The Electron Transport System and Hydrogenase of *Paracoccus denitrificans*

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I. Introduction

One feature that characterizes bacteria is the ability to adapt to environmental changes and nutritional conditions. At the molecular level the versatility of bacterial systems can be explained, at least in part, by adaptive changes in their respiratory chains. *Paracoccus denitrificans*, formerly *Micrococcus denitrificans* Beijerinck, is a respiratory "jack-of-all-trades" and a very good example of adaptation of the electron-transport chain to changing growth conditions. Changes in bacterial respiratory chains occur most often at the level of the terminal oxidases [for well documented reviews on bacterial respiratory chains and terminal oxidases, see Gel'man *et al.* (1975), Jurtshuk *et al.* (1975), and Haddock and Jones (1977)], and this is exemplified in *P. denitrificans*. Although it is an aerobic bacterium, *P. denitrificans* can use nitrate, nitrite, and nitrous oxide as terminal electron acceptors (Kluyver, 1956). It is, however, unable to use organic compounds as electron acceptors for anaerobic growth, and so is nonfermentative. Besides utilizing a variety of terminal electron acceptor molecules, *P. denitrificans* can grow on a diverse range of carbon compounds from methanol to sucrose (Aragno *et al.*, 1977; Pichinoty *et al.*, 1977). In addition, *P. denitrificans* is able to grow autotrophically with CO_2 as the source of carbon and with H_2 as the source of reducing equivalents with either O_2 or NO_3^- as electron acceptor (Kluyver and Verhoeven, 1954b). Under these specific autotrophic growth conditions, *P. denitrificans* synthesizes a membrane-bound hydrogenase that is part of the respiratory chain (Sim and Vignais, 1978).

In vivo the hydrogenase of *P. denitrificans* acts essentially as a "hydrogen-uptake hydrogenase" that feeds electrons to the respiratory chain. In the present review special attention is given to the role of hydrogenase and its interaction with the electron transport chain of *P. denitrificans*. Although it can function reversibly *in vitro*, it is not known whether or not hydrogenase can act *in vivo* as an "H_2 valve" and, by evolving H_2, can dispense with an excess of reducing equivalents (Gray and Gest, 1965) or regulate the intracellular pH (Raven and Smith, 1976).

II. Aerobic Respiration in Heterotrophically Grown Cells

The excellent review articles of John and Whatley (1975, 1977a,b) pointing to the similarities existing between mitochondria and *P. denitrificans* stirred great interest and boosted research on the bioenergetics of this bacterium.

This section will deal with the most recent data concerning electron transfer and redox components of *P. denitrificans* that have appeared since the articles by John and Whatley (1975, 1977a,b) were published.

Newly available information confirms the analogies existing between the aerobic electron-transport chain of *P. denitrificans* and that of the inner mitochondrial membrane and points to some additional similarities as developed below.

A. The Redox Components

1. A Linear Respiratory Chain?

The plasma membrane of *P. denitrificans* contains essentially the same constitutive enzymes and redox components as mitochondria except during autotrophic growth on methanol (van Verseveld and Stouthamer, 1978a) or on H_2 (Porte and Vignais, 1980) or in different aerobic growth conditions (Cox *et al.*, 1978). The cytoplasmic membrane is widely thought to possess a single route of electron transfer to O_2, where cytochrome aa_3 acts as the sole terminal electron carrier that reacts directly with O_2 to form water. This conclusion is based both on spectrophotometric studies and on oxygen uptake measurements with different sources of electron donors and respiratory chain inhibitors. However, it has been suggested (van Verseveld and Stouthamer, 1978a) that cytochrome *o* is the main terminal oxidase in aerobic heterotrophically grown cells. Furthermore, in some exceptional conditions, for example, in the presence of cyanide, a new cytochrome (cytochrome *d*) acting as a second terminal oxidase is synthesized to supplement the nonoperational cytochrome aa_3 (Henry and Vignais, 1979).

The oxidation of NADH by isolated cytoplasmic membranes is markedly inhibited by rotenone, antimycin A, and cyanide, but is less sensitive to 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) (Henry and Vignais, 1979). In this sense *Paracoccus* differs from other bacterial species, which often prove to be rather resistant to antimycin A and rotenone.

Titration curves of NADH oxidation with antimycin A and cyanide are monophasic and therefore indicate only one site titrated by the inhibitors (Henry and Vignais, 1979). The two inhibitors fully inhibit respiration at concentrations within the range used for mitochondria. The results of Scholes and Smith (1968) show that succinate oxidation in *P. denitrificans* is about 15% less sensitive to antimycin than is NADH oxidation; also membrane vesicles prepared from cells harvested near the stationary phase of growth are more resistant to antimycin than those obtained from cells collected in the early exponential phase of growth.

The use of different substrates and suitable respiratory inhibitors has thus enabled the dissociation of the respiratory chain into different segments, which have been studied separately for their composition and assayed for energy transduction.

2. *Iron–Sulfur Proteins*

Until recently, little was known about the nature and functional role of iron-sulfur centers in bacterial systems, especially in *P. denitrificans*.

Electron paramagnetic resonance (EPR) studies performed at the temperature of liquid helium on membranes isolated from aerobically grown cells of *P. denitrificans* indicate the presence of signals whose g values, temperature dependence, power saturation, and half-reduction potentials are quite similar to those found in mammalian mitochondria (Meijer *et al.*, 1977b; T. Ohnishi, personal communication).

