7.3, 5 mM magnesium acetate, 0.6 mM NAD⁺, 50 μg of yeast alcohol dehydrogenase, 1% (v/v) ethanol, and initially 0.2 mM ADP. The incubations were carried out at 30°C in a thermostatically controlled cell for 5 min with the reaction mixture open to atmospheric oxygen, and then 2 ml of the reaction mixture was withdrawn and added to 0.2 ml of ice-cold 40% HClO₄. The acid extracts from Expts 1 to 4 were left on ice for 10 min, and then the precipitated protein was removed by centrifugation at 2000g. The supernatants were neutralised by addition
of the predetermined amount of 0.25 M Tris/10% (w/v) KOH, and EDTA was also added to a final concentration of 2 mM. ATP in the neutralised extracts was determined with hexokinase and glucose-6-phosphate dehydrogenase, and ADP was assayed with pyruvate kinase and lactate dehydrogenase (Chapter 3). Pi was measured by the method of Hurst (1964) in a Technicon Autoanalyser.

In calculating $\Delta G_p$, a value for $\Delta G^0$ of 30.1 kJ mol$^{-1}$ (7.2 kcal mol$^{-1}$) was used (Rosing & Slater 1972).
Fig 2.7
Fig. 5.7  Effect of FCCP concentration on respiratory rate of P. denitrificans vesicles under controlled and phosphorylating conditions

Respiration was monitored polarographically in a 2 ml reaction mixture containing 10 mM $^{32}$P$_1$-Tris (1 mCi/mmol) pH 7.3, 5 mM magnesium acetate, 1% v/v ethanol, 0.1 mg alcohol dehydrogenase, 0.6 mM NAD$^+$, 10 mM glucose, 10 units hexokinase, 1.63 mg vesicle protein, 20 μM KSCN and the appropriate concentration of FCCP. Basal respiratory rates were measured (○) before the addition of 0.2 mM ADP (●). Respiratory rates are expressed as a percentage of the rate under controlled conditions in the absence of FCCP. This respiratory rate, corresponding to 100%, was approximately 650 n atom O/min/mg protein.
Fig. 5.8. Effect of FCCP concentration on various activities of oxidative phosphorylation catalysed by _P. denitrificans_ vesicles

The Figure displays the effect of FCCP concentration upon a number of activities related to oxidative phosphorylation catalysed by the _P. denitrificans_ vesicles. The rate of ATP synthesis was determined as described in the legend to Fig. 5.9. The P/O was determined as the ratio between the rate of ATP synthesis and the rate of oxygen uptake (Fig. 5.7). The membrane potential, which under these incubation conditions quantitatively accounts for the protonmotive force, was determined by parallel flow dialysis experiments using the reaction mixture described in the legend to Fig. 5.7, except that glucose and hexokinase were omitted and the thiocyanate used was labelled with $^{14}$C. The phosphorylation potentials obtained at three uncoupler concentrations are given in kcal/mol adjacent to the membrane potential measurements, and were determined as described in the legend to Table 5.3. The reaction mixture for the phosphorylation potential determinations was the same as that for the flow dialysis measurements, except that thiocyanate was omitted.
Fig 5.9
Fig. 5.9. Time course of $\text{ATP}^{32}$ synthesis by $P$. denitrificans vesicles in the presence of various concentrations of FCCP

The incubation mixture was the same as that given in the legend to Fig. 5.7. After the addition of ADP to the reaction mixtures indicated in the legend to Fig. 5.7 0.2 ml samples were taken and added to 1 ml of the phosphate-precipitating reagent described by Sugino and Miyoshi (1964), in an Eppendorf Microfuge tube. After centrifugation in an Eppendorf Microfuge for 2 min, 1 ml of supernatant was taken and counted for radioactivity in 10 ml of water. Radioactivity counts were converted to phosphate concentrations using an internal standard of known phosphate concentration. Curves A to G contained, respectively, the following FCCP concentrations (micromolar): 0.0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.5. The rates obtained from this experiment are also included in the previous figure.
Determination of $\Delta G_p$

Table 5.3 shows the $\Delta G_p$ values obtained with the P. denitrificans vesicles during the oxidation of NADH or succinate. The $\Delta G_p$ obtained with NADH was not significantly altered when 10 mM KNO$_3$ was included in the reaction mixture (Table 5.3).

The effect of low concentrations of uncoupler on $\Delta G_p$, on the rate of ATP synthesis and on $\Delta \rho$

In view of the current interest in the relationship between the rate of electron transport and the steady-state protonmotive force generated by energy-transducing membranes (e.g. Schönfeld and Neumann 1977; Nicholls 1977b; Johnson and Hansford 1977; Baccarin-Melandri et al., 1977) it was of interest to titrate the particles with uncoupler, in order to examine this relationship. Figures 5.7 to 5.9 indicate the effects of increasing uncoupler concentration on (a) the respiratory rate (Fig. 5.7), (b) the protonmotive force in the absence of phosphate acceptor (Fig. 5.8), (c) the $\Delta G_p$ attained at equilibrium (Fig. 5.8), (d) the rate of ATP synthesis with a glucose/hexokinase trap present (Figs. 5.8 and 5.9) and (e) the P/O ratio (Fig. 5.8). Both $\Delta \rho$ and $\Delta G_p$ are unaffected (Fig. 5.8) by a concentration of FCCP sufficient to inhibit the rate of ATP synthesis (and to decrease the P/O ratio) by over 50%. Thus there does not appear to be the strict relationship between either $\Delta \rho$ or the equilibrium $\Delta G_p$ and either the rate of ATP synthesis or the P/O ratio that would be expected for a reversible system in which the oxidative and phosphorylative systems of the membranes were in kinetic equilibrium. However, as noted above,
the particles have a very low rate of ATP synthesis by hydrolysis (Ferguson et al., 1976b), which is barely (less than 2-fold) stimulated by uncouplers (Ferguson, S. J. & John, P., personal communication). Thus it seems that the redox-reduction and hydrodehydration systems of these particles are not in kinetic equilibrium either with each other or with the protonmotive force, for changes in the rates of both oxidation and phosphorylation can occur without changes in the protonmotive force when attempts are made to change the latter by the addition of uncoupler. However, the ability of the particles to generate an unchanged \( \Delta G^\circ \) even when the rate of ATP synthesis is severely diminished by uncoupler is predicted from the known kinetic irreversibility of the \( P. \) denitrificans ATPase (Ferguson 1977). The possible significance of an irreversible ATPase in vivo has been discussed by Ferguson (1977). (see also Chapter 7). A similar titration of these activities has recently been reported by Azzone et al., (1978) for submitochondrial particles, with broadly similar results.

DISCUSSION

The \( \Delta G^\circ \) determined by the flow dialysis method during NADH respiration by the phosphorylating vesicles from \( P. \) denitrificans is similar to the \( \Delta G^\circ \) determined with submitochondrial particles (Chapter 4) and is greater than the \( \Delta G^\circ \) determined for chromatophores from \( R. \) rubrum (Chapter 3). However the \( \Delta G^\circ \) values determined for all three of these systems are similar to the \( \Delta G^\circ \) values which have been claimed for intact cells of a variety of bacteria (see Hamilton 1977 and Chapter 7), and for right-side-out vesicles
of *Escherichia coli* (Ramos & Kaback 1977a,b). The $\Delta p$ values obtained by this method are lower, however, than values obtained for chromatophores from *Rhodopseudomonas capsulata* (Casadio et al., 1974a) and for intact mitochondria (Mitchell & Moyle, 1969b; Nicholls 1974). The general validity of this method has been discussed in Chapters 3 and 4. In the case of the *P. denitrificans* vesicles it appears unlikely that I have failed to detect a significant $\Delta$ pH since a $\Delta$ pH was measurable when KCl plus valinomycin were added to convert $\Delta\psi$ to a $\Delta$ pH. Furthermore there was no significant increase in $\Delta\psi$ when nigericin and potassium acetate were added to convert any $\Delta$ pH to $\Delta\psi$. In aerobic bacteria, it is believed that the internal pH is held constant (Thauer et al., 1977), and since the internal pH of *P. denitrificans* cells is claimed to be 7.2 - 7.4 (Scholes & Mitchell 1970a), it is to be expected that no pH gradient would be detected at a pH of 7.3, as found by other authors (e.g. Ramos et al., 1976).

The $\Delta G_p$ value of 53.6 kJ mol$^{-1}$ (12.8 kcal mol$^{-1}$) determined for the phosphorylating vesicles of *P. denitrificans* is similar to the $\Delta G_p$ values previously determined for *Azotobacter vinelandii* vesicles (Eilerman & Slater 1970, recalculated by Ferguson & Sorgato 1977), *Rhodopseudomonas capsulata* (Casadio et al., 1974a), *Rhodospirillum rubrum* chromatophores (Chapter 3), chloroplasts (Kraayenhof, 1969) and rat-liver mitochondria (Cockrell et al., 1966; Slater et al., 1973), but is higher than the $\Delta G_p$ value of about 43.9 kJ mol$^{-1}$ (10.5 kcal mol$^{-1}$) determined for submitochondrial particles (Ferguson and Sorgato 1977 and Chapter 4). The significance of these similarities and this difference has been discussed.
at length by Ferguson & Sorgato (1977). If it were assumed that $\Delta p$ and $\Delta G_p$ come into equilibrium in state 4 (as defined by Chance & Williams, 1956), then from equation 3.3, using values for $\Delta p$ and $\Delta G_p$ given in Tables 5.2 and 5.3 for NADH oxidation in the absence of NO$_3^-$, an $\rightarrow H^+/ATP$ ratio of 3.8 is derived for the $P$. denitrificans ATPase. However, this value for the $\rightarrow H^+/ATP$ ratio must be a minimum value since, as noted above, $\Delta p$ is not in effective equilibrium with $\Delta G_p$. The chemiosmotic hypothesis envisaged an $\rightarrow H^+/ATP$ ratio of 2 (Mitchell 1966a), but the $\rightarrow H^+/ATP$ ratio of more than 3 derived from the experiment described in this chapter is in line with recent findings with mitochondria (Wiechmann et al., 1975; Rottenberg 1975; Brand 1977; Nicholls 1977b), submitochondrial particles (Rottenberg & Gutman 1977; Chapter 4), chloroplasts (McCarty & Portis 1976; Hauska & Trebst 1977; Junge 1977) and chromatophores (Chapter 3). An $\rightarrow H^+/ATP$ ratio of 3-4 for $P$. denitrificans would be in agreement with the conclusions of Meijer et al., (1977a), which were based on the measurements of the $\rightarrow H^+/O$ ratio and growth yields of intact cells of $P$. denitrificans. Lawford (1978) has recently reported that the respiration-driven proton extrusion from cells of $P$. denitrificans is consistent with an $\rightarrow H^+/2e^-/site$ ratio of 3.

If the P/O ratio for $P$. denitrificans oxidising NADH is less than 3 (John & Whatley 1977b, and see Chapter 1) then the results of Lawford suggest that $\rightarrow H^+/ATP$ is indeed 3 or more.

The observation of an undetectable $\Delta p$ and an undiminished $\Delta G_p$ when 10 mM KNO$_3$ is present during NADH oxidation, and the previous observation of respiratory control and oxidative
phosphorylation in the presence of nitrate (John & Whatley 1970), suggest that \( \Delta p \) does not represent the sole mode of membrane energization in this organism. However, it would be premature to call into question the essential nature of \( \Delta p \) for ATP synthesis in other systems until this curious effect has been more fully characterised.

The widely observed absence of ATP synthesis coupled to ascorbate oxidation by \textit{P. denitrificans} vesicles with TMPD as electron mediator has led some authors to conclude that the flow of reducing equivalents along the terminal region of the respiratory chain is not coupled to ATP synthesis (see John & Whatley 1977b). It has been shown in this chapter that oxidation of ascorbate with either DAD or TMPD as electron mediator can be linked to the generation of a protonmotive force. This finding does not necessarily mean that ascorbate oxidation will be able to drive ATP synthesis, though, since the protonmotive force is lower than when succinate or NADH are substrates, and may therefore be below a critical threshold that is needed to activate ATP synthesis (cf. Avron 1977; Baccarini-Melandri et al., 1977; Junge 1977). However, when reducing equivalents flow down the whole respiratory chain the contribution of the terminal region to ATP synthesis may prove to be of more importance than when antimycin is present and ascorbate (plus TMPD or DAD) is the reductant, since the charge separation at the terminal region of the respiratory chain would be additional to the charge separations occurring at the other regions of the chain. The finding that on occasion the oxidation of ascorbate plus DAD did not generate a protonmotive force with vesicles in which succinate oxidation did generate a
protonmotive force, suggests that the presence of an energy coupling site in the terminal region of the respiratory chain may depend very finely on the exact environmental conditions and the phase of growth, as suggested by Edwards et al., (1977) from the results of growth yield experiments. Nevertheless the irreproducibility of energy-linked ascorbate oxidation indicates that when a proton-motive force is generated by ascorbate oxidation, proton translocation is linked to electron flow at the terminal region of the respiratory chain. This conclusion parallels the arguments made in Chapter 4, that ascorbate, with either DAD or TMPL as mediator, does not donate electrons to a putative proton-pumping redox carrier in the cytochrome bc₁ region of the respiratory chain.

The lack of correlation between the rate of oxidation, the rate of phosphorylation and the efficiency of oxidative phosphorylation (P/O ratio) on the one hand, and the proton-motive force on the other hand, suggests that in the P. denitrificans membrane vesicles the bulk protonmotive force measured by the ion-distribution method may not represent the functional high-energy intermediate between oxidation and phosphorylation. This conclusion is strengthened by the finding that the addition of 10 mM KNO₃ to vesicles oxidising NADH dissipates the bulk protonmotive force without any uncoupling effect, as judged by respiratory control, or the ability to generate a large ΔGₚ. The implications of these findings for current theories of the functional proton current in oxidative phosphorylation are discussed at greater length in Chapter 7.
METHODS

The preparation of membrane vesicles (Chapter 2), the determination of oxygen uptake and protein (Chapter 2), adenine nucleotides and inorganic phosphate (Chapter 3), the determination of $K_3^{14}CN$ and $^{14}C_7$ methylamine uptake using a flow dialysis technique (Chapter 3) and the calculation of $\Delta p$, $\Delta \psi$, $\Delta p_H$ and $\Delta \gamma_p$ (Chapter 3) were all as described previously.

The fluorescence of 1-anilino naphthalene-8-sulphonate was measured as described by Ferguson et al., (1976a). Esterified $^{32}P$ was estimated by the method of Sugino and Miyoshi (1964).
CHAPTER 6
CONTINUOUS MONITORING OF THE PROTONMOTIVE FORCE USING ION-SELECTIVE ELECTRODES; APPLICATION TO CHROMATOPHORES, SUBMITOCHONDRIAL PARTICLES AND P. DENITRIFICANS MEMBRANE VESICLES
INTRODUCTION

In the last three chapters it has been demonstrated that the protonmotive force generated in Pi-Tris media of low osmolarity by three different energy-transducing membrane systems may be quantitatively accounted for by the electrical component $\Delta \psi$ alone, since $\Delta \text{pH}$ was not detectable. I therefore felt that ion-sensitive electrodes would allow a continuous monitoring of SCN$^-$ or NO$_3^-$ uptake by these membrane vesicles, and thus provide a continuous monitor of $\Delta p$.

In this chapter I describe the use of commercially available anion-selective electrodes for the real-time determination of $\Delta \psi$ (and thus $\Delta p$) in bovine heart submitochondrial particles, R. rubrum chromatophores and P. denitrificans membrane vesicles. Although the flow dialysis method may be used to follow the uptake of any substances, it is a lengthy assay, does not provide results quickly and requires radioactive incubation mixtures. For the routine determination of $\Delta \psi$, the ion-selective electrode method possesses a number of advantages over this and other (Rottenberg 1975) methods currently available for the quantitative determination of $\Delta \rho$.

RESULTS

Fig. 6.1 shows that the response of the Orion SCN$^-$ electrode is linear rather than Nernstian at the low concentrations of SCN$^-$ that are used as probes for $\Delta \psi$. The $t_\text{1/2}$ for the response of the electrode is near the limit of response of the chart recorder (1-2 seconds). On addition of a respiratory
Fig. 6.1. **Energy-dependent uptake of SCN⁻ by bovine heart submitochondrial particles monitored with an ion-sensitive electrode**

Submitochondrial particles (18.8 mg protein) were incubated in 5 ml of a reaction medium containing 10 mM Pi-Tris, pH 7.3, 5 mM magnesium acetate, 1% ethanol, 0.2 mg alcohol dehydrogenase and 10 µM KSCN. The temperature was 23°C. The free SCN⁻ concentration was monitored potentiometrically as described in Methods. Calibrating additions of KSCN were made as indicated by the arrows. NAD⁺ (0.2 mM), ADP (0.2 mM) and FCCP (2.5 µM) were added as indicated. The electrode response after addition of FCCP did not quite return to the level observed before adding NAD⁺. This effect was due to a slight interference with electrode by NAD⁺ which was also seen when NAD⁺ was added to the particles after FCCP.
Fig 6.2

Respiration (% control) vs. [Rotenone] (nmol mg⁻¹ protein)
Fig. 6.2. The relationship between the respiratory rate and the membrane potential of bovine heart submitochondrial particles

$\Delta \psi$ was measured from the extent of SCN$^-$ uptake which was monitored either by the SCN$^-$ electrode (•••••) or by the flow dialysis method (○○○○○). When the electrode was used the reaction mixture was as described in the legend to Fig. 6.1 except that 12.5 mg submitochondrial particle protein were used. For flow dialysis measurements the upper chamber of the flow dialysis cell contained in a total volume of 1 ml, submitochondrial particles (6.0 mg protein), 10 mM P$_i$-Tris pH 7.3, 5 mM magnesium acetate, 1% ethanol, 0.05 mg alcohol dehydrogenase, 0.2 mM NAD$^+$ and 20 uM KSCN (60 mCi mmol$^{-1}$). At the two lowest respiratory rates the uptake of SCN$^-$ was undetectable, and the limit of detection is indicated on the figure. Respiration was monitored using an oxygen electrode with a 3 ml reaction mixture which contained: submitochondrial particles (2.1 mg protein), 10 mM P$_i$-Tris, pH 7.3, 5 mM magnesium acetate, 1% ethanol, 0.1 mg alcohol dehydrogenase and 50 $\mu$M KSCN. Respiration was initiated by the addition of 0.2 mM NAD$^+$. Respiration rates (■■■■■) are plotted as a percentage of the rate in the absence of rotenone, which was 366 natom O/min/mg protein. In all experiments the submitochondrial particles were preincubated for 2 minutes with the appropriate concentration of rotenone. The temperature throughout was 23$^\circ$C.
substrate, SCN⁻ is taken up into the lumen of submitochondrial particles with pseudo-first order kinetics, \( t_\frac{1}{2} \) about 45 seconds (cf. Mitchell and Moyle 1969b, Lehninger 1974), until a steady state is attained (Fig. 6.1). On addition of 0.2 mM ADP, the steady state uptake of SCN⁻ decreases transiently (for less than one minute) while the ADP is phosphorylated. All the SCN⁻ taken up is released upon addition of 2.5 \( \mu \)M FCCP as uncoupler.

Fig. 6.2 shows the effect of increasing rotenone concentrations on \( \Delta \psi \), obtained both from experiments similar to that shown in Fig. 6.1, and from a parallel assay of \( S^{14}CN^- \) uptake using flow dialysis (Chapters 3, 4, 5). Also plotted are the results of parallel experiments in which the steady state rate of oxygen uptake was determined. (Rates of oxygen uptake were linear only after a pre-incubation with rotenone (cf. Hinkle et al., 1975). Except at the highest respiratory rates, the relationship between the respiratory rate and \( \Delta \psi \) is essentially ohmic, as found, for example, in rat liver mitochondria (Nicholls 1974), and as suggested by Hinkle et al., (1975) for submitochondrial particles. The results using the potentiometric assay of SCN⁻ are very similar to those obtained with the flow dialysis method (Fig. 6.2).

When NO₃⁻ is used as the permeant ion (Montal et al., 1970), the respiration-driven uptake into submitochondrial particles (Fig. 6.3), monitored with the NO₃⁻ electrode, again displays pseudo-first order kinetics, with \( t_\frac{1}{2} \) approx. 2 minutes, almost threefold that for SCN⁻ uptake under the same conditions. Nevertheless, \( \Delta \psi \) calculated from the extent
Energy-dependent uptake of \( \text{NO}_3^- \) by bovine heart submitochondrial particles monitored with a \( \text{NO}_3^- \) electrode

Submitochondrial particles (5.9 mg protein) were incubated in 5 ml of a reaction medium containing 10 mM \( \text{P}_1\)-Tris, 5mM magnesium acetate, 1% ethanol, 0.2 mg alcohol dehydrogenase and 50 \( \mu \text{M} \) \( \text{KNO}_3 \). The temperature was 23°C. The free nitrate concentration was monitored with a \( \text{NO}_3^- \) electrode as described in methods. \( \text{KNO}_3 \) (10 \( \mu \text{M} \)), \( \text{NAD}^- \) (0.2 mM) and FCCP (2.5 \( \mu \text{M} \)) were added as indicated by the arrows.
of NO\textsubscript{3}\textsuperscript{−} uptake (Fig. 6.3) is between 145 and 150 mV, the same as that calculated from the extent of SCN\textsuperscript{−} uptake (Figs. 6.1 and 6.2). The use of equation 3.3 in calculating $\Delta \psi$ assumes that the anions passively equilibrate with an electrical potential across the membrane, and thus the steady-state level of accumulation of an anion should be independent of the nature of the anion. The finding that measurement of the uptake of either SCN\textsuperscript{−} or of NO\textsubscript{3}\textsuperscript{−} gives the same value for $\Delta \psi$ thus provides important support for use of equation 3.1.

Fig. 6.4 illustrates the versatility of the electrodes, and the generality of their applicability to the study of energy-transducing membrane vesicle preparations. Fig. 6.4 shows the use of the Orion NO\textsubscript{3}\textsuperscript{−} electrode to sense the SCN\textsuperscript{−} ion (to which it is sensitive in the absence of NO\textsubscript{3}\textsuperscript{−}) in a reaction mixture containing R. rubrum chromatophores. A marked, light-induced, pseudo-first order uptake of SCN\textsuperscript{−} is observed (Fig. 6.4). This uptake corresponds to a $\Delta \psi$ of about 100 mV (cf. Schuldiner et al., 1974; Chapter 3). The NO\textsubscript{3}\textsuperscript{−} electrode, unlike the SCN\textsuperscript{−} electrode, is insensitive to light and so can be used to determine light-dependent uptake of either NO\textsubscript{3}\textsuperscript{−} or SCN\textsuperscript{−}. Indeed, a considerable number of anions may be sensed by liquid membrane electrodes of this kind (e.g. Coetzee and Freiser 1968, 1969; Reinsfelder and Schultz 1973; Jyo et al., 1974). NO\textsubscript{3}\textsuperscript{−} does not interfere with the SCN\textsuperscript{−} electrode and so this electrode can be used to follow SCN\textsuperscript{−} uptake in the presence of NO\textsubscript{3}\textsuperscript{−}. 
Fig. 6.4. Light-induced uptake of SCN\(^-\) by *Rhodospirillum rubrum* chromatophores monitored with a NO\(_3\)\(^-\) -selective electrode

*R. rubrum* chromatophores (0.63 mg bacteriochlorophyll) were incubated in 5 ml of a reaction medium containing 10 mM P\(_i\)-Tris pH 8.0, 5 mM magnesium acetate, 20 mM sucrose, 0.2 mM sodium succinate and 10 \(\mu\)M KSCN. Free SCN\(^-\) concentration was monitored with a NO\(_3\)\(^-\) electrode as described in Methods, and calibrating additions of KSCN were made as indicated. At the open arrow, the illumination (a 300 W projector filtered with a Kodak Cinemoid Deep Orange filter and 5 cm of water) was turned on. It was turned off at the full arrow. The (saturating) light intensity at the centre of the reaction vessel was approximately 3 \(\times\) 10\(^5\) erg cm\(^{-2}\) s\(^{-1}\). When the light was off, the reaction mixture was covered with aluminium foil. The temperature throughout was 27°C.
Fig 6.5

The diagram shows the relationship between the respiration rate (O2 consumption) and the membrane potential (mV) as a function of Rotenone concentration (nmol/mg protein). The respiration rate decreases as the concentration of Rotenone increases, indicating inhibition of respiration. The membrane potential remains relatively constant across the range of Rotenone concentrations shown in the graph.
Oxygen uptake was determined polarographically and thiocyanate uptake potentiometrically in parallel 5 ml reaction mixtures, which contained 10 mM P_i-Tris pH 7.3, 5 mM magnesium acetate, 1% v/v ethanol, 0.2 mg alcohol dehydrogenase, 0.6 mM NAD+, 2.65 mg vesicle protein and 20 μM KSCN. Aliquots of rotenone were added from a stock solution in ethanol to the concentrations indicated. The membrane potential was calculated from the extent of thiocyanate uptake as described in Chapter 5. Similar results were obtained when a number of experiments were performed, each preincubated for 2 minutes before initiation of respiration (with NAD+) with the appropriate concentration of rotenone.
Fig. 6.5 displays the results from an experiment similar to that displayed in Fig. 6.2, except that *P. denitrificans* membrane vesicles were substituted for bovine heart submitochondrial particles. In this case (Fig. 6.5), the relationship between the rate of electron transport and the protonmotive force is far from ohmic, as found for the FCCP-titration described in the previous chapter. This contrasts with the results for bovine heart submitochondrial particles, and suggests that there is a significant difference in the functional (and presumably structural) organization of the energy-transducing membranes of *P. denitrificans* and beef heart mitochondria. This effect in *P. denitrificans* vesicles, that decreasing the rate of respiration with an electron transport inhibitor had no effect on the protonmotive force, was also found when Antimycin A was used instead of rotenone (data not shown). A fuller theoretical discussion of titrations of this type is given in Chapter 7.

**DISCUSSION**

The use of ion-selective electrodes in biochemical studies has been widely reported, as testified by the current interest in potentiometric methods of analysis (e.g. Moody and Thomas 1971; Koryta 1975; Bailey 1976; Lakshminarayariah 1976). In studies of bioenergetics, ammonium (e.g. Rottenberg and Grunwald 1972; Rottenberg and Lee 1975) and potassium-selective electrodes (e.g. Jackson *et al.*, 1968; Mitchell and Moyle 1969a; Papa *et al.*, 1973a; Collins and Hamilton 1976) have been used previously, as well as the electrodes sensitive to lipophilic ions developed by Skulachev and co-workers (Grinius *et al.*, 1970), the latter of which are unfortunately
not commercially available. Papa et al., (1973a) reported the use of a SCN\(^{-}\) electrode, but found that the response was too slow to be of use in their kinetic studies. Possibly the electrodes that were used in the present work do not suffer from this disadvantage (Figs. 6.1, 6.3, 6.4). Neither electrode was significantly interfered with by lipophilic ionophores (valinomycin, nigericin), by electron transport inhibitors (antimycin, rotenone) or by low concentrations of uncouplers (FCCP, 1799). Millimolar concentrations of succinate, however, did interfere with the electrodes when they were sensing micromolar concentrations of NO\(_3^{-}\) or SCN\(^{-}\), as did the radical-forming electron mediator TMPD.

I believe that these electrodes possess a number of clear advantages over methods currently used for the monitoring of transmembrane electrical potentials in microscopic systems:

1. Quantitative probing of bulk transmembrane electrical potentials by following ion distribution is a method which possesses a sound theoretical basis, in contrast, for example, to the use of fluorescence probes, for which the basis of response to \(\Delta \Psi\) remains unclear (e.g. Njus et al., 1977, and see Chapter 7).

2. The electrodes can sense low activities of ions, and avoid the use of radioisotopes of high specific activity.

3. The electrodes are constructed of biologically inert materials.

4. A direct read out in real time is provided.

5. A large number of ions may be sensed.

6. The method is non-destructive, allowing further analysis of samples.
7. The size and high sensitivity of the electrodes is such that only small amounts of biological materials are required.

Electrodes should also be of particular value in extending studies on the relationship between the rate of electron flow and the magnitude of $\Delta \psi$ (see earlier), as titrations with an uncoupler or electron transport inhibitor may in principle be performed on a single sample.

**METHODS**

Ion-selective electrode measurements were made in a thermostatted 25 ml beaker using an Orion Series 94-58 solid-state SCN$^-$ electrode or an Orion Series 93-07 liquid membrane NO$_3^-$ electrode. The potential developed by the SCN$^-$ or NO$_3^-$ electrode was measured against a double junction reference electrode (model 90-02, Orion Research, M.S.E., Manor Royal, Crawley, Sussex, U.K.) using a pH meter (Pye Unicam model 290 mK II) set on the mV mode and connected in parallel with a 100 Ohm variable resistor to a Servoscribe chart recorder, essentially as described by John (1977). The 5 ml reaction mixtures, which are given in the legends to the figures, were stirred with a magnetic bar, and both the stirrer speed and the variable resistor were adjusted so that the noise level did not exceed 1% of a full scale deflection. Where appropriate, a jet of oxygen, saturated with water at 30°C, was blown at the surface of the reaction mixture. The bridging solution in the double junction reference electrode was 10% KF. Electrodes were soaked in a 0.1 M solution of the ion to be sensed for at least one hour before use.
Measurement of the extent of ion uptake together with estimates of the internal volume enclosed by the vesicles, namely 1.3 µl per mg protein for submitochondrial particles (Chapter 4) and 50 µl per mg bacteriochlorophyll for chromatophores (Chapter 3), enabled \( \Delta \psi \) to be calculated from equation 3.1, as described previously.