To define the role of the iron–sulfur centers involved in complex I (NADH–ubiquinone oxidoreductase) of the aerobic respiratory chain, Meijer *et al.* (1977b) and Lawford (1978) used cultures of *P. denitrificans* grown under sulfate-limited conditions. The technique of sulfate or iron limitation was originally designed by Haddock and Garland (1971) to investigate the changes that occurred in the mitochondrial electron transport chain of the yeast *Candida utilis*. As a result of limitation of iron or sulfate, Garland and his colleagues (Light and Garland, 1971; Clegg and Garland, 1971; Haddock and Garland, 1971) observed a reversible loss of piericidin A sensitivity associated with NADH dehydrogenase and a loss of phosphorylation site I activity. Similar experiments carried out with *P. denitrificans* by Meijer *et al.* (1977b) and Lawford (1978), however, gave conflicting results. According to Meijer *et al.* (1977b) a consequence of sulfate limitation during aerobic growth of *P. denitrificans* is the disappearance of an EPR signal with lines at $g_z = 2.05, g_y \simeq g_x \simeq 1.92$. The loss of this signal, similar to iron–sulfur center N-2 of mitochondrial complex I (Ohnishi, 1976), is accompanied by a concomitant decrease in both NADH oxidase activity and rotenone sensitivity and by the loss of phosphorylating site I, as was observed by Haddock and Garland (1971). In contrast, Lawford (1978) found that sulfate limitation resulted neither in an appreciable decrease in activity of NADH oxidase nor in the loss of piericidin A sensitivity. The apparent stoichiometry of proton translocation associated with L-malate oxidation also remained unchanged. In further experiments, Meijer *et al.* (1978), using cells of *P. denitrificans* grown in the presence of rotenone, confirmed their previous results and again observed that NADH oxidase activity was not inhibited by rotenone, but was decreased by 80% under these growth conditions and that there was no EPR signal corresponding to iron–sulfur center N-2. These authors concluded that a close relationship exists between rotenone sensitivity, the presence of iron–sulfur center N-2 and an active coupling site I, a point elaborated upon in Section II,B,4.

3. *Cytochrome aa_3*

Cytochrome oxidase, the terminal component of the respiratory chain, mediates electron transfer from cytochrome *c* to O_2. It has been identified so far in all mitochondrial systems, and it is also present in a few bacterial species (for a review on bacterial terminal oxidases, see Jurtshuk *et al.*, 1975).

Cytochrome aa_3 has been detected in whole cells and in membranes from aerobically grown cultures of *P. denitrificans* by its absorption bands at 605 nm in the α region and at 445 nm in the Soret region in reduced-*minus*-oxidized difference spectra at room temperature (Imai *et al.*, 1967; Scholes and Smith, 1968).

It has been claimed that cytochrome aa_3 is also present, although in lesser amounts, when *P. denitrificans* is grown anaerobically on nitrate as added terminal electron acceptor (Scholes and Smith, 1968; Lam and Nicholas, 1969a). However, the presence of cytochrome aa_3 is sometimes barely detectable (Sapshead and Wimpenny, 1972) in cells grown under denitrifying conditions.

Even though Meijer *et al.* (1977b) could detect neither the copper signals nor the ferric heme groups of oxidized cytochrome aa_3 by EPR spectroscopy, copper could be measured by proton-induced X-ray fluorescence (Vis and Verheul, 1975); thus it appears that the cytochrome oxidase of *P. denitrificans*, like that of mitochondria, contains two Cu atoms and two Fe per minimal unit (Ludwig and Schatz, 1980). Moreover, recent EPR studies by Erecińska *et al.* (1978) on the orientation of the hemes of mitochondrial and *P. denitrificans* cytochrome oxidases in hydrated oriented multilayers indicated that the oxidized heme of *Paracoccus* liganded to azide or to sulfide has essentially the same orientation as the reactive heme from mammalian cytochrome oxidase. Also, several lines of evidence seem to indicate a very close relationship between the two subunits of the *Paracoccus* cytochrome oxidase and subunits I and II of the mitochondrial cytochrome oxidase (Ludwig and Schatz, 1980). The bacterial cytochrome oxidase can also react with mammalian cytochrome *c* (Smith *et al.*, 1966; Ludwig and Schatz, 1980), which is a further indication of the similarities between the bacterial and mitochondrial enzymes.

The kinetics of reaction of cytochrome aa_3 of *P. denitrificans* with O_2 and CO were studied using the triple trapping method of Chance *et al.* (1975). As with mitochondria, formation of compound "A" is elicited between $-80°$ and $-100°C$ when the cells are grown aerobically. This observation is another indication of the similarities of *P. denitrificans* and the mitochondrion. If *P. denitrificans* is grown with nitrate as oxidant, no

formation of compound A is observed between −80° and −100°C (Henry *et al.*, 1979), which is further strong evidence for the almost complete absence of cytochrome aa_3 in anaerobically grown cells.