Mg-ATP submitochondrial particles were prepared as described by Ferguson and Sorgato (1977), except that all chlorides were replaced by the corresponding acetate salts. The preparation of \textit{R. rubrum} chromatophores, the flow-dialysis measurements, oxygen uptake and protein determinations are detailed in previous chapters.
CHAPTER 7

ON THE FUNCTIONAL PROTON CURRENT OF ELECTRON TRANSPORT PHOSPHORYLATION: AN ELECTRODIC VIEW
"Natural selection in particular has operated to prevent any increase in entropy because it has eliminated any disorder which tended to arise and promoted an increase of more efficient and often more complex structural and functional orders."

Rensch (1971)

"Electrodics is the study of processes which occur at the surface of an electronic conductor in contact with a liquid phase."

Bockris and Reddy (1970)

INTRODUCTION

It is generally believed (e.g. Boyer et al., 1977) that the chemiosmotic hypothesis of biological energy transduction (Reviews: Mitchell 1976a, 1977a,b and references therein) represents the most useful working hypothesis for experiments designed to gain an insight into the molecular mechanisms underlying the coupling between electron transport and the phosphorylation of ADP. Indeed, the experiments reported in the previous 5 chapters were explicitly designed using the chemiosmotic hypothesis as a heuristic tool. I should now like to review the extent to which the experimental observations I have so far reported, particularly those of Chapters 3-5, are consistent with this (chemiosmotic) paradigm.

It is appropriate to begin with a brief review of the premises upon which the chemiosmotic hypothesis is based (Mitchell 1966a, 1968, and see Chapter 1), and which I have used as a working hypothesis for the purposes of experimentation:

(a) Electron transport or ATP hydrolysis in an energy-
transducing biological membrane results in the transmembrane
electrogenic translocation of protons from one bulk aqueous
phase to another, such that in the steady state an electro­
chemical potential difference of protons is set up across
the membrane.

(b) The magnitude of the transmembrane electrochemical proton
gradient, \( \Delta \mu H^+ \) or protonmotive force \( \Delta p \), is given
by the sum of an electrical \( \Delta \psi \) and a chemical \( \Delta pH \)
component, according to the equation

\[
\Delta p = \Delta \mu H^+ = \Delta \psi - \frac{\Delta pH}{F} \cdot 2 \cdot \frac{RT}{F}
\]

where \( \Delta p \) and \( \Delta \psi \) are mV, \( \Delta pH \) is in pH units and the symbol
\( \Delta \) signifies a difference between 2 transmembrane aqueous
phases. \( R, T \) and \( F \) have their thermodynamic meaning, and
the factor \( RT/F \) is approximately equal to 60 mV at 25°C.

(c) Since proton movements are primary, the electrical and
chemical terms of the equation \( \Delta p = \Delta \psi - 60 \Delta pH \) are energetically
interchangeable under appropriate conditions of charge- or
pH-neutralisation.

(d) The proton gradient generated by electron transport may
be utilised to drive the endergonic synthesis of ATP from ADP
and \( P_i \) such that, under 'static head' conditions, the free
energy stored in ATP is poised against the protonmotive force
according to the relationship

\[
\Delta \mu = -zF \cdot \Delta p
\]

where the phosphorylation potential \( \Delta \mu \) \( = \Delta \mu^{\circ} \) + \( RT \cdot \ln \frac{[\text{ATP}]^7}{[\ADP]^7[\Pi]^7} \)
and \( z \) = the number of protons translocated across the membrane
for each ATP molecule synthesised, the \( H^+/ATP \) ratio.
Additionally, $z$, the $\rightarrow H^+/ATP$ ratio is also related to the stoichiometry of proton- and ATP-generation according to the relationship

$$\rightarrow H^+/ATP = z = \frac{\rightarrow H^+/e^-}{ATP/e^-}$$

Although the actual stoichiometries themselves are not an integral part of the chemiosmotic hypothesis, the numbers obtained should be constant under given reaction conditions.

In the experiments described in Chapters 3-5 I have used a flow dialysis assay of the distribution of hydrophilic solutes to determine the magnitude of the components of the protonmotive force generated by three different biological membrane vesicle preparations under different conditions. An extensive validation of the methods used, and of the homogeneity of the vesicle preparations, has been given in Chapters 3 to 5, and in the following discussion it will be assumed that the parameters measured by the flow dialysis method are indeed the components of the bulk, transmembrane protonmotive force as defined by Mitchell (1966, 1968, and see Rottenberg 1975).

Results from chromatophores: chemiosmotic interpretation

In the experiments with *Rhodospirillum rubrum* chromatophores described in Chapter 3 it was found that light-driven electron transport caused the formation of a transmembrane electrical potential of magnitude 100 mV in a $P_i$-Tris/magnesium acetate medium. Increasing concentrations of thiocyanate caused a conversion of the electrical component of the proton-
motive force $\Delta \psi$ into a transmembrane pH gradient $\Delta \text{pH}$ such that their sum (in mV) remained essentially constant. Comparison of this value (100 mV) of the protonmotive force with the free energy stored in ATP under static head conditions indicated that 5 or 6 protons must be translocated across the membrane for each molecule of ATP synthesised. Arguments were presented in an attempt to rule out the possibility of systematic error in the measurements. The only other quantitative measurement of the $\Delta \text{pH}/\text{ATP}$ ratio of chromatophores (of *Rhodopseudomonas spheroides*) (Jackson et al., 1975) for which no technical criticism could be offered gave a value of 2. Although an octamolecular reaction for the *R. rubrum* ATPase ($6\text{H}^+, 1\text{ADP}, 1\text{P}_i$) seems very unlikely, no explanation, based upon reasoning within the chemiosmotic idiom, could be offered for the discrepancy between the kinetic work of Jackson et al., (1975) and the present thermodynamic measurements.

**Results from submitochondrial particles: chemiosmotic interpretation**

Experiments similar to those described for chromatophores were carried out using bovine heart submitochondrial particles (see Chapter 4). Under a variety of conditions it was established that the bulk phase protonmotive force could serve as a thermodynamically competent intermediate between electron transport and ATP synthesis provided that an $\Delta \text{pH}/\text{ATP}$ ratio of 3 was accepted. The electrical and chemical components of the protonmotive force were essentially interchangeable when increasing concentrations of the permeant nitrate ion were added
to the usual reaction mixture. It was concluded, despite some controversy in the literature concerning this point, that the results were broadly consistent with a chemiosmotic interpretation in which the $\rightarrow H^+/ATP$ ratio was equal to 3.

Two additional points emerged from the results of Chapter 4. First, the value of $\Delta p$ generated by a given substrate varied markedly depending upon the incubation medium used, without a corresponding variation in $\Delta G_p$. This result is not expected if a bulk phase transmembrane electrochemical proton gradient constitutes the sole and obligatory link between electron transport and ATP synthesis. Secondly, an independence between the measured bulk protonmotive force and the $\Delta G_p$ generated by submitochondrial particles was observed when the substrate was varied, in particular when ascorbate/TMPD was the substrate. Further, there did not seem to be any relationship between the value of the steady-state protonmotive force and the rate of ATP synthesis.

Whereas the chemiosmotic hypothesis requires a constant ratio between $\Delta p$ and $\Delta G_p$, at least under conditions of constant pH, it was found that the value of the $\rightarrow H^+/ATP$ ratio calculated from a comparison of $\Delta p$ and $\Delta G_p$ in fact varied significantly. Additionally, it was found that concentrations of the permeant nitrate ion sufficient to raise $\Delta pH$ at the expense of $\Delta \psi$ when NADH was the substrate were unable to convert the $\Delta \psi$ generated by TMPD-mediated ascorbate oxidation into a $\Delta pH$. Again this behaviour would not be expected if a delocalised bulk phase transmembrane protonmotive force is the sole obligatory functional intermediate between electron transport and phosphorylation.
Results from *P. denitrificans* vesicles: chemiosmotic interpretation

In the case of the phosphorylating *P. denitrificans* vesicles, measurement of the protonmotive force generated by NADH oxidation in the usual P$_i$-Tris/magnesium acetate reaction medium suggested that the protonmotive force consisted of a transmembrane electrical potential of 145 mV with no contribution from a transmembrane pH gradient (Chapter 5). Comparison of this value with the phosphorylation potential attained was consistent with the idea that at least 4 protons must be translocated across the membrane per ATP synthesised if a chemiosmotic type of mechanism is operative. However, the addition of 10 mM nitrate to this system caused a decrease in \( \Delta y \) to an undetectable level with no increase in the pH component of the protonmotive force. Under these latter conditions there was no decrease in the \( \Delta G_p \) generated by the particles, and it was concluded that under such conditions a bulk transmembrane electrochemical proton gradient did not constitute a thermodynamically competent intermediate between electron transport and ATP synthesis.

In summary, therefore, it would seem that although a number of the experimental observations described above may be accommodated within the chemiosmotic framework of biological energy transduction, there are a number of observations which cannot be so accommodated. Particularly in view of the large magnitude and the variability of the \( \rightarrow H^+ / ATP \) ratio calculated from these measurements it seems appropriate to seek an interpretation of these observations that is alternative to the chemiosmotic viewpoint.

Accepting the widely held view that proton gradients are intimately involved as an intermediate in electron
transport phosphorylation, I present first a qualitative statement of 4 postulates of a working hypothesis that I believe does incorporate the most attractive features of other current models of energy-coupling in electron transport phosphorylation (Boyer et al., 1977), and which may be of some explanatory and predictive value. This hypothesis is based upon (a) the application of current ideas concerning electrode processes to energy-transducing biological membranes, (b) the recognition that charge separation is a primary result of electron transport, and (c) the belief that evolutionary factors will tend to promote a subtle and comprehensive structural and functional organisation within biological membranes. I shall refer to the present formulation, for the purposes of semantic distinction, as an electrodic view.

An electrodic model for electron transport phosphorylation

Postulate 1: Transmembrane electrochemical proton gradients of some kind do provide a functional link between electron transport and phosphorylation, but the electrochemical proton gradient across the membrane is greater than that measured across the bulk aqueous phases.

Postulate 2: The functional proton current of electron transport phosphorylation does not enter the bulk phase opposite to that in which ADP is phosphorylated, but is carried along localised and specific channels at the surface of the membrane in a specific interphase 's phase'. Specific proton conduction, both across the membrane and within the interphase is effected by charge-relay systems, Grotthus transfer along chains of adsorbed water molecules and by proton tunnelling. Therefore the electrochemical proton potential at the F_0 part of the ATPase (Racker 1970), which is itself within the interphase
'S phase', is displaced from equilibrium with that in the bulk phase which it faces.

**Postulate 3:** Treatments which act to decrease the native proton current flow within the S phase affect electron transport phosphorylation in a manner which may be predicted from their known effects on surface potentials described by the Gouy-Chapman-Stern-Grahame theory of the double layer (Bockris and Reddy 1970).

**Postulate 4:** The generation of surface charges is an integral part of electron transport phosphorylation. It is brought about by changes in ionisation and conformation of electron transport carriers (and the ATPase enzyme) which are responsible for vectorial proton adsorption and release. Although the term 'surface' is of somewhat arbitrary nature, I shall retain it to describe the specific 'interphase' phase S, within the Stern-Grahame layer, in which the postulated functional proton current is carried.

Before considering experimental evidence in favour of such a formulation I think it worthwhile to review some concepts concerning electricity, electrodics and proticity, for I believe that very fruitful analogies may be drawn between the processes of electrochemistry and biological energy transduction by proticity (e.g. Mitchell 1976a).

**Electricity and proticity, and the importance of interfaces**

Electricity is the flow of electrons from regions of higher electrical potential to regions of lower electrical potential. In an analogous fashion (Table 7.1), proticity is the flow of protons from regions of high protonic potential to regions of lower protonic potential. Now neither electrical or protonic
| Table 7.1 | Electrical concepts and their protical equivalents |
| Flow of current | Electrical term | Protical term |
| Thermodynamic cause of current flow | Electromotive force (emf) | Protonmotive force (pmf) |
| Interface between emf and aqueous solution for the purpose of performing chemical work | Electrode | Protode |
| Free energy store | Electrical potential difference $\Delta G = -eF\Delta V$ | Protonic potential difference $\Delta \mathcal{P}_{H^+} = \Delta V - 2.3 R T \Delta \rho H / F$ |
| Aqueous conducting solution | Electrolyte | Buffer solution |
| Current-carrying entity in aqueous solution | Ions | Protons |
| Current-carrying entity in external circuit | Electrons | Protons or "H" |
| Site of high potential | Positive ions | 'Acidic' moieties |
| Site of low potential | Negative ions | 'Basic' moieties |
| Ability to store energy | Capacitance | Buffering power |
Potentials may be defined in absolute terms, but must either be related to an arbitrary reference point of zero potential or expressed as a difference in potential between the point of interest and another point in space. The electrical potential difference between two points is given by

$$\Delta G_e = -zF\Delta V$$ \hspace{1cm} \text{Eqn 7.1}$$

where $\Delta G_e$ is the electrical potential energy difference, in kcal mol$^{-1}$, between the two points, $z$ is the number of charges on an electron (-1) and $\Delta V$ is the potential difference in volts between the two points. $F$ is the Faraday constant (23.08 kcal volt$^{-1}$).

Similarly, the electrochemical proton potential difference between two points in space is given by (Mitchell 1968)

$$\Delta \mu^+ = \Delta \psi - 2.3 \frac{RT}{F} \Delta \text{pH}$$ \hspace{1cm} \text{Eqn 7.2}$$

where $\Delta \mu^+$ is the electrochemical potential difference of protons (in volts), $\Delta \psi$ is the electrical potential difference (in volts) and $\Delta \text{pH}$ is the pH difference (in pH units). $R$ and $T$ have their usual thermodynamic significance and $RT/F$ has a value of approximately 60 mV at 25°C. Mitchell has used the symbol $\Delta p$ for the electrochemical proton gradient between two bulk phases separated by an energy-transducing membrane.

In this chapter I shall reserve the $\Delta p$ symbol for this specific (transmembrane bulk phase) meaning, and use the symbol $\Delta \mu^+$ in more general terms to signify an electrochemical potential difference of protons between any two regions. The particular region may be identified by a superscript, using the bulk phase of lower protonic potential as a reference point.
The diagram represents a phospholipid membrane M phase separating two aqueous phases L and R. Interphases SL and SR exist between the M phase and the two bulk phases L and R respectively. The M phase contains a protonmotive electron transport complex (ETC) and a protonmotive ATPase of the appropriate polarities, and the radial and axial flow of the proton current, i.e. a two-dimensional proton current flow, between them is indicated by the arrows.

Most of the discussion in this chapter will be concerned with such a protic circuit, and it will be argued that in real systems the bulk of the proton current flow is confined within the S phases. The electrical equivalent of this circuit, and the resulting one-dimensional protonic potential profile across the system, are given in Fig. 7.9.
Thus for the protic circuit of Figure 7.1, the symbol $\Delta \mu^L_{H^+}$ refers to the electrochemical protonic potential difference between the surface phase adjacent to phase L and the bulk aqueous phase R.

Now since our interest in proticity lies in its ability to be reversibly coupled to the performance of useful chemical work, a comparison between electricity and proticity should necessarily begin with a consideration of the manner and mechanism by which electricity can be coupled to the performance of useful chemical work. Therefore I shall begin this comparison of electricity and proticity by the consideration of certain electrochemical phenomena, for a most lucid, and more rigorous, derivation of which the reader is referred to Bockris and Reddy (1970).

If we consider an aqueous solution of chemical entities, together with a source of electrical energy, work can be done by the latter on the former only by the intercession of electrodes. An electrode may be defined for the present purposes as an interface between a source of electrical energy and an aqueous solution, at which the electrical energy may be used to carry out useful chemical work. In order for continuing chemical work to be done, however, two electrodes are required, to serve as a connection between the two poles of the electrical energy source. Thus we may consider the system shown in Figure 7.2, which represents a source of electrical energy, the battery, with its negative pole connected to a plane-parallel Pt electrode (cathode) and its positive pole connected to a plane-parallel silver electrode (anode), with the two electrodes immersed in a solution of acidified water. As soon as the
Fig 7.2
Fig. 7.2  An electrolytic circuit

The minimal configuration for an electrolytic cell. For discussion, see text. The variation in electrical potential around the circuit is given in Figure 7.3.
Fig 7.3
Fig. 7.3  Potential-distance diagram for the system of Fig. 7.2

The diagram illustrates the potential variation in the circuit shown in Figure 7.2. It is of particular interest that the major potential drops occur across the electrode-electrolyte interfaces. The reason for this is discussed in the text.
switch is turned on (Figure 7.2) an electric field is set up, given by the potential difference across the solution divided by the distance between the electrodes. The electrical potential difference between the electrodes takes the form shown in Figure 7.3. The system is not at equilibrium, and in an attempt to restore equilibrium positive ions move toward the cathode and negative ion toward the anode. Such a tendency towards gross charge separation means that electroneutrality is upset in the bulk of the solution; the separated charge causing the lack of electroneutrality tends to set up its own electric field, of an opposite polarity to that of the applied field. If the two fields were to become equal in magnitude the net result would be that the solution was an ideal capacitor, and no chemical work would have been done. However, in the circuit described by Figure 7.2 there are two types of current-carrying entity; within the metal wires of the external circuit current is carried by electrons, whereas in the aqueous solution the current is carried by hydronium $\text{H}_3\text{O}^+$ and hydroxyl $\text{OH}^-$ ions. A steady flow of current round the circuit can be maintained only if there is a change of charge-carrier at the electrode/electrolyte interface. The transfer of electrons from the ions of the solution to the external circuit results in chemical changes (in the valence state of the ions), and under such conditions the flow of current continues in response to the applied potential and useful chemical work is done. What has happened to the energy stored in the battery? Neglecting losses in the wires of the external circuit and in the internal resistance of the battery, two sites of energy dissipation, or the doing of work, may be
identified. Work has been done in moving the ions from the bulk of the solution to the electrode/electrolyte interfaces and work has been done, necessarily associated with chemical changes, in causing electron flow between the ions of the solution and the wires of the external circuit.

Two particularly important points emerge from Figure 7.3. The potential drop across the electrode/electrolyte interface is much greater than that across the electrolyte solution and furthermore is the only potential drop directly associated with the performance on useful chemical work. The study of electrified interfaces, and of their molecular properties is called Electrodics. In the definition by Bockris and Reddy (1970), "Electrodics concerns the region between an electronic and an ionic conductor and the transfer of electric charge across it". Now, electron transport phosphorylation concerns the generation of electrochemical proton gradients across a lipophilic membrane containing electronic conductors, separating two aqueous phases containing ionic conductors. I therefore consider it a properly electrodic study.

The model for biomembrane energy transduction by proticity that I shall be putting forward in more explicit detail in the following sections contains a major departure from the more traditional chemiosmotic view of these processes. This departure is the suggestion that there is a significant change in protonic potential across the interfaces between energy-transducing biomembranes and the adjacent aqueous phases.

In defending this departure, therefore, I will describe certain salient features of electrodic theory and practice that I believe are of relevance to the present considerations.
Fig 7.4
Different regions in the interphase between a bulk aqueous phase on a charged surface which may be distinguished by their physical properties. The Stern layer (see also Fig. 7.5) extends for a few tenths of a nm from the surface, while the extent of the Gouy-Chapman region, also known as the region of diffuse charge, may extend for 10-50 nm at low ionic strengths, although at high ionic strengths it is severely reduced. The Nernst potential, or phase boundary potential, is equal to the total interfacial potential. The shear boundary separates the free water from the water of hydration, and the potential at this point is called the zeta potential. It may be obtained from electrokinetic measurements.
Fig 7.5
A schematic illustration of the constitution of the Stern-Grahame layer adjacent to a charged membrane in contact with an aqueous solution. The symbol $\text{\textsuperscript{\rightarrow}}$ represents a water molecule with the arrow indicating the positive pole of the water dipole. $\text{M}^+$ and $\text{H}_3\text{O}^+$ represent alkali metal cations and hydronium ions respectively, and the position of the inner (IHP) and outer (OHP) Helmholtz planes is indicated by dotted lines. Thus, the IHP is found at the boundary of the primary layer of adsorbed water molecules, while the OHP delineates the midpoints of the primary layer of solvated ions adjacent to the OHP.
Electrodics; the mechanistic kinetics of electrode processes

Consider again the electrical circuit of Figure 7.2.

The initial chemical change at the cathode is

\[ \text{M}^{\text{pe}} + e^- + H^+ \rightarrow \text{M} \ldots \ldots H(\rightarrow \text{M} + \frac{1}{2}\text{H}_2) \]  
Eqn 7.3

and at the anode

\[ \text{M}^{\text{Ag}} + \text{OH}^- \rightarrow \text{M} \ldots \ldots \text{OH} + e^- (\rightarrow \text{M} + \frac{1}{4}\text{O}_2 + \frac{1}{2}\text{H}_2\text{O}) \]  
Eqn 7.4

Now the sole action of the electromotive force in bringing about these chemical changes is due to its effect in charging the electrode surfaces relative to the bulk phase. If we consider the cathode, the electrical potential drop from the metal surface to the bulk phase may be split into three distinct regions, and takes the form shown in Figure 7.4.

In media of high ionic strength the Gouy-Chapman region of diffuse charge is relatively insignificant, and for the present purposes may be conveniently left out of consideration.

The important events take place within the Stern-Grahame layer, which extends for 1 nm or so from the metal surface. The constitution of the Stern-Grahame layer, consisting of inner (IHP) and outer (OHP) Helmholtz planes, is given in Figure 7.5. The inner Helmholtz plane is populated by contact-adsorbed water molecules, whose dipole orientations, induced by the negative charge on the electrode surface, are as given in the Figure 7.5. Adjacent to the IHP is a layer of solvated protons, consisting of molecules of the general formula \((\text{H}_{2n+1}\text{O}_n)^+\) together with other solvated cations. In understanding the reason for the dramatic potential drop across the Stern-Grahame layer we must consider the molecular forces acting on the hydronium ions in the OHP that cause them to donate their excess protons to the charged electrode. The
Fig 7.5
Fig. 7.6.  **Free energy and potential profiles for an electrode-electrolyte interface**

The free energy and potential profiles across the interface between a charged electrode and an electrolytic solution. Only the Stern layer, represented by the IHP and OHP as in the previous Figure, is considered. The electrical work necessary to activate the ion is determined by the potential difference across which the ion has to be removed to reach the top of the free energy-distance relation.
free energy and potential profiles across the Stern layer under the circumstances of the reaction under consideration are given in Figure 7.6. The factor $\beta$ is an extremely important one in electrodics (Bockris and Reddy 1970), and to understand its significance the Butler-Volmer equation will be introduced.

When no net current is flowing the forward reaction of equation 7.3, the electronation of protons, is taking place at a rate equal and opposite to the back reaction, or deelectronation of hydrogen atoms. Under such conditions therefore (no applied field) we may write a rate equation for the electronation current density, $\dot{I}$, and the deelectronation current density, $\dot{I}$. These values are equal and opposite in the absence of a field; they are then called the equilibrium exchange current density $i_0$.

Thus,

$$i_0 = \dot{I} = -\dot{I} \quad \ldots \quad \text{Eq 7.5}$$

where

$$\dot{I} = Fk_eC_A - \alpha \exp\left(-\beta \frac{F \Delta \phi}{RT}\right) \quad \ldots \quad \text{Eq 7.6}$$

and

$$\dot{I} = Fk_eC_D \alpha \exp\left((1-\beta) \frac{F \Delta \phi}{RT}\right) \quad \ldots \quad \text{Eq 7.7}$$

$\beta$ is the symmetry factor of Figure 7.6, $C_A$ and $C_D$ are the concentrations of electron acceptor (protons) in the aqueous phase and the electron donor at the electrode surface (Pt) respectively. $k_e$ and $k$ are rate constants derived from the absolute theory of reaction rates (Glasstone et al., 1941; Laird 1965) and are given by

$$k_e = \frac{kT}{h} \exp\left(-\frac{\Delta \xi^0}{RT}\right) \quad \ldots \quad \text{Eq 7.8}$$

where $k$ is Boltzmann's constant, $h$ is Planck's constant and $\Delta \xi^0$ is the standard free energy of activation. $k_e$ and $k_e$ have the
When an ionmotive electrical field or 'overpotential' is applied across the interface, however, equilibrium is upset, there is a change in free energy across the interface and net current flows. The value of the net current density is given by the Butler-Volmer equation:

\[ i = \dot{i} - \dot{i} = F \dot{k} C_D \exp \left( (1 - \beta) \Delta \phi \frac{F}{RT} \right) - F \dot{k} A \exp \left( -\beta \Delta \phi \frac{F}{RT} \right) \]

.........Eq 7.9

This equation may be more conveniently written in terms of the exchange current density \( i_0 \) and the current-producing field or overpotential \( \eta \):

\[ i = i_0 \sqrt{\exp \left( (1 - \beta) F \eta \frac{RT}{F} \right) - \exp \left( -\beta F \eta \frac{RT}{F} \right)} \]

.........Eq 7.10

If the symmetry factor \( \beta \) is equal to \( \frac{1}{2} \), as is in fact frequently the case, the Butler-Volmer equation reduces to

\[ i = i_0 \sinh \left( F \eta \frac{RT}{2F} \right) \]

.........Eq 7.11

and the \( i \) versus \( \eta \), or current-voltage, plot is of the form of a hyperbolic sine function. Two limiting cases of equation 7.10 are of interest. The first is one in which the overpotential is relatively large (say \( > 100 \text{mV} \)), and

\[ \exp \left( F \eta \frac{RT}{2F} \right) \gg \exp \left( -F \eta \frac{RT}{2F} \right) \]

such that the equation 7.10 reduces to:

\[ i = i_0 \exp \left( (1 - \beta) F \eta \frac{RT}{F} \right) \]

.........Eq 7.12

and thus the current density increases exponentially with the overpotential. If the field is very small, however, (say \( \eta \ll 100 \text{ mV} \)) one can consider that \( F \eta /2RT \ll 1 \) and use the approximation \( \sinh (F \eta /2RT) \approx \frac{F \eta}{2RT} \).

The low-field approximation therefore reduces the Butler-Volmer equation to

\[ i = \frac{i_0 F \eta}{RT} \]

.........Eq 7.13
\( \eta \) (OVERPOTENTIAL IN VOLTS)

\[ \begin{align*}
0.15 \\
0.10 \\
0.05 \\
\end{align*} \]

\[ \log i_0 \quad \log i \]

**Fig 7.7**
Fig. 7.7. A Tafel plot

A typical Tafel line for a one-electron-transfer electrode reaction is given, showing the exponential relationship at high overpotentials, which makes the relation between $\eta$ and log $i$ linear.
with the result that a linear relationship apparently exists between the driving overpotential and the current density. A typical plot of the current/voltage relations of an inorganic electrode, a Tafel plot, is shown in Figure 7.7. Its significance will be clarified when we consider the relationship between the rate of performance of chemical work by a transmembrane proton potential gradient and the size of that gradient. As I have indicated above, the contribution of any rate terms in the bulk of the solution or in the diffuse charge region has been neglected in this treatment; the reason for this will be indicated in the section on zeta potentials.

**A summary of the digression into electrodics**

Two salient points emerged from a consideration of the structure of the electrode-electrolyte interface and its influence on the kinetics of electrode processes. The first was that the movement of protons between the OHP and the electrode surface was associated with the only transformation of chemical into electrical energy (Figs. 7.2, 7.3), and the second was that the large electrical potential drop across this region could be viewed in terms of the energy required to break the H-0 bonds in H₃O⁺ ions and carry them over the 'hump' of the activation energy barrier (Fig. 7.6).