4. *Cytochrome b*

At least two *b*-type cytochromes can be identified in membrane vesicles of *P. denitrificans* by absorption difference spectroscopy at room temperature. The two pigments absorb, respectively, at 560 and 566 nm in the α region of the spectrum (Imai *et al.*, 1967; Scholes and Smith, 1968). Fourth-order finite difference spectra at liquid nitrogen temperature indicate that in the exponential phase of aerobic growth, *P. denitrificans* contains three spectrally distinguishable cytochromes of the *b* type, with absorption maxima at 554, 558, and 563 nm, respectively (Shipp, 1972). Whether these maxima actually correspond to three distinct components or to a split α peak remains to be demonstrated. Indeed, some confusion exists as to the number of *b*-type cytochromes present in membrane preparations from aerobically grown cells of *P. denitrificans*. Low-temperature absorption difference spectra of a membrane preparation reduced with dithionite *minus* an oxidized sample yielded only two peaks with maxima at 556 and 562 nm, which could correspond to the b_{560} and b_{566} observed at room temperature (Cox *et al.*, 1978; Henry and Vignais, 1979). On the other hand, potentiometric titrations indicated the presence of three distinct *b*-type cytochromes with half-reduction potentials of +245, +75, and −60 mV, respectively (Cox *et al.*, 1978). A more recent set of experiments using the redoxostat technique (Wilson *et al.*, 1979) at three separate wavelength pairs, gave only two midpoint potentials whose values were +240 and +50 mV (Henry and Wilson, 1981).

As with mitochondria, a pulse of oxygen given to an anaerobic suspension of membrane particles from aerobically grown *P. denitrificans* that had been reduced by a substrate in the presence of antimycin A produces a rapid reduction of the *b*-type cytochromes, especially of cytochrome b_{566}. When oxygen is exhausted, reoxidation of the *b* cytochromes occurs (John and Papa, 1978). 2-Heptyl-4-hydroxyquinoline *N*-oxide could not substitute for antimycin in this antimycin-enhanced, oxygen-induced reduction of cytochromes *b*. Similar results were obtained with membranes from anaerobically grown cells.

5. *Cytochrome o*

In contrast to mammalian mitochondria, under certain physiological conditions *P. denitrificans* possesses another component, cytochrome *o*, which in other bacteria can act as a terminal oxidase.

Cytochrome *o* has been defined as a *b*-type cytochrome capable of forming a light-sensitive complex with CO in the reduced state (Castor and Chance, 1959). The difference absorption spectra (dithionite reduced + CO *minus* dithionite reduced) yield in the Soret region a peak at about 418 nm and a trough near 434 nm, characteristic of a *b*-type component.

In several bacterial species, cytochrome *o* is induced under O_2-deprivation or when a culture reaches the stationary phase of growth, although the latter condition can also be attributed to a sharp decrease in the O_2 tension in the culture medium. Scholes and Smith (1968) found no *b* species reacting with CO in 12-hour cultures of aerobically grown *P. denitrificans*, whereas this component was observed when the cells were harvested in the stationary phase of growth.

Alterations of the aerobic growth conditions allowed Cox *et al.* (1978) to obtain cells of *P. denitrificans* containing either both cytochromes aa_3 and *o* or only one of those two components. A potentiometric titration of the *b*-type cytochromes indicated an E'_0 value of + 120 mV for cytochrome *o*.

Up to now, the available evidence is still insufficient to assign a definite physiological role *in vivo* to cytochrome *o* in *P. denitrificans*. Although its presence was recognized by spectroscopic studies, its kinetic competence as a terminal oxidase in aerobically grown cells is still questionable (Lawford *et al.*, 1976). However, Willison and John (1979) using cytochrome *c*-deficient mutants of *P. denitrificans*, found that a branched respiratory chain linked to a kinetically competent cytochrome *o* was the best scheme compatible with their data (Fig. 1).

6. *Cytochrome d*

Several bacterial species are able to grow in a medium supplied with cyanide (Knowles, 1976; Henry, 1981). The presence of cyanide results in

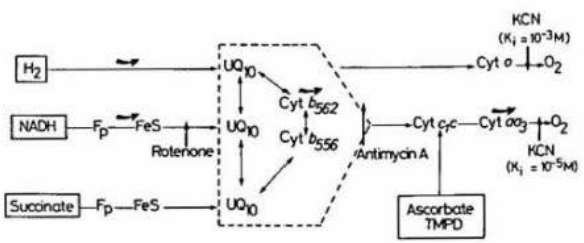

FIG. 1. Proposed electron-transfer chain in aerobically grown cells of *Paracoccus denitrificans*. The curved arrows indicate the sites of energy conservation; UQ, ubiquinone, TMPD, *N*, *N*, *N'*, *N'*-tetramethyl-*p*-phenylenediamine. From van Verseveld and Stouthamer (1978a), Willison and John (1979), Porte and Vignais (1980).

important modifications among the terminal oxidases and in the development of a respiration relatively insensitive to cyanide.

Paracoccus denitrificans can also grow aerobically in the presence of up to 300 μM KCN (Henry and Vignais, 1979). As a consequence, the bacterium has a branched respiratory system together with two components in addition to the normal set of cytochromes, i.e., a CO-binding *b*-type cytochrome (cytochrome *o*) and a pigment absorbing at 627 nm, usually referred to as cytochrome *d* (formerly a_2). Under these particular conditions, cytochrome aa_3 is almost absent. The branched nature of the electron-transport chain is indicated by the biphasic inhibition curve with KCN of NADH oxidation and by the incomplete inhibition of respiration by antimycin, which is in contrast to aerobic heterotrophic cells grown without KCN. Cytochrome *d* may perhaps play the role of the cyanide-resistant oxidase, since growth in the presence of cyanide also lowers the K_m for oxygen, with an enhancement of the affinity for O_2 by about 10-fold (Henry and Vignais, 1979).