We shall consider later one or two other aspects of electrodics, and their relevance to biomembrane energy transduction by proticity, in particular the notion of the protonic capacitance of energy-transducing membranes. However, it is first appropriate to consider in some detail the nature of proton transport and of proticity.
Interphases and the kinetic control of the flow of proticity

Nearly twenty years ago, Williams (1961, 1978a, b) independently of Mitchell, surmised that localised proton gradients might be involved in electron transport phosphorylation, and drew particular attention to the energetics of protons within the phospholipid membrane phase. Williams has more recently laid emphasis upon the concept of kinetic control of proton current flow (Williams 1978a, b). Certainly a reduction of the volume occupied by the functional proton gradients, which volume might be affected by the conditions of incubation, would neatly account for all the problems associated with magnitude and the variability of the proton-motive force that I have discussed earlier. Whilst postulates 1-4 (above) concerning the possible functional proton current during electron transport phosphorylation are in broad agreement with certain of the proposals of Williams (1978a,b), I have chosen to lay particular emphasis upon the events at the membrane surface in this process. A number of previous authors have emphasised the importance of the membrane surface and its electrical charges during electron transport phosphorylation (e.g. Bockris 1969; Azzi 1969; Dell'Antone et al., 1972; Montal and Gitler 1973; Young 1974; Iberall 1975; Schäfer and Rowohl-Quisthoudt 1975; Archbold et al., 1976; Kraayenhof and Arents 1976; Barber et al., 1977; Rumberg and Muhle 1976; Kraayenhof 1977; Nishi et al., 1976; Njus et al., 1977), and it is within this context that I now present this framework for the role of the membrane surface in exercising a kinetic control on proton transfer during electron transport phosphorylation.
FIGURE 7-8

MATRIX SIDE

ATP Synthetase complexes

Electron transport complex

Uninterrupted Bilayer areas

INTERMEMBRANE SPACE
A fluid mosaic model of the structure of energy-transducing membranes. The diagram depicts the composition of an energy-transducing membrane as variable between organized regions that have hydrophobic domains, probably in lipid bilayer configuration, and areas of the membrane in which the hydrophobic domains are interrupted by proteins or lipoproteins.
Fig 7.9
The upper half of the figure shows the electrical equivalent circuit of the 5-phase protic circuit given in Figure 7.1. The significance of the batteries, resistors and capacitances is given in the text. Other symbols are given in the legend to Fig. 7.1. The capacitance of the M phase per se and any resistive 'leak' across the membrane have been omitted. The lower half indicates the variation in protonic potential perpendicular to the plane of the system, and the relationship between $\Delta p$ and $\Delta \tilde{\nu}^+$. The lower half of the figure serves only to indicate the general trend of the protonic potential, for the microscopic nature of the diagram is not appropriately represented by macroscopic potential-distance relationships.
Fig. 7.8 shows a fluid mosaic model of the structure of an energy-transducing membrane (after Packer 1974), containing electron transport complexes and ATP synthase complexes embedded in a proton-impermeable phospholipid bilayer membrane. Figure 7.9 indicates the electrical equivalent of the electrostatic view of two protonmotive cells embedded in a phospholipid membrane (cf. Fig. 7.1). The "batteries" ET and "ATPase" represent protonmotive electron transport complex and ATPase species, respectively. The wires indicate proton transport pathways that occur with an insignificant loss in free energy, while the resistances indicate the activation energy barrier that must be overcome in bringing protons from the bulk phases to the membrane surfaces, just as in the electrode case in Figures 7.2, 7.3 and 7.6. The capacitances represent the buffering powers of the different phases.

In the following I shall be equating the interphase S phase, in which the functional proton current is postulated to be carried, with the Stern layer (cf. Figures 7.4, 7.5), and in order to defend this view it is necessary to review current ideas concerning the kinetics of proton transfer processes (e.g. Eigen 1964; Zimmerman 1964; Faraday Society 1965; Caldin 1969; Bockris and Reddy 1970; Bell 1973, Bamford and Tipper 1978).

**The kinetics of proton transfer processes**

As a direct result of its extremely small size, and thus of its very high charge density, the proton does not exist free in solution, but as the hydronium H₃O⁺ ion. This hydronium ion may be further solvated to give species of general formula (H₂n⁺ Oₙ), where n is commonly equal to 4. These extra water molecules attached to a given proton are termed the secondary
Fig 7.10
In the initial state (1), 3 water molecules and a hydronium ion are lined up favourably for proton transfer along the chain by an essentially concerted mechanism. Such a transfer leads to state 2, and is accomplished in approximately $10^{-14}$ seconds. For another proton to be bound to the water molecule which was a hydronium ion in state 1, a reorientation is necessary, and under normal conditions takes place in approximately $2 \times 10^{-13}$ seconds. Such a Grotthus transfer mechanism accounts for the anomalously high mobility of the proton in aqueous solutions. (e.g. Bockris and Reddy 1970).
hydration sheath. In aqueous solution the rate-limiting step for the transfer of a proton from one water molecule to an adjacent water molecule is the re-orientation time necessary for Grotthuss transfer of the type shown in Figure 7-10.

However the mobility of protons in ice crystals exceeds even the proton mobility in aqueous solution by one to two orders of magnitude. In this case the rate-limiting step for proton transfer is the non-classical quantum mechanical 'tunnelling' (Caldin 1969) under the activation energy barrier. The reason for this change in rate-limitation is that in the case of ice the individual water molecules are both less concentrated and are 'structured' in a manner favourable to the rapid classical intermolecular transfer. Thus any sort of favourable 'structuring' of water molecules will effect a specific kinetic control upon the pathway of proton current.

There is one other type of mechanism which is responsible for significantly affecting the rate of proton transfer, and that is the presence of chains of acid or base centres (other than water molecules). The rate of the transfer of protons between acid/base molecules is given by the product of a rate constant and the concentration of acid/base centres. This rate constant is itself dependent upon the differences in pK between the donor and acceptor molecules (Elgen 1964). Thus the nature and concentration of different acid/base centres will significantly affect the (vectorial) rate of a proton transfer process. Direct proof of this in the case of a biological molecule has recently been given for the enzyme carbonic anhydrase \( \mathcal{C} \), in which it has been shown
(Campbell et al., 1975) that the rate of proton translocation to the active site of the enzyme, as visualised by high-resolution proton NMR, is greatly increased by a chain of acid/base centres on the enzyme surface (cf. Williams 1978a). It is therefore of great interest, in the context of the present model of proton transfer during electron transport phosphorylation, to enquire whether such a mechanism might operate during the latter process. Such systems have been called charge-relay systems (Blow et al., 1969). In the discussion earlier in this chapter of the structure of the electrode surfaces, it was shown that an important role was played by water molecules adsorbed to the electrode surface which constituted the Inner Helmholtz Plane. It was shown how the IHP formed a kinetic barrier to proton uptake during current flow from the bulk phase. Additionally their rather 'structured' nature suggests that they might play a further key role in the context of a rapid channelling of the flow of proticity in the sense that lateral flow, in contrast to the flow of protons to and from the aqueous phases, would be encouraged by this type of structure. I shall therefore now consider very briefly the types of evidence that suggest that energy-transducing biological membranes do indeed have 'structured' water molecules adsorbed to their surfaces just as do electrodes, and that the properties of such water molecules are rather different from the water molecules of the bulk aqueous phases. For a comprehensive overview of this topic the reader is referred to Drost-Hansen (1971).

'Bound water' at biological interfaces

A large number of authors have drawn attention to the fact that biological membranes possess adsorbed or 'bound'
water molecules which possess considerably different physical properties from the water molecules of aqueous solutions (e.g. Bockris and Reddy 1970; Schultz and Asunmaa 1970; Drost-Hansen 1971; Walters & Hayes 1971; Lumry 1974; Cooke and Kuntz 1974; Yamamoto & Nishimura 1976; Hazlewood 1977; Ling 1977; Hagler and Moult 1978). Evidence that such 'bound' water molecules exist has come from 2 main types of experiment. The first concerns measurements of the physical properties of membranes, such as thermal phase transitions, with anomalous changes in slope at certain temperatures, or infrared (Zundel 1976) and magnetic resonance (see Drost-Hansen 1971; Hazlewood 1977) spectroscopic studies. The other type of study involves measurements of the rate of diffusion of solutes across biological membranes under various conditions (see Drost-Hansen 1971). The purpose of the present chapter is not to review the enormous body of evidence that is consistent with the possibility of 'bound water' at biological interfaces, but to draw attention to the importance that such a water structure would have for the relative kinetics of proton flow along the membrane surface and into the bulk aqueous phases during electron transport phosphorylation. The importance of 'bound' water molecules in crystalline proteins has been discussed more generally by Scotton and Eisenberg (1975) and by Hagler and Moult (1978). Therefore whilst Williams (1978a,b) emphasises the possible role of 'structured' water molecules in the F0 part of the ATPase (and see Okamoto et al., 1977), I should prefer to stress the role of structured water molecules in more general terms within the context of electron transport phosphorylation, namely as a medium, which is itself not in
equilibrium with the bulk phase water for the conduction of protons, liberated by electron transport or ATP hydrolysis (cf. Zundel 1976).

Now, ions can affect the degree of organisation of water such that dipole changes and changes in hydrogen bonding occur. The sequence of activity of some anions in decreasing the degree of organisation of water is

$$\text{BPh}_4^- > \text{SCN}^- \approx \text{ClO}_4^- > I^- > \text{Br}^- > \text{NO}_3^- > \text{Cl}^- > \text{OH}^- > \text{F}^-$$ (e.g. Jain 1972)

The ions at the head of this series are known as chaotropic anions (Hatefi and Hanstein 1969; McLaughlin et al., 1975). In line with the observation that the rate-limiting step in the transmembrane movement of many lipid-soluble ions is passage across the 'unstirred' interphase layer (e.g. Ciani et al., 1975), such chaotropic ions are most rapidly able to permeate biological membranes (cf. Chapter 6). Therefore, in the context of the ability of ions such as SCN$^-$ to permeate biological membranes, it should be remembered that they are equally effective in disrupting the structure of 'ice-like' water in the interphase S phase. Significantly, Yamamoto and Nishimura (1976), in a study of the effect of temperature on the kinetics of proton movements in spinach chloroplast thylakoids, stressed the possible role of structured water molecules in determining the rate of dark efflux of protons into the outer aqueous phase following a period of illumination. Regrettably current theories of the structure of water at the surfaces of energy-transducing membranes are insufficiently developed to permit quantitative comparison with experimentation. Whatever the nature of the shuttle mechanism by which electron-transport-derived protons may be moved across the surface of the membrane, such a mechanism offers two important advantages:
(a) a subtle and effective partitioning may be made of the energy generated by electron transport between ATP synthesis and other energy-requiring reactions such as active substrate transport,

(b) if the functional proton current involved in electron transport phosphorylation does pass along specific membrane-associated channels not in equilibrium with the bulk proton potential in phase L, there is no problem associated with an interpretation of the ability of marine or alkalophilic bacteria (see Garland 1977) to exist in environments in which ATP synthesis would be thermodynamically impossible if a chemiosmotic system is operative.

It is appropriate at this juncture to review experimental evidence in the literature that has been, or may be, interpreted to suggest that there is indeed a kinetic diffusion barrier to proton flow between an energy-transducing membrane surface and the adjacent bulk aqueous phases under conditions approximating those in vivo.

Evidence for a diffusion barrier to protons near the surface of energy-transducing membranes

Direct evidence for the existence of a proton-diffusion barrier at the inner surface of the thylakoid membrane, observed using the hydrophilic pH-indicating dye cresol red, has been presented by Junge and Ausländer (1973; see also Ausländer and Junge 1973). The rapid binding of protons to the surface of light-energy transducing membranes, followed by their slower equilibrium with a bulk aqueous phase, has also been indicated by the observations of Rumberg and Muhle (1976); Ort et al., 1976; Kraayenhof (1977) and Nishi et al., (1977).
The slow rate and extent of proton ejection into bulk phase L induced by the addition of a small pulse of oxygen to anaerobic suspensions of mitochondria (e.g. Mitchell and Moyle 1967b) or bacteria (e.g. Scholes and Mitchell 1970b; Gould and Cramer 1976) in the absence of permeant ions has also been noted. The ability of permeating ions such as K\(^+\) plus valinomycin (Mitchell and Moyle 1967b) or SCN\(^-\) (Scholes and Mitchell 1970b) to increase the rate and extent of H\(^+\) ejection by these systems has been explained on the basis of an electrophoretic migration of these ions acting to neutralise the thermodynamic back-pressure of electrical potential caused by the rapid build-up of a protonmotive force (Mitchell and Moyle 1967b; Mitchell 1968; Jackson et al., 1968; Scholes and Mitchell 1970b; Papa 1976). However, this interpretation has been questioned on the basis of some measurements of bacterial \(\rightarrow\) H\(^+\)/O ratios in the absence of permeant count­erions, in particular the observation (Gould and Cramer 1977) that the addition of a second oxygen pulse immediately following the first one is not subject ot this back-pressure. There is evidence from work with liposomes of different surface potential, that both thiocyanate (McLaughlin et al., 1975) the valinomycin-K\(^+\) complex (McLaughlin et al., 1970; Hsu and Chan 1973; Ciani et al., 1975) and other ions (Ketterman et al., 1971; Andersen et al., 1978) interact with phospholipid membranes to alter the surface potential of such membranes. The rate-limiting step in valinomycin-mediated transmembrane ion conductance, from one bulk aqueous phase to another, at saturating valinomycin concentrations, is the diffusion of the charged valinomycin-cation complex in the unstirred layers adjacent to the membrane (e.g. Ciani et al., 1975; McLaughlin & Eisenberg 1975; cf. Winne 1973).
Thus it is easy to see that protons liberated by electron transport at the surface of a charged membrane, and which are kinetically restricted from entering the bulk phase by a diffusion barrier in the form of an 'unstirred' interphase, may enter the bulk phase if the diffusion barrier is lifted by an alteration of the surface potential of the charged membrane. In this regard, it might be expected that the electrophoretic mechanism of charge-neutralisation (at saturating counterion concentrations) should operate equally satisfactorily with co- or counter-migration of appropriately charged ions. However if surface potential effects in the interphase are of importance in causing stoichiometric proton ejection into the bulk aqueous phase, then it need not be expected that saturating concentrations of thiocyanate of $K^+$/valinomycin would have the same effect as each other on the proton current pathway in phase L. Such a lack of correspondence between the stoichiometry of $H^+$ liberation in the presence of the two types of permeant ion has indeed been noted (Scholes and Mitchell 1970b; Ort and Dilley 1976). This mechanism for an action within the surface layers of SCN$^-$ in stimulating proton ejection is indicated in Fig. 7-11. Such a mechanism is fully consistent with the observation that both buffering in phase L and the presence of permeant ions are required either to uncouple electron transport from phosphorylation (e.g. Montal et al., 1970; Leiser and Gromet-Elhanan 1973; Azzone et al., 1976; Gromet-Elhanan 1977) or to drive it during 'acid-bath' experiments (e.g. Jagendorf and Uribe 1966; Jagendorf 1972; Thayer and Hinkle 1975; Wilson et al., 1976; Gromet-Elhanan 1977), since thiocyanate would speed proton equilibration across the S phases in both directions.
Glass electrode

Fig 7.11
Dual mechanisms by which membrane-permeable ions stimulate proton ejection into the bulk aqueous phase during oxygen pulse experiments. As well as an electrophoretic migration of SCN\(^-\) across the membrane, contact-adsorbed SCN\(^-\) ions decrease the capacitance of the S phase, by substituting for the adsorbed water molecules of the IHP. According to the calculation given in the text, approximately 90% of the stimulation of H\(^+\) ejection into the bulk aqueous phase must be accounted for by the decrease in capacitance of the S phase(s), while about 10% may be ascribed to a charge-neutralising electrophoretic migration of the SCN\(^-\) ion. According to this electrodic view, therefore, all membrane-permeable ions, such as the K\(^+\)/valinomycin complex, will exhibit chaotropic behaviour, a fact in accordance with experimental observation.
A further prediction stemming from this mechanism is that the stoichiometry of proton ejection for various systems, measured in the presence and the absence of charge-neutralising ions, should be influenced in a predictable manner by treatments affecting their surface charge. Appropriate systems might include bacteriorhodopsin-containing liposomes of differing surface charges, or neutral membranes whose fixed surface charges has been modified by such compounds as biguanides (Schafer 1974; Schafer et al., 1974; Schafer and Rowohl-Quisthoudt 1975; Schafer 1976a,b; cf. Schafer and Rowohl-Quisthoudt 1976) or salicylates (McLaughlin 1973).

Thus in electrochemical terms the action of membrane-permeable ions in stimulating proton ejection into the bulk aqueous phases is, by substituting for the adsorbed water molecules of the I.H.P., to effectively decrease the capacitance and to increase the resistance to proton current flow within the S phases, just as in electrode kinetics is found for such contact-adsorbed ions (Bockris & Reddy 1970). The insufficiency of the electrophoretic mechanism to account for the stimulation by thiocyanate of $H^+$ ejection by $P. denitrificans$ cells may be put on a more quantitative footing using the data of Scholes and Mitchell (1970b). All relevant data are those cited by these authors except for the following: the $t_{1/2}$ for SCN$^-$ uptake by $P. denitrificans$ vesicles, which was obtained by the ion electrode method described in Chapter 6, was 45 seconds, and it is assumed that this holds true for SCN$^-$ efflux from intact cells of the organism. Additionally, the volume enclosed by the cells, measured by the sucrose-impermeable space method described in Chapter 3, was 2 ml per g dry weight.
Scholes and Mitchell (1970b) found an apparent $\frac{H^+/0}{4}$ ratio of 4 in the absence of SCN$^-$, which was increased to 7.5 when 17.5 mM SCN$^-$ was present, at pH 6.0 - 6.1. According to the electrophoretic mechanism, therefore, the extra 3.5 per O$^+$ ejected into the outer aqueous phase must be accompanied by a similar number of negatively charged SCN$^-$ molecules during the time of reduction of the pulse of oxygen added. The conditions used by Scholes and Mitchell (1970b) were 20 mg dry weight of Paracoccus cells per 4 ml reaction mixture, and 23.5 natom O were injected. The uncoupled respiration rate was 1.5 ng atom/mg dry weight/second (Scholes and Mitchell 1970a,b). Thus the respiratory burst induced by the pulse of O$_2$ lasts 0.784 s.

The pseudo-first order rate constant for transmembrane electrophoretic movement of SCN$^-$ is given by \((\ln{2/45}) \, s^{-1} = 0.0154 \, s^{-1}\). The actual rate of SCN$^-$ movement across the membrane is given by \(k \times (\frac{[SCN^-]}{7})\), where \(k\) is the pseudo-first order rate constant. Therefore the rate of SCN$^-$ movement is given by 0.0154 x 17.5 nmol/µl/s, = 0.27 nmol/µl/s.

The internal volume of the cells in the suspension is 40 µl, and thus the total SCN$^-$ movement during the oxygen-induced burst of respiratory activity = 0.27 x 40 x 0.784 = 8.5 nmol.

Now the amount of O added was 23.5 ng atom, and therefore the extra H$^+$ which must be accommodated = 3.5 x 23.5 = 82.3 H$^+$. Thus under the conditions described by Scholes and Mitchell (1970b), it would appear that an electrophoretic mechanism of charge neutralisation may only account for 10% of the extra H$^+$ released. It is concluded that a reexamination of the role of SCN$^-$ in increasing the stoichiometry of H$^+$ ejection measured during pulses of electron transport activity may indeed be
warranted, both in the case of \textit{P. denitrificans} cells and more generally.

**Ion lipophilicity, membrane potentials and proton movements**

An important and specific prediction stemming from the present formulation is therefore that both proton and ion movements should be dramatically affected by the concentration of contact-adsorbing permeant ions in the interphase S phases. If the argument is restricted to vesicular systems in which electron transport or ATP hydrolysis causes the lumen of the vesicle to become acidic or positive with respect to the medium, it is necessary to consider proton and anion uptake.

In such 'inverted' systems is is well known (e.g. Jackson \textit{et al.}, 1968; Leiser and Gromet-Elhanan 1973) that electron-transport-induced proton uptake is stimulated by permeant anions, and this is explained, within the chemiosmotic framework, by an electrophoretic migration of the permeant ion tending to neutralise the membrane potential component of the protonmotive force, concomitantly replacing it with a steady-state pH gradient (e.g. Mitchell 1968). Two effects may be distinguished (see also Chapters 2 to 5). At low concentrations of hydrophilic permeant ion (10-20 \( \mu \text{M} \)), electron transport- or ATP-induced ion uptake occurs to an extent governed by the Nernst potential, and the ions may be used as a probe for the Nernst potential. At much higher concentrations of hydrophilic permeant ion, however (approx 10 mM), ion uptake causes a decrease in the Nernst potential to energetically insignificant values, together with a large stimulation of proton uptake and the formation of a pH gradient. If under the present model it is considered that these effects are mediated within the interphase S phases, however, the effects
of hydrophilic and lipophilic permeant ions may be dis-
tinguished, for the concentration of contact-adsorbed lipo-
philic ion in the S phases will be much greater than the
concentration of hydrophilic ions in the S phases for a
given added ion concentration, and it may be expected that
lipophilic ions will be more effective in both stimulating
proton uptake and being taken up themselves in response to
electron transport. I will restrict consideration here to
the extent of ion uptake induced by electron transport,
as a function of ion lipophilicity.

It was shown in Chapter 6 that the extent of ion uptake
induced by NADH oxidation in submitochondrial particles was
identical for the hydrophilic nitrate and thiocyanate ions.
However other more lipophilic ions, in particular the tetra-
phenylborate ion (Grinius et al., 1970), would be expected to
be taken up to a greater extent on energisation than
hydrophilic ions such as nitrate or thiocyanate. Such behaviour
has indeed been reported for a number of lipophilic anions
in submitochondrial particles (Azzone et al., 1978c).
Chloroplast thylakoids are of special interest in this respect,
for it is well known that the movement of Cl⁻ and Mg²⁺ across
the thylakoid membrane in response to electron transport
results in the neutralisation of a transmembrane potential
(e.g. Hind et al., 1974), and the expression of the proton-
motive force solely in the form of a transmembrane pH gradient
(e.g. Hauska and Trebst 1977; Junge 1977). However pea
chloroplasts have been shown to take up the lipophilic phenyl
decarbaundecaborane ion in response to electron transport
under steady-state conditions (Grinius et al., 1972), consistent
with the view, advanced here, that the electrical potential at the surface of the membrane is greater than that within the bulk phase L. It is of particular interest in this regard that the magnitude of the field-indicating carotenoid absorption change is consistent with the idea that an electrical potential of approx. 100 mV exists across the pigment molecules in the steady state (e.g. Witt 1971; Graeber & Witt 1976; Hauska and Trebst 1977; Junge 1977). It will be of interest therefore to test the prediction that stems from these findings: that tetraphenylborate, but not thiocyanate, should increase the magnitude of the light-induced uptake of protons by chloroplast thylakoids, and that a given concentration of tetraphenylborate should enhance the light-induced proton uptake of *R. rubrum* chromatophores more than the same concentrations of thiocyanate.

**An electrogenic explanation of the mechanism of action of uncouplers**

It is widely believed (but cf. Hanstein 1976) that weakly acidic lipophilic uncouplers of electron transport phosphorylation act by virtue of their ability to conduct protons across natural (Mitchell and Moyle 1967a; Scholes and Mitchell 1970a) and synthetic (e.g. Chizmadzhev et al., 1971; Haydon and Hladky 1972; Jain 1972; Cohen et al., 1977) bilayer membranes. However, the rate of proton translocation catalysed by a variety of uncouplers is strongly dependent upon the surface potential of charged membranes. This has been noted, for example, in the case of dinitrophenol (McLaughlin 1972), 5,6-dichloro-2-trifluoromethylbenzimidazole (McLaughlin 1977) and FCCP (Haydon and Hladky 1972). Bakker et al., (1975) have also shown that the binding of a variety of uncouplers to both liposomes and mitochondria both affects and is
According to the electrodic view, weakly acidic uncoupler of oxidative phosphorylation may exert 2 types of action. They may conduct protons across the membrane, either by diffusion or by a relay mechanism, and they may also contact adsorb, and, like other permeant ions, speed the equilibration of protons between the S phases and the bulk aqueous phase. The relative effectiveness of the second type of uncoupling mechanism will be governed by the buffering power of the bulk aqueous phases. $\text{AH, A}^-$ represent neutral and ionised uncoupler molecules.
affected by the surface potential and the bulk pH in a manner consistent with the predictions of double layer theory. Therefore whether or not there is a kinetic barrier to protons entering and leaving the bulk phases from the Stern layer S phases, the potent ability (Muraoka et al., 1975; Terada 1975) of uncoupler molecules to short-circuit a proton current between the S phases is to be expected. The mechanism is illustrated schematically in Figure 7-12. The uncoupling activity of non-acidic molecules such as the phenylisothiocyanates (Miko and Chance 1975) and pentachloronitrobenzene (Seaston et al., 1976) would be ascribed, in the electrodc formulation, to an effect on the dipoles of the water molecules of the IHP. The ability of thiocyanate to reduce the uncoupling effectiveness of FCCP in P. denitrificans cells (Scholes and Mitchell 1970b) was in fact ascribed to a "space-charge effect", although it could perhaps be as easily explained by a lowering of the proton and uncoupler 'reservoir' within the S phases (cf. Figs. 7.1, 7.9). Conventional explanations of the mechanism of uncoupling action (cf. Chapter 2) of ionophores of the valinomycin and nigericin types (e.g. Jackson et al., 1968; Mitchell 1968; Pressman 1976) are also as easily applicable to situations in which the functional proton current of electron transport phosphorylation occurs within the S phase region as if it occurs between the two bulk phases. Therefore, although the seminal observations that one gramicidin molecule per thylakoid (see Witt 1971) or per chromatophore (Saphon et al., 1975b) are sufficient to give full uncoupling have been widely interpreted to indicate that the functional proton gradients of electron transport
Table 7.2

$\Delta H^+/ATP$ ratios measured under various conditions

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>Pulse technique $\Delta H^+/ATP$ with membrane permeable &quot;charge-neutralising&quot; ions present</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>4</td>
<td>A</td>
</tr>
<tr>
<td>Yes</td>
<td>3-4</td>
<td>B</td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>C</td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>C'</td>
</tr>
<tr>
<td>SMP</td>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
<td>D</td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>E</td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>F</td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>G</td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>H</td>
</tr>
<tr>
<td>Chromatophores</td>
<td>No</td>
<td>6</td>
</tr>
<tr>
<td>Yes*</td>
<td>2</td>
<td>J</td>
</tr>
<tr>
<td>No*</td>
<td>2-3</td>
<td>K</td>
</tr>
</tbody>
</table>


Estimates of the $\Delta H^+/ATP$ ratio for the ATPase of various energy transducing organelles. Although the situation in mitochondria is complicated by the operation of substrate carriers, the results from the other organelles are fully consistent with the idea that apparent $\Delta H^+/ATP$ stoichiometries are smaller when measured during 'pulse' experiments in the presence of a high concentration of charge-neutralising ions. Chloroplasts have not been included.
Table 7.2 continued

above owing to the controversy over the existence of a steady-state membrane potential. (See Hauska and Trebst 1977; Junge 1977). Estimates derived from fluorescent probe measurements have not been included (see text). * indicates that the electrochromic shift in carotenoid absorption was used.
phosphorylation occur across the bulk aqueous phases, they are fully consistent with the present 5-phase formulation. Thus, although the protonic potentials in the $S$ phases are kinetically displaced from equilibrium with those of the adjacent bulk aqueous phases, the protonic potential throughout a given $S$ phase is essentially constant, and a single uncoupler molecule per vesicle may be expected to change it.

**Thermodynamic and kinetic measurements of proton movements in vesicular systems; the resolution of a controversy?**

The disquieting problem (Table 7.2) of the lack of harmony between the $\rightarrow H^+/ATP$ ratio measured for submitochondrial particles by kinetic methods in the presence of "charge-neutralising" membrane-permeable ions (Moyle and Mitchell 1973a, Thayer and Hinkle 1973) ($\rightarrow H^+/ATP = 2$) and by thermodynamic comparison of $\Delta p$ and $\Delta G_p$ (Wiechmann et al., 1975; van Dam et al., 1977; Chapter 4) ($\rightarrow H^+/ATP \geq 3$) has been alluded to earlier (Chapter 4). Similar controversies exist for both chromatophores (Jackson et al., 1975; Baccarini-Melandri et al., 1977; Chapter 3) and chloroplasts (Graber and Witt 1976; Portis and McCarty 1976; Hauska and Trebst 1977; Junge 1977).

In each of these systems the ATPase faces the medium, and thus there are no complications from energy-linked movements of adenine nucleotides (Rottenberg and Klingenberg 1977) or of phosphate (Brand 1977). I would suggest that the most economical resolution of this problem lies in the mechanism elaborated here: (a) that the functional proton current of electron transport phosphorylation occurs not within phases $L$ and $R$ but in $S$ phase within the double layer, (b) that the proton-motive force measured across the bulk phases is not in kinetic equilibrium with this, but that the addition of high concen-
trations of "charge-neutralising" ions such as thiocyanate or valinomycin/K⁺ changes the electrical (and thus proton electrochemical) potential at phase S with expulsion of protons into the bulk of phase L. If such an explanation is correct, it would be expected that correspondence might be found between (a) the → H⁺/ATP ratio calculated from the thermodynamic relationship between Δp and ΔGₚ, measured in the presence of high concentrations of charge-neutralising ions, and (b) the → H⁺/ATP ratio calculated by dividing the → H⁺/O ratio by the P/O ratio under such conditions. An 'inverted' system with an unbranched respiratory chain, and with a fully reversible ATPase, would be required to test this hypothesis, and it would appear that bovine heart submitochondrial particles constitute such a system. On the basis of the present analysis it is therefore concluded that the true → H⁺/ATP ratio of the mitochondrial ATPase is 2, and that that measured in Chapter 4 is an overestimation, since the functional proton current is carried in the interphase region.