B. Energy Yield of Respiration

The energy released by respiration is assumed to be recovered in the form of ATP (oxidative phosphorylation) through the functioning of membrane-bound ATP-generating respiratory systems. The chemiosmotic theory proposed by Mitchell (1961, 1966, 1976a) has focused attention on the role of membranes in vectorial metabolism in general and on the role of H^+ in energy transduction in particular.

The methods used to determine the efficiency of energy conservation during oxidative phosphorylation are of three types.

1. The traditional procedure consists of determining the amount of P_i esterified into ATP during the reduction of O_2. The quantitative relationship between ATP formed (in moles) and O_2 consumed (in gram atoms) is expressed as a P/O ratio. If electron acceptors other than O_2 are used, the stoichiometric relationship is related to the number of electron pairs transferred (P/2 e) or to the amount of oxidant reduced (e.g., P/NO_3^-). This type of assay involves the coupled functioning of complex systems, namely the electron carriers and the ATP-synthetase complex. Owing to the absence of an ATP/ADP carrier in most bacterial plasma membranes, this method necessitates the use of cell-free extracts or inverted membrane particles. In some cases whole resting cells have been used and their content of endogenous adenine nucleotides measured and correlated with O_2 consumption (Hanselmann, 1974).

2. A second procedure derived from the application of the chemiosmotic hypothesis is the measurement of respiration-linked proton translo-

cation. This method does not involve directly the ATPase enzyme but, as an *in vitro* test, it requires, as does the preceding one, that the membrane does not leak protons (well coupled membrane).

3. A third method, more indirect, is based on physiological experiments under energy-limited growth conditions in which bacterial growth yields are measured and compared to the theoretical ATP requirements for the formation of microbial biomass.

The three types of methods have been applied to the study of respiration-linked energy-yielding reactions in *P. denitrificans*. They are presented in the order of their chronological use with *P. denitrificans*.

1. Determination of P/O Ratio

The classical method of P/O ratio determination was adopted in early work. Adenosine triphosphate was determined in cell extracts either directly (e.g., with the luciferin–luciferase system; Knobloch *et al.*, 1971) or from the amount of glucose 6-phosphate formed in the presence of hexokinase and glucose as an ATP-trapping system using either nonlabeled or ^{32}P-labeled phosphate (Imai *et al.*, 1967; John and Whatley, 1970).

As mentioned above, unlike mitochondria, *Paracoccus* does not possess an ADP/ATP carrier (cf. Vignais, 1976). Thus, everted membrane particles have to be used to measure phosphorylation of externally added ADP. The method of membrane preparation has a very strong effect on the phosphorylating capacity of isolated membranes. Lysozyme treatment followed by osmotic lysis (Scholes and Smith, 1968; John and Whatley, 1970) or cell breakage by French pressure cell (Knobloch *et al.*, 1971) are milder treatments than sonication (Imai *et al.*, 1967) and produce higher P/O ratios (Table I). A method for preparing tightly coupled membrane vesicles from *P. denitrificans* has been described (Nichols and Hamilton, 1978).

When the oxidizable substrate is nonpermeant and has its binding site on the cytoplasmic face of the membrane, as does ATP synthetase, only inside-out particles contribute to both respiration and phosphorylation. However, with permeant substrates such as hydrogen gas (Porte and Vignais, 1980) both inside-out and right-side-out particles can oxidize the respiratory substrate, but only the former can phosphorylate externally added ADP. Under these conditions, the P/O ratio does not reflect the true coupling capacity of the membrane (Table I).

The proportion of inside-out particles depends on the nature of the carbon source used for growth. After osmotic lysis of spheroplasts of *P. denitrificans*, Burnell *et al.* (1975a) obtained membrane vesicles believed to consist predominantly of inverted vesicles when cells were grown on

TABLE I

P/O RATIOS OF *Paracoccus denitrificans* PARTICLES[a]

Growth of cell			P/O ratios				
Substrate	Terminal electron acceptor	Preparative technique	NADH	Succinate	Ascorbate TMPD[b] Cyt. *c*	H_2	References
Succinate	O_2	Sonication	1.02	0.40	0	—	Imai *et al.*, 1967
Succinate	NO_3^-	Osmotic lysis	1.46	0.48	0	—	John and Whatley, 1970
Succinate	O_2	French press	1.44	0.41	0	—	Knobloch *et al.*, 1971
Succinate	O_2	Osmotic lysis	1.21	0.60	0	—	van Verseveld and Stouthamer, 1976
Glucose	O_2	Osmotic lysis	1.38	0.84	—	—	van Verseveld and Stouthamer, 1976
Ethanol	O_2	Osmotic lysis	0.79	0.59	—	—	van Verseveld and Stouthamer, 1976
Propanol	O_2	Osmotic lysis	0.45	0.47	—	—	van Verseveld and Stouthamer, 1976
H_2	O_2	French press	0.95	1.00			Knobloch *et al.*, 1971
H_2	O_2	Osmotic lysis	0.40	0.40	0.01	0.04	Porte and Vignais, 1980

[a] Reproduced and adapted from John and Whatley (1977b).
[b] TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

succinate and nitrate, and of right-side-out vesicles when cells were grown autotrophically on hydrogen. Ferguson *et al.* (1978) could effect a partial separation of the two types of particles by passing the vesicle preparation through an ADP-agarose column that retained inside-out particles in which the ATPase faces outward. Van Verseveld and Stouthamer (1976) also concluded that growth conditions are critical for the preparation of membrane particles showing efficient phosphorylative activity and prepared membrane particles from cells grown on different carbon sources. The isolated particles yielded average P/O ratios of 1.38, 1.21, 0.79, and 0.45 with NADH as electron donor and 0.84, 0.60, 0.59, and 0.47 with succinate as electron donor when the carbon source was glucose, succinate, ethanol, and propanol, respectively. However, when the P/O ratio was determined in intact, resting cells (in that case the changes in the amount of internal ATP, ADP, and AMP was related to O_2 consumed), there was no significant difference between the P/O values (around 1.0) whether the cells had been grown on succinate or on propanol. It was suggested that the carbon source influenced the structure of the plasma membrane and hence the percentage of inside-out vesicles, and it was concluded that the P/O ratio determined with membrane particles is not a good indication of the *in vivo* P/O value.