Detection of surface potentials with fluorescent probes: ANS

In the mechanism of proton current flow during electron transport phosphorylation described here, emphasis has been placed on the effect of surfaces on local electrochemical proton potentials, and it is therefore of interest to know how surface potentials may be detected in energy-transducing membranes. 1-anilinonaphthalene-8-sulphonate (ANS) has been widely used as a reporter of the 'energised state' of energy-transducing membranes (e.g. Radda 1971; Radda and Vanderkooi 1972; Azzi 1975; Njus et al., 1977). It responds to the energisation of submitochondrial particles by an increase in its binding to the
particles, with no change in fluorescence yield (at least with ATP-energisation), the bound form having a larger fluorescence than the free form (see Njus et al., 1977). Although the molecular basis for the 'ANS response' remains unclear (Njus et al., 1977), it has been shown (Montal and Gitler 1973) that the optical response of anionic dyes is subject to a membrane surface potential. ANS in particular (Azzi 1969; Haynes 1974; Schafer 1974; Schafer and Rowohl-Quisthoudt 1975; McLaughlin and Harary 1976; Njus et al., 1977) has been shown to report changes in surface or, more particularly, Stern (McLaughlin and Harary 1976) potentials. Although attempts have been made (Ferguson et al., 1976a; Leiser and Gromet-Elhanan 1977) to calibrate the ANS response in terms of a transmembrane potential by means of K⁺/valinomycin diffusion potentials, it has been shown (Ferguson et al., 1976a; Njus et al., 1977) that the kinetic characteristics of such diffusion potential-generated ANS responses are different from those induced by electron transport or by ATP hydrolysis. Of particular interest in this context is the suggestion (Ferguson et al., 1976a; see also Ernster 1977) that ANS responds to potential energy gradients of a more localised nature than transmembrane electrical potentials.

Detection of surface potentials with fluorescent probes: 9AA

The energy-linked fluorescence quenching of 9-aminoacridine (9AA) has also been used as a probe of membrane energisation, in chloroplast thylakoids (Schuldiner et al., 1972), bacterial chromatophores (Casadio et al., 1974a,b) and submitochondrial particles (Rottenberg and Lee 1975). It was originally proposed
that there was a direct correspondence between the interaction of 9AA with the thylakoid membrane and the 'bulk' trans-
membrane of pH gradient measured by hydrophilic amine distribution
(Sculdiner et al., 1972). However, it is now believed that
in many systems (Fiolet et al., 1974; Kraayenhof and
1976; Kraayenhof et al., 1976; Kraayenhof 1977; Searle et al.,
1977) the 'energy-linked' quenching of 9AA fluorescence arises
from binding of the protonated from of the probe at a surface
of the membrane. In this respect, it is of interest that the
results presented earlier (Chapters 4, 5) showed that the
pH gradients determined by the 9AA method were always approx­
imately 1.5 pH units higher than those dextemined by methyl-
amine distribution. A similar conclusion has been reached, after
a more systematic analysis, by De Benedetti and Garlaschi
(1977). It may be concluded that there is good evidence that
the 'energised' state probed by both 9AA and ANS is intimately
associated with the generation of membrane surface charges and
with a change in Stern potential.

Direct measurement of energy-linked changes in surface charge
distribution

Figure 7.4 indicates the electric charge profile between a
charged membrane surface and an adjacent 'bulk' aqueous phase. Although this is based upon the Gouy-Chapman and Stern-Grahame
theories, with their attendant approximations, it serves to
illustrate how changes in the membrane surface charge dis-
tribution may be reflected at different points in space. In
particular, the zeta potential, the electrical potential at
the plane of shear, is a parameter which is directly accessible
to experimental analysis using microelectrophoretic mobilities,
at least in semi-quantitative terms. Measurements of the zeta
potential of biological membranes, and of its changes upon membrane energisation, have been reported by e.g. Davies et al. (1956), Nobel and Mel (1966), Kamo et al. (1976), Aiuchi et al. (1977) and Quintanilha and Packer (1977). However, although changes in the zeta potential of energy-transducing membranes do indeed accompany membrane energisation, such changes are energetically very small, and it is for this reason that I have suggested that the functional proton current occurs within the Stern layer, i.e. within the plane of shear.

It may be concluded from the previous 3 sections, therefore, that although the fluorescent probe methods may be regarded as providing an accurate monitor of membrane energisation as indicated as changes in Stern potential and membrane charge distribution, such changes are not readily apparent at the plane of shear.

The electrical capacitance of energy-transducing membranes

According to the chemiosmotic view of biological energy transduction, the sole function of the proton-impermeable phospholipid membrane is to separate the bulk aqueous phases across which the electrochemical proton gradient is generated. The electrical capacitance of the membrane itself constitutes the only part of the system across which a potential energy drop takes place. According to the electrodic view presented here, however, the electrical (or, more properly, protonic) capacitance of the system resides additionally in the S phases on either side of the membrane. Thus, as in the study of electrochemistry (Bockris and Reddy 1970) analysis of
the current-voltage relationships of the membrane may lead to an understanding of the nature of the electrical capacitors involved in energy transduction. In particular, a consideration of the properties of a series array of capacitors allows a resolution of the 'Pacific Ocean' controversy, and it is to this topic that I now turn.

According to Mitchell, the lipoprotein membrane surrounded on either side by an aqueous phase may be regarded as a 3-phase system, consisting of a lipophilic membrane M phase forming an osmotic barrier between 2 aqueous phases L and R, and it was for this reason that the term 'chemiosmotic' was conceived. On this basis, any protons moved 'across' the membrane must necessarily move from one bulk aqueous phase to another. This conception has been repeatedly criticised on theoretical grounds by Williams as involving an unacceptable loss of free energy if the buffering power in one or both aqueous phases is sufficiently large (e.g. Williams 1978a), the so-called 'Pacific Ocean' effect. This criticism is rejected by Mitchell (e.g. Mitchell 1977a), but in the following I shall show that, although functional proton gradients do occur across the plane of the M phase, provided that the functional proton current under normal conditions is confined to the interphase region between the lipophilic M phase and the aqueous phases on either side of it, bacteria may indeed live in the Pacific Ocean (Williams 1978a) or in regions of extreme alkalophilicity (e.g. Garland 1977), without a loss in free energy, despite the generation of a transmembrane electrochemical proton gradient.
According to the chemiosmotic view (e.g. Mitchell 1977a), the differential buffering capacity of a protonmotive system is given by:

\[
\frac{1}{B} = \frac{1}{B_o} + \frac{1}{B_i}
\]

where \(B_o\) and \(B_i\) represent the external and internal phase buffering powers. Thus if either \(B_o\) or \(B_i\) is made infinitely large, although the differential buffering capacity of the system will be affected, the system will still be able to store protic energy. In the context of the present formulation the protonic capacitance of the membrane system is given in the same way by:

\[
\frac{1}{C} = \frac{1}{C_L} + \frac{1}{C_M} + \frac{1}{C_R} + \frac{2}{C_{SL}} + \frac{1}{C_{SR}}
\]

where the subscripts refer to the 5 phases described in Figure 7.1. For mercury electrodes the double layer capacitance is approximately 20 \(\mu\)F cm\(^{-2}\) (Bockris and Reddy 1970) whilst the capacitance of the M phase is approximately 1 \(\mu\)F cm\(^{-2}\) (Mitchell 1966; Packham et al., 1978). Thus, even if the buffering power in each aqueous phase is made infinitely large, the capacitance of the system will be reduced to only 0.9 \(\mu\)F cm\(^{-2}\). In other words the existence of large buffering capacities in either the bulk phases or (one of) the S phases will have a negligible effect upon the capacitance of the whole membrane system. Only when the S phase capacitances are severely reduced, e.g. by the presence of contact-adsorbing ions (Anderssen et al., 1978), can the storage capacity of the membrane be affected by the buffering power of the bulk aqueous phases. It is worth noting in this context that Young (1974) has calculated surface capacitances for the chloroplast thylakoid membrane of 140 \(\mu\)F cm\(^{-2}\), whilst a value
of 50 \mu F \text{ cm}^{-2} \text{ is given by Feldberg and Delgado (1978) for phospholipid bilayers. Therefore the suggestion (Mitchell 1966) that translocation across the mitochondrial cristae membrane (capacitance 1 \mu F \text{ cm}^{-2}) \text{ of } 1 \text{ ng ion H}^+ \text{ per mg protein should lead to a transmembrane potential of } 250 \text{ mV is not correct within the context of the present formulation, for the large double layer capacitances will have the effect of reducing the effective transmembrane potential for a given quantity of charge released into the intramitochondrial S phase. A similar (incorrect) analysis has been given for chromatophores by Packham et al., (1978).}

In this section it is appropriate to draw attention finally to a study by Coster and Simons (1970). These authors used a Wayne-Kerr conductance bridge to study the capacitance changes in phospholipid bilayer membranes. Whilst they enjoined caution in the quantitative acceptance of their results, they showed that there were indeed layers of water adjacent to the membrane surface with capacitance properties very different from those of bulk phase water.

The current-voltage relationships of protonmotive circuits

In the introduction to Electrodics presented earlier, particular emphasis was laid upon the relationship between the potential across an electrode and the current flow through it. It is appropriate, therefore, in the context of the present formulation, to discuss the relationships observed between these parameters in systems catalysing electron transport phosphorylation.

It has been known for some time that the absorption spectrum of membrane carotenoids undergoes a red shift upon membrane
'energisation', which may be mimicked (at least partially) by ionic diffusion potentials (Jackson and Crofts, 1969). It is now generally accepted that the 'carotenoid band shift' and the '515 nm shift' of thylakoids is indeed electrochromic in nature (e.g. Jackson et al., 1968; Witt 1971; Saphon et al., 1975a; Junge 1977; Symons et al., 1977; Witt 1977), and the extent and decay rate of the shift has been utilised to provide a 'molecular voltmeter and ammeter' in both chromatophores and chloroplast thylakoids. The purpose of this section, inter alia, is to draw attention to the similarity of the 'current-voltage' plots obtained by this method and the Tafel plots (Fig. 7.7) of electrochemistry, and to suggest that such a similarity may be interpreted to constitute further evidence for the electrolic analogy which I have drawn in this chapter.

In both the chemiosmotic and electrolic formulations the rate of electron transport, the rate of ATP synthesis and the rate of proton current flow are related by: rate of proton transport = rate of electron transport \( x \rightarrow H^+/e^- = \) rate of ATP synthesis \( x \rightarrow H^+/ATP \).

For a protic circuit the rate of current flow is equal to the rate of proton transport (in the steady state) whilst the current-producing field of 'overpotential' may be equated with the protonmotive force across the M phase. Thus the protic equivalent of the Butler-Volmer equation (Eqns 7-9, 7-10) is:

\[
i_{H^+} = i_o \exp (1 - \beta ) \frac{\Delta \tilde{\mu}^+_{H^+}}{F/RT} - \exp(-\beta \Delta \tilde{\mu}^+_{H^+}/F/RT) \]

Eq. 7-14

where \( i_{H^+} \) is the rate of proton current flow and \( \Delta \tilde{\mu}^+_{H^+} \) is the electrochemical proton gradient between phases SL and SR.
The significance of $\beta$ is the same as that in the Butler-Volmer equation (see above), where it has the effect of reducing the proportion of free energy which is available for doing useful chemical work. For proton current flow within the S phases it is assumed that $\beta$ is vanishingly small, but for other proton diffusion pathways which may be considered there will be different values of $\beta$. A case of particular interest, within the context of present-day bioenergetics and the 5-phase formulation outlined here, is the comparison between the protonmotive forces between the 2 bulk aqueous phases on either side of the membrane and the 'true' protonmotive force between the 2 interphases. In this case the relationship

$$\Delta p^L-R = \beta \Delta \tilde{\mu}^{H+}_{SL-SR}$$

holds.

As in the case of the electrode it is predicted that $\Delta \tilde{\mu}^{H+}_{SL-SR}$ and the logarithm of the rate of ATP synthesis (or electron transport) will be proportional to each other for values of $\Delta \tilde{\mu}^{H+}_{SL-SR}$ over 100 mV. Such a relationship has been found, for example, by Gräber and Witt (1976).

What are the current-voltage relationships when the protonmotive force is measured across the 2 bulk aqueous phases? The most complete study of this question has been that of Azzone et al. (1978a,b,c), who have used an ion-distribution method to examine the current-voltage relationships of the inner mitochondrial membrane. They found no relationship between the size of the protonmotive force and the rate of protic current flow, and concluded that a bulk protonmotive force was not involved as an intermediate in ATP synthesis (cf. Chapters 4-6). This may be explained by substituting the value of $\beta = \Delta p / \Delta \tilde{\mu}^{H+}_{SL-SR}$
into equation 7-14, giving:

$$i_{H^+} = i_o \exp \left( \frac{\Delta \mu_{H^+}}{RT} - \Delta p \right) \frac{F}{RT} \exp \left( - \frac{\Delta \mu}{RT} \right) \text{ Eq 7-15}$$

Thus it is not to be expected that there should be a relationship between $\Delta p$ and the rate of protic current flow, as Azzone et al., (1978a,b,c) demonstrated.

How localised are the proton circuits within the S phases?

Several authors (e.g. Ferguson et al., 1976a; Ernster 1977) have drawn attention to the possibility that the functional proton current between electron transport complexes and ATP synthase enzymes may be 'localized' in the sense that the 'high-energy' intermediate generated by the former is not equally distributed among all of the latter. Within the framework of the electrodic view of energy transduction and of theories stressing the importance (Williams 1978a,b) of a kinetic control of proton current flow, this hypothesis seems very attractive, and offers a simple explanation for the puzzling finding (Chapter 4) that even when the protonmotive forces generated across submitochondrial particles by the oxidation of NADH or of reduced TMPD were of similar magnitude, a phosphorylation potential was generated by only the former substrate. This, if the energy generated by the oxidation of reduced TMPD were distributed among all ATPase molecules on a given submitochondrial particle it would be expected that the magnitude of the protonmotive force alone should be the determinant of the phosphorylation potential generated if a bulk chemiosmotic mechanism were operative. If, however, the energy generated by ascorbate oxidation at the third 'site' were made available to only a restricted fraction of the ATPase enzymes on a given submitochondrial particle, it would be expected that the other ATPase molecules would be
able to hydrolyse the ATP generated at the 'active' ATPases.

Two testable predictions stem from this: 1) addition of ADP, glucose and hexokinase to submitochondrial particles respiring on reduced TMPD would not lower the time-averaged membrane potential, in contrast to what is observed in the case of NADH oxidation (Chapter 4), (2) Inhibition of ATP hydrolysis activity by adenylylimidodiphosphate should allow expression of a phosphorylation potential generated. It should be noted that the submitochondrial particles used in the present work are devoid of ATPase-inhibitor (see Chapter 4).