Although particles derived from *P. denitrificans* yield P/O values that are relatively high for a bacterial system, these values are significantly lower than those obtained with mitochondria. Therefore, it is difficult to estimate the number of coupling sites really involved. To overcome this problem, comparative studies with substrates supplying electrons at different levels in the respiratory chain have been carried out. For example, with membrane fragments prepared from cells grown heterotrophically either aerobically or anaerobically, P/O ratios of 1.0–1.5 were obtained with NADH as substrate. For succinate oxidation P/O values were 0.4–0.5 and there was no phosphorylation for the oxidation of ascorbate + TMPD (Imai *et al.*, 1967; John and Whatley, 1970; Knobloch *et al.*, 1971; van Verseveld and Stouthamer, 1976) (Table I). It was concluded by the above-mentioned authors that the third energy-conservation site is lacking in aerobic heterotrophic cells.

2. *Estimation of Oxidative Phosphorylation Efficiency from the Determination of Growth Yields*

This method is based on the classical observations of Bauchop and Elsden (1960), who obtained with various fermentative bacteria a constant relationship between ATP production and cell yield (10.5 g dry weight per mole of ATP produced). The aim of yield studies is to establish the relationship between the formation of new cell material, substrate utilization, and ATP synthesis (Stouthamer, 1969; Stouthamer and Bettenhaussen, 1973). If the energy-yielding metabolism is tightly coupled to the energy-consuming reactions of biosynthesis, the observed (or apparent) growth yield can be expressed by the following equation (Pirt, 1965): $1/Y = m_e/\mu + 1/Y^{max}$, where Y is the observed growth yield, Y^{max} is the true growth yield, m_e is the so-called maintenance energy coefficient, which is probably not, in fact, independent of growth rate (Neijssel and Tempest, 1976), and μ is the specific growth rate. With the knowledge of the elementary cell composition [it is $C_6H_{10.8}N_{1.5}O_{2.9}$ for *P. denitrificans* (van Verseveld and Stouthamer, 1978b)], equations concerning material balances can be written. The true growth yield (Y^{max}) or growth yield corrected for maintenance energy should be proportional to the energy yield for energy-limited cells. This implies that substrates are degraded via known catabolic pathways in which the ATP generating reactions are known. Many factors influence the molar growth yield of a microorganism on a given growth substrate (Stouthamer, 1976a), e.g., the complexity of the medium, the nature of the anabolic pathways, the pH and temperature, the nature of the nitrogen source, the macromolecular composition of the microbial cells, and the occurrence of energy-requiring processes other than the formation of new cell material (transport processes,

motility, energy of maintenance). The interplay of these factors has been discussed extensively by Stouthamer (1979). For example, this author calculated that 21 g of cell material can be formed per mole of ATP from pyruvate and preformed monomers, and only 13.5 g from pyruvate and inorganic salts; for growth on acetate, theoretically not more than 10 g of cell material can be formed per mole of ATP. From the observation that the possible rate of ATP production by catabolism does not necessarily correspond to the rate of ATP consumption by anabolism, Tempest and Neijssel (1978) and Stouthamer (1979) pointed out that organisms must adjust the rate of ATP production to the needs of anabolism. One form of adjustment may be deletion of sites of oxidative phosphorylation as observed for site I in the presence of toxic concentrations of accumulated nitrite (van Verseveld *et al.*, 1977). Other forms of energy dissipation may be the synthesis of branched respiratory chains with one chain limb being nonphosphorylating, or the use of energy-spilling reactions (such as H_2 evolution in anaerobic hydrogenase-containing organisms). These adjustments will be reflected by lower energy recovery efficiencies, i.e., lower overall P/O ratios, or variable energy coupling (see, e.g., Stucki, 1978).

The overall efficiency of aerobic energy conservation (number of ATP equivalents per mole of O_2 consumed) has been determined by Edwards *et al.* (1977) with cells of *P. denitrificans* grown in continuous culture under carbon-limited conditions. Average values of 6.2, 5.9, 5.6, and 4.0 mol of ATP equivalents per mole of O_2 were calculated for growth on glucose, glycerol, lactate, and acetate, respectively. Corresponding values of 4.3, 4.0, 3.7, and 2.9 mol of ATP equivalents per mole of O_2 have been previously reported by Farmer and Jones (1976) for growth of *Escherichia coli* W under identical carbon-limited conditions. Comparison of these values indicate an approximately 45% higher efficiency in *P. denitrificans* than in *E. coli* W. Since *E. coli* lacks site III (cf. Haddock and Jones, 1977), the extra phosphorylation site in *P. denitrificans* was assigned by Edwards *et al.* (1977) to site III.