Thus it may be concluded that a certain degree of localisation of the proton current between particular electron transport complexes and ATPase molecules does indeed take place. However it should be noted that the ability of the uncoupler SF 6847 (Muraoka et al., 1975) to exert a full uncoupling effect at a concentration of 0.2 molecules per respiratory chain suggests that such localisation may not be complete. Alternatively SF 6847 could act to induce 'negative cooperativity' in the protonic coupling between electron transport and ATPase complexes in the same sense that the binding of Antimycin A to mitochondria exhibits positive cooperativity (Slater 1973).

Is specific channelling a property shared by protons and other metabolites?

The mechanism of electron transport phosphorylation outlined here contrasts with the bulk phase chemiosmotic formula-
tion elaborated by Mitchell in the sense that while chemiosmosis predicts an equilibrium of the protons involved in electron transport phosphorylation with bulk phase proton potentials, the present mechanism suggests a specific channelling of protons along a multi-enzyme (membrane) surface. Such ideas concerning specific channelling by multienzyme complexes using more conventional substrates have also been evolved, and I believe that a comparison of the present ideas with those evolved to account for 'metabolite microcompartmentation' is worthwhile. The theoretical and experimental arguments which favour the occurrence of metabolite microcompartmentation will be reviewed, in the belief that similar approaches to the analysis of the present model of electron transport phosphorylation will prove fruitful.

Metabolite microcompartmentation: theory

It is becoming increasingly clear that the cytoplasm of the living cell can not adequately be explained by simple solution chemistry. Atkinson (1969) drew attention to the undesirability of large solution concentrations of metabolic intermediates in the living cell, and of the need to conserve solvent capacity. He suggested that relatively high enzyme-substrate ratios, together with low metabolite concentrations, near the $K_m$ of the enzymes for which they were substrates, would be an effective way of avoiding large changes in intracellular metabolite concentrations whilst preserving adequate metabolite fluxes. This analysis was lucidly extended by Sols and Marco (1970), who showed, inter alia, that the number of intracompartamental molecules of certain tricarboxylate and glycolytic intermediates are probably barely in excess of their protein-binding sites. Currently a new paradigm is evolving (e.g. Ling 1969, 1977; Welch 1977a,b) to meet the
challenge of describing and explaining the organisation of the internal milieu of the living cell. In the belief that the physicochemical basis for these ideas is similar to that upon which the present model of electron transport phosphorylation must rest, I shall indicate some of the supposed advantages of metabolite microcompartmentation and 'channelling'.

The evolutionary, and hence (Huxley, J. 1974) energetic, advantages of the maintenance of a high degree of spatial order in the cellular cytoplasm have been comprehensively reviewed by Welch (1977a,b). The advantages inherent to all multienzyme complexes of increased efficiency and control potential have been repeatedly pointed out (e.g. Reed and Cox 1966; Katchalski et al., 1971; Srere and Mosbach 1974; Welch 1977a). Examples of series of reactions which are catalysed by spatially ordered multienzyme complexes include tryptophan biosynthesis (Gibson and Pittard 1968; Srere and Mosbach 1974; Welch 1977a), fatty acid synthesis and oxidation (Green and Allman 1968a,b), glycolysis (Mowbray and Moses 1976) and the oxidation of pyruvate to acetyl CoA (Reed and Cox 1966). In each of these cases, metabolic intermediates generated during the synthesis of the end-product of the pathway remain protein-bound, and do not equilibrate with the bulk of the cellular compartment. Welch (1977a) has emphasised the possible extensiveness of this phenomenon, and it has been suggested (Mowbray and Moses 1976) that the protein-bound nature of glycolytic and other low molecular weight metabolites is largely responsible for the finding (e.g. Harris 1957; Ling and Cope 1969; Cope 1976; Hazlewood 1977) that the apparent
mobilities and diffusion coefficients of these compounds within the cell are several orders of magnitude different from those for the same substance in aqueous solution. "Thus the use of multienzyme complexes with restricted diffusion paths may be forced on the cell by the need to achieve efficient function at very low free metabolite concentrations" (Mowbray and Moses 1976).

The possible advantages of both soluble and membrane-bound multienzyme aggregates which 'channel' metabolic intermediates, compared with those which allow metabolite equilibration with the bulk intracellular pool include (Srere and Mosbach 1974):

1) A decreased diffusion time for the intermediates concerned,
2) Competition with other pathways is minimised by keeping an intermediate in a limited microenvironment,
3) The restriction to a microenvironment of a few molecules of an intermediate can present an effectively high concentration to the next active site,
4) The protein-protein interactions of a complex may be necessary for control features of the system,
5) Unstable intermediates can be protected,
6) Specific environments of varying nature (hydrophobicity) can be created to enhance specific reactions.

The application of these heuristic principles to delineating and investigating the present model of electron transport phosphorylation and its protonic intermediate is thus analogous and obvious. It may be noted that the major 'competing reaction' (2, above) in which protons are involved is their neutralisation by acids and bases of the appropriate pH (cf. Ort et al. 1976). Therefore, whereas Mitchell (1961, 1966, 1970), in recognising the importance of the membrane in
electron transport phosphorylation, stressed that this importance was in separating electro-osmotic gradients, reasoning based on the supposed advantages of multi-enzymes complexes (1 to 6 above) has led me to postulate that such membrane-bound systems possess an important and intrinsic additional advantage: that of allowing subtle control and channelling of the protons released by electron transport and consumed by ATP synthesis and other energy-requiring processes.

**Metabolite microcompartmentation: evidence**

Turning to the evidence concerned with the demonstration of intracellular compartmentation in unicellular and other 'single-compartment' systems, I would begin by noting that it is based on 3 types of experiment. The first type studies the specific rate of incorporation of an isotopically labelled metabolite into one or more metabolic pathways sharing a common intermediate which is supposed to exist in 'free' solution in the cytoplasm of the cell. The expected specific activity for each intermediate may be calculated as a function of time from the actual activity of label, the metabolite fluxes and the pool sizes using standard methods. It is clear that the specific rate of incorporation into the pathway will vary if the
endogenously synthesised intermediate does not equilibrate
with the intracellular intermediate pool derived from the
exogenous (labelled) source. Such an approach has been used,
for instance, by Kemp and Rose (1964); McBrien and Moses
(1968); MacNab et al., (1973); Subramanian et al., (1973);
Tokumitsu and Ki (1973); Vignais et al., (1975); and Cronholm
et al., (1976). In each of these studies (and see Srere and
Mosbach 1974; Welch 1977a) it was established that a single
cellular compartment (e.g. cytosol, mitochondrion, intact
unicell) with a homogeneously dispersed pool of metabolites
could not account for the partitioning of isotope label into
different fractions. This 'competition' approach has been
utilised in the case of energy-linked proton current generation
in spinach thylakoids by Ort and Dilley (1976) and Ort et al.,
(1976).

The second type of experiment is based upon magnetic
resonance studies of intact cells. Briefly, the nuclear
magnetic resonance (NMR) spectrum of a particular atom reflects
the molecular and cellular environment in which that atom
resides, and both the sharpness and the transverse relaxation
time(s) of the signal reflect the homogeneity of this environ­
ment (Dwek 1973). Thus for a given metabolite, it is
possible to study, in a non-destructive manner, the micro­
heterogeneity of a supposedly homogeneous metabolite pool.
Such methods (e.g. Cope 1976) have been used to determine the
extent of water-structuring and alkali metal cation-complex
formation in living tissues. It has been claimed from such
studies (Cope 1976) that the idea of a homogeneous pool of
metabolites within a given membrane-bound cellular compartment
is no longer tenable. It is of particular interest in this
context that Lange et al., (1975) have used high-resolution proton NMR to establish that phosphatidylethanolamine micelles may channel protons above their surface without permitting their equilibration with the bulk phase solvent protons.

The third type of experiment is based upon the analysis of the rates of metabolite fluxes catalysed by carefully isolated enzyme aggregates compared with those catalysed by their component enzyme. In each case, the channelling of metabolites along the enzyme's surface, within the double layer (De Simone 1977), results in a much more rapid, and hence efficient, transformation of substrates. For examples of such analyses, the reader is referred to the papers of Matchett (1974), Mowbray and Moses (1976), Koch-Schmidt et al (1977) and Welch (1977a).

I now conclude with a reinterpretation of some of the findings presented in earlier chapters, which I tried to interpret at the beginning of this chapter within the chemiosmotic theory, by considering them within the electrodynamic theory set out above.

An electrodynamic explanation of some anomalous results in this thesis

In the case of chromatophores it was noted above that there was a significant discrepancy between the value of the $\frac{\Delta H^+}{\Delta ATP}$ ratio calculated from the thermodynamic comparison of $\Delta p$ and $\Delta G$ and that obtained by Jackson et al. (1975) using spectroscopic analysis of ADP on the decay of the pH changes following single turnover flashes of light. The latter measurements were carried out in the presence of a high concentration of charge-neutralising ions such that it was to be expected that all protons generated by electron transport entered
the bulk phase external to the chromatophore suspension. However, if the native proton current flow occurs within the postulated phase S adjacent to the chromatophore membrane it would indeed be expected that under conditions in which charge-neutralisation was not complete there would be no correspondence between the value of the $\Delta^{H^+}/\text{ATP}$ ratio measured by the two types of method.

In the case of bovine heart submitochondrial particles, the $\Delta^{H^+}/\text{ATP}$ value of 3 calculated by comparing the $\Delta p$ and $\Delta G_p$ values generated by NADH oxidation (Chapter 4) was greater than that obtained by direct glass electrode measurement during the hydrolysis of a pulse of ATP (Thayer and Hinkle 1973; Moyle and Mitchell 1973a). This anomaly is economically explained by the suggestions outlined in the current model: that the functional proton current during electron transport phosphorylation occurs in the interphase close to the membrane surface, and that charge-neutralising ions act to cause the ejection of such protons into the bulk phase, while comparison of $\Delta G_p$ with a $\Delta p$ measured by assessing the distribution of permeant hydrophilic solutes leads to an erroneously high value for the $\Delta^{H^+}/\text{ATP}$ ratio.

Further, in the case of bovine heart submitochondrial particles (Chapter 4), it was found that the value of $\Delta p$ determined varied markedly with the type of incubation medium used, without a corresponding variation in $\Delta G_p$. This behaviour is not expected if bulk phase ion gradients are important in determining the thermodynamics of ATP synthesis. If, however, the functional proton current does not enter the bulk phases, the parameter measured by the distribution of hydrophilic solutes would not be expected to bear a constant relationship
to the phosphorylation potential attained, for changes in the structure of the Gouy-Chapman layer, or of the buffering power of the bulk phases, would seriously change the bulk phase protonmotive force without necessarily affecting the protonmotive force across the membrane itself. An independence between the measured bulk protonmotive force and the phosphorylation potential generated by bovine heart submitochondrial particles was also noted in the case of proton movements generated by electron transfer in the terminal region of the electron transport chain. Even on those occasions in which the measured protonmotive force generated by the TMPD-mediated oxidation of ascorbate was as high as that generated by NADH oxidation, a significant phosphorylation potential was not generated. This observation is economically explained if the functional proton current of electron transport phosphorylation occurs not via the bulk phase but along specific channels at the membrane surface. The slow leak into the bulk phase(s) which is what is measured by the flow dialysis assay of the distribution of hydrophilic solutes, would be expected under varying conditions of protolytic electron transport, and could occur quite independently of the specific current of protons along the surface of the membrane, which was suggested in this chapter to be the functional intermediate between the oxidoreduction and hydrodehydration reactions of electron transport phosphorylation. (This latter phenomenon was also observed in the case of *P. denitrificans* vesicles (Chapter 5)). 10 mM nitrate did not cause a transformation of the membrane potential component of the protonmotive force generated by TMPD-mediated
ascorbate oxidation by submitochondrial particles into a transmembrane pH gradient, although the addition of this concentration of the permeant nitrate ion was sufficient to cause a significant pH gradient across submitochondrial particles oxidising NADH under similar incubation conditions. This result is fully consistent with the postulate that a specific proton pathway, close to the membrane, is responsible for providing the intermediate driving force in ADP phosphorylation, and, as with the inability of TMPD-mediated ascorbate oxidation to generate a significant $\Delta G_p$, suggests that the functional proton pathways are different for the oxidation of NADH and ascorbate/TMPD.

If the ratio of protons released by the oxidation of 1 NADH molecule to those released by the oxidation of one succinate molecule is 3:2 (Mitchell 1966a), and the rate of succinate oxidation to NADH oxidation is 0.7:1 (Ferguson and Sorgato 1977) in these particles, the rate of proton translocation when NADH is substrate would be about twice that when succinate is substrate. This ratio of the rates of proton translocation with NADH and succinate as substrate (of 2:1) is paralleled by the relative rates of ATP synthesis with these substrates by these particles (Ferguson and Sorgato 1977). However, both the phosphorylation potential attained and the protonmotive force generated during the oxidation of these substrates, under 'static head' conditions, are similar with the two substrates. This result can again be explained by the existence of specific proton pathways between electron transport and phosphorylation, but is not expected if a bulk protonmotive force is the link between electron transport and ADP phos-
phorylation in a reversible system. The variability of $\Delta p$ without a corresponding variation in $\Delta G_p$ was also observed in the case of $P. \text{denitrificans}$ vesicles (Chapter 5). The ability of 10 mM nitrate in this system to decrease the protonmotive force measured by ion distribution to an undetectable level whilst having no effect on the phosphorylation potential generated can not be explained on thermodynamic grounds, even in an apparently 'irreversible' system (Ferguson et al., 1976b) if a bulk protonmotive force is required for the synthesis of ATP. The ability of low concentrations of uncouplers to raise the apparent $\Delta \rightarrow H^+/ATP$ ratio has been observed by other authors in bacterial chromatophores (Baccarini-Melandri et al., 1977) and in submitochondrial particles (Azzone et al., 1978c), whilst low uncoupler concentrations affected the rate of ATP synthesis without decreasing the protonmotive force in the $P. \text{denitrificans}$ vesicles (Chapter 5). None of these observations are easily accommodated within the framework of a requirement for a bulk transmembrane proton gradient as a functional intermediate between electron transport and ADP phosphorylation, but may be explained within the context of an electrodic view, for in the latter formulation no strict relationship is expected between bulk phase transmembrane proton gradients and either the rate or extent of ATP synthesis.

**Summarising remarks**

In this chapter I have offered some speculations concerning the molecular mechanism of the transmission of electrochemical proton gradients. It leans heavily upon the chemiosmotic and 'localised proton' theories developed by Mitchell
and Williams and represents an attempt to bring together a number of controversial experimental observations which appear mutually inconsistent. The electrodic view, as I have called it, is 'chemiosmotic' in the sense that transmembrane phase proton gradients are involved, but is not chemiosmotic in the sense that the functional proton current is not carried in the bulk phases on either side of the membrane. Local surface effects, based on generally accepted physical chemical principles, were stressed, and a number of predictions that are susceptible to experimental analysis were presented.
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