3. *Measurement of Respiration-Dependent Proton Translocation*

According to the chemiosmotic hypothesis (Mitchell, 1961, 1966, 1968, 1976a; Mitchell and Moyle, 1967, 1979), measurements of $\leftarrow H^+/O$ ratios associated with the oxidation of different respiratory substrates by depleted cells can be a means of determining the number of energy-conserving sites in the respiratory chain. Like the inner mitochondrial membrane (Mitchell and Moyle, 1967), the cytoplasmic membrane of *P. denitrificans* exhibits a relatively low proton conductance (Scholes and Mitchell, 1970a), and whole cells have been shown to eject protons

outward during respiration (Scholes and Mitchell, 1970b; Lawford *et al.*, 1976).

a. Difficulty in Defining Spans of the Respiratory Chain with Proton Translocating Activity. One of the problems in interpreting measurements of the stoichiometry of vectorial protons is the presence of endogenous substrates in whole cells and spheroplasts. Even "starved" cells still contain a certain amount of endogenous substrates and it is difficult to evaluate their contribution when an external substrate is being oxidized. Furthermore, the endogenous substrates are not always identified, and it is not known at what level of the respiratory chain they donate electrons. One way to eliminate the contribution of endogenous substrates is to use site-specific inhibitors to block electron transport in a well defined part of the respiratory chain. For example, rotenone, known to inhibit specifically NADH-dehydrogenase in *P. denitrificans* (Imai *et al.*, 1967; Scholes and Smith, 1968), and piericidin, an insecticide discovered by Tamura *et al.* (1963) and shown to inhibit NADH-dehydrogenase in membrane particles of *P. denitrificans* (Imai *et al.*, 1968), have been used to block electron transport at the level of site I. Another method adopted by Porte and Vignais (1980) to minimize the contribution of endogenous substrates to respiration-driven proton translocation has been the use of small enough amounts of spheroplasts so that no proton ejection could be observed in oxidant pulse experiments without added substrate.

Another difficulty is the assessment of the portion of the respiratory chain really involved in proton translocation. One classical way is to use electron donors or acceptors that will interact with well-identified components of the respiratory chain. The couple ascorbate (electron donor) + TMPD (mediator), which feeds electrons to the respiratory chain via cytochrome *c*, has been widely used to evaluate the energy-coupling capacity of cytochrome oxidase in *P. denitrificans* (see below).

b. Stoichiometry of Translocated Protons per "Site." The efficiency of free energy conservation can theoretically be assessed in terms of the stoichiometry of respiration-driven proton translocation. However, the stoichiometry of the proton translocating systems is still controversial. Earlier estimates by Mitchell (1968) indicated that 2 H^+ were extruded from mitochondria or bacteria during the transfer of 2 electrons in a redox loop, and it was suggested that 2 H^+ were pumped into mitochondria or bacteria per mole of ATP synthesized. These conclusions have been challenged recently on theoretical and experimental grounds (cf. Brand, 1977, for review), and it would appear that in mitochondria a stoichiometry of 3 H^+ per "high-energy phosphate bond" synthesized could over-

come these thermodynamic difficulties and fit better with the newer experimental data.

The problem of the stoichiometry of charge and proton translocation linked to mitochondrial redox and hydrolytic proton pumps is the subject of current animated debates (Brand, 1979; Wikström and Krab, 1979b; Mitchell and Moyle, 1979). It is complicated by the fact that the three energy-conserving "sites" in the electron-transport chain between NADH and oxygen may not be energetically equivalent (Wikström and Krab, 1979a; Brand, 1979). Tables II and III give the conclusions reached by some experimenters involved in this type of research.

In *P. denitrificans,* Scholes and Mitchell (1970b) and Edwards *et al.* (1977) measured 8 g-ions of H^+ outwardly ejected per gram-atom of oxygen consumed for the oxidation of endogenous substrates. Assuming a H^+/site ratio of 2, these authors concluded that there occur four coupling sites (site 0 corresponding to the energy-linked transhydrogenase reaction) in the respiratory chain of aerobic, heterotrophically grown *P. denitrificans*.

On the other hand, other authors (Meijer *et al.*, 1977a; Lawford, 1978, 1979; Porte and Vignais, 1980) concluded that in *P. denitrificans* the $\leftarrow H^+$/site ratio is 3–4, a value that contrasts with that observed in other species of bacteria, where a value of $\leftarrow 2$ H^+/site has been, up to now, consistently recorded (Haddock and Jones, 1977; Jones, 1977). This difference between *P. denitrificans* and other bacteria is not due to a possibly variable rapid phosphate transport (Cox and Haddock, 1978) as found in mitochondria (Brand, 1977; Wikström and Krab, 1979a). One possible reason (S. J. Ferguson, personal communication) is that in other organisms some of the extruded protons might be used for ATP synthesis during the oxygen (or reductant) pulse itself, whereas the ATPase in *Paracoccus,* which apparently contains a high titer of inhibitor protein (Ferguson *et al.*, 1976), is "turned on" rather slowly upon membrane energization compared with that in other bacteria. Harris *et al.* (1979) recently showed that the rate-limiting step for the initiation of phosphorylation by respiration in bovine heart submitochondrial particles was the release from the enzyme of the inhibitor protein first discovered by Pullman and Monroy (1963). Therefore, if other organisms do make ATP during an O_2 pulse experiment, this may well be the reason for the lower $\leftarrow H^+$/site ratios observed relative to those observed in intact cells (or spheroplasts) of *Paracoccus*. We are not aware of any experiments designed to test this suggestion, but it would seem to us of importance to attempt to exclude this line of reasoning as the explanation of the discrepancy between the $\leftarrow H^+$/site ratio observed in *Paracoccus* and that observed in other species of bacteria, presumably by the use of an appropriate ATPase inhibitor. It should be mentioned that

TABLE II

PROPOSED H^+/e^- AND CHARGE/e^- RATIOS IN MITOCHONDRIAL RESPIRATION[a,b]

	Segment of respiratory chain										
	NADH → UQ "Site 1"		UQ (or succinate) → cyto-chrome *c* "Site 2"		Cytochrome *c* → O_2 "Site 3"		UQ (or succinate) → O_2 "Sites 2 + 3"		NADH → O_2 "Sites 1 + 2 + 3"		
Experimenters	$\leftarrow H^+/e^-$	$\leftarrow q^+/e^-$	$\leftarrow H^+/e^-$	$\leftarrow q^+/e^-$	$\leftarrow H^+/e^-$	$\leftarrow q^+/e^-$	$\leftarrow H^+/e^-$	$\leftarrow q^+/e^-$	$\leftarrow H^+/e^-$	$\leftarrow q^+/e^-$	References
Hinkle, Mitchell, Papa	1	1	2	1	0	1	2	2	3	3	Hinkle and Yu, 1979; Mitchell and Moyle, 1967; Papa, 1976
Brand *et al.*	—	—	—	—	—	—	3	3	4.5	4.5	Brand *et al.*, 1976
Wikström and Krab	—	1	2	1	1	2	3	3	—	—	Wikström and Krab, 1979a
Azzone, Lehninger	2	2	2	1	2	3	4	4	6	6	Azzone *et al.*, 1979; Pozzan *et al.*, 1979b; Lehninger *et al.*, 1978

[a] Adapted from Wikström and Krab (1979a,b).

[b] Abbreviations: $\leftarrow H^+/e^-$ = number of H^+ ions ejected from the mitochondria per transferred electron; $\leftarrow q^+/e^-$ = net number of electrical charge equivalents translocated (as positive charge from the M side to the C side) across the mitochondrial membrane per electron transferred.

TABLE III

MITOCHONDRIAL ATPASE H^+ PUMP[a]

Experimenter	H^+/ATP (synthesis+ transport)	ATP/site			P/O	References
Mitchell	2 + 0	1	1	1	3	Mitchell *et al.*, 1978
Hinkle	2 + 1	0.67	0.67	0.67	2	Hinkle and Yu, 1979
Wikström	2 + 1	0.67–1	0.67	1.33	2.67–3	Wikström and Krab, 1979b
Lehninger, Azzone	3 + 1	1	1	1	3	Lehninger *et al.*, 1978 Azzone *et al.*, 1979 Pozzan *et al.*, 1979b
Brand	2 + 1	0.67	0.67	1.33	2.67	Brand, 1979

[a] Adapted from Azzone (1980).

in chloroplast thylakoids, whereas valinomycin blocks the initial stages of photophosphorylation, phosphorylation driven by a pH gradient starts in much less than 0.5 second after the initiation of electron transport (Ort *et al.*, 1976; Vinkler *et al.*, 1980; Davenport and McCarthy, 1980), the exact time of the start of phosphorylation also depending upon the release of the inhibitor protein (Harris and Crofts, 1978). Since an O_2 pulse usually lasts for from 0.5 second to more than 1 second (Scholes and Mitchell, 1970b; Kell, 1979), it is easy to see that there is, in fact, a strong possibility that ATP synthesis does occur in this time scale in some organisms, and that there may thus be a variation between the observed and "true" $\leftarrow H^+$/site ratios dependent upon this effect.

c. Number of Coupling Sites. Although the concept of true coupling sites with fixed stoichiometry is being questioned and the formalism of linear nonequilibrium thermodynamics (e.g., Rottenberg, 1979; Westerhoff and van Dam, 1979) may be a more appropriate tool for analyzing energy transduction in bacterial systems, we will keep the old terminology of "site," for it is convenient in reporting published work.

Experimentally, one measures the total amount of protons ejected during the oxidation of either endogenous or added substrate (e.g., succinate) (cf. Tables II and III). The number of coupling sites can then be calculated if either the stoichiometry of protons per site or of protons per ATP synthesized is known.

i. $\rightarrow H^+/ATP$. To determine a minimal $\rightarrow H^+$/ATP ratio, Kell *et al.* (1978) using phosphorylating membrane vesicles from *P. denitrificans* compared the proton-motive force (Δp) generated by the oxidation of

NADH or succinate with the established phosphorylation potential: $\Delta G_p = \Delta G^{\circ\prime} + RT \ln [ATP]/([ADP] + [P_i])$. According to the chemiosmotic hypothesis (Mitchell, 1966, 1968), Δp and ΔG_p are linked by the following relationship: $\Delta G_p = -zF\,\Delta p$, where F is the Faraday constant and z is the number of protons translocated via the ATPase for each mole of ATP synthesized. From an experimentally determined Δp value of about 125–145 mV and a ΔG_p around 53 kJ/mol (12.5–12.9 kcal/mol) an $\rightarrow H^+/ATP$ ratio of more than 3 was derived for the ATPase of *P. denitrificans*. As pointed out by Kell *et al.* (1978), this may even be an underestimate, for Δp and ΔG_p may not actually reach equilibrium (Ferguson *et al.*, 1976).

ii. $\leftarrow H^+/site$. To evaluate $\leftarrow H^+$/site stoichiometries, authors have compared the capacity for proton translocation of bacterial cells in which the synthesis of a "site" was either induced or inhibited.

Loss of site I. Under conditions of carbon limitation, Meijer *et al.* (1977a) obtained $\leftarrow H^+/O$ ratios of 7–8 for the oxidation of endogenous subtrates, which were approximately halved under sulfate limitation, while oxidation of succinate was accompanied by an outward proton translocation of about 4 g-ions of H^+ per gram-atom of O_2 consumed, an amount that was not markedly changed under sulfate limitation (Table IV). On the basis of previous work (see Section II,B,2) that suggested the occurrence of only two coupling sites in the aerobic chain of heterotrophically grown cells (van Verseveld and Stouthamer, 1976), Meijer *et al.* (1977a) concluded that site I phosphorylation is lost under sulfate-limited conditions and that the H^+/site ratio is about 3–4 instead of 2 as postulated by the chemiosmotic hypothesis.

These results and conclusions are at variance with those of Lawford (1978), who observed that sulfate limitation in continuous culture of *P. denitrificans* did not alter appreciably the stoichiometry of respiration-driven H^+ translocation in intact cells oxidizing endogenous substrates (Table IV). This author concluded that site I was not lost under sulfate-limited growth. To eliminate the contribution of the first coupling site in cells grown under succinate limitation, Lawford (1978) used piericidin, which was able to inhibit completely pyridine nucleotide-linked respiration in whole cells of *P. denitrificans*. Lawford *et al.* (1976) had previously observed that the $\leftarrow H^+/O$ quotient associated with malate oxidation in *P. denitrificans* was significantly decreased in the presence of piericidin. Measurement of the stoichiometry of proton translocation associated with the oxidation of endogenous substrates yielded an observed $\leftarrow H^+/O$ of 8.55 and 8.42 for succinate-limited and sulfate-limited cells, respectively; this decreased to 5.85 and 5.75, respectively, in the presence of piericidin. If aerobic heterotrophically grown cells of *P. denitrificans* possess only

TABLE IV

STOICHIOMETRIES OF PROTON TRANSLOCATION FOR CELLS OF *Paracoccus denitrificans* GROWN AEROBICALLY

Growth of cells; substrate	Electron donor	$\leftarrow H^+/O$	References
Succinate-limited	Endogenous	7.5	Meijer *et al.*, 1977a
	Succinate	3.7	Meijer *et al.*, 1977a
	Succinate + rotenone	3.0	Meijer *et al.*, 1977a
Sulfate-limited	Endogenous	3.4	Meijer *et al.*, 1977a
	Succinate	3.9	Meijer *et al.*, 1977a
	Succinate + rotenone	3.4	Meijer *et al.*, 1977a
Methanol-limited	Endogenous	7.2	van Verseveld and Stouthamer, 1978a
	Succinate	6.2	van Verseveld and Stouthamer, 1978a
	Succinate + rotenone	5.1	van Verseveld and Stouthamer, 1978a
	Methanol + rotenone + antimycin	3.5	van Verseveld and Stouthamer, 1978a
Formate-limited	Endogenous	6.3	van Verseveld and Stouthamer, 1978a
	Succinate	3.9	van Verseveld and Stouthamer, 1978a
	Succinate + rotenone	3.5	van Verseveld and Stouthamer, 1978a
Succinate-limited	Endogenous	8.6	Lawford, 1978
	Endogenous + piericidin	5.9	Lawford, 1978
	Succinate	7.3	Lawford, 1978
Sulfate-limited	Endogenous	8.4	Lawford, 1978
	Endogenous + piericidin	5.8	Lawford, 1978
Succinate-limited	Endogenous	2.4	Lawford, 1979
	Endogenous + rotenone	2.1	Lawford, 1979
	Succinate	6.3	Lawford, 1979
	Succinate + rotenone	5.3	Lawford, 1979
	Ubiquinol	5.2	Lawford, 1979
	Ubiquinol + rotenone	5.2	Lawford, 1979
H_2	Endogenous	10.6	Porte and Vignais, 1980
	Endogenous + rotenone	7.2	Porte and Vignais, 1980
	H_2	6.8	Porte and Vignais, 1980
	H_2 + rotenone	6.2	Porte and Vignais, 1980
	Succinate	7.0	Porte and Vignais, 1980
	Succinate + rotenone	7.0	Porte and Vignais, 1980
	Ascorbate + TMPD[a]	2.7	Porte and Vignais, 1980

[a] TMPD, *N,N,N',N'*,-tetramethyl-*p*-phenylenediamine.

two coupling sites as proposed by several authors (Imai *et al.*, 1967; Knobloch *et al.*, 1971; van Verseveld and Stouthamer, 1976), the $\leftarrow H^+/O$ of about 6 obtained by Lawford (1978) with piericidin-poisoned cells should be attributed to a single energy-conservation site. On the other hand, if one assumes two coupling sites in the cytochrome region, the $\leftarrow H^+$/site ratio drops to 3. Assuming a $\leftarrow H^+$/site of about 3, Lawford (1978) concluded that *P. denitrificans* grown under either succinate or sulfate limitation possess three potential energy-coupling sites including one in the cytochrome oxidase region.

Presence of site III. A functional assay involving energy-coupled transport has been used to demonstrate the existence of an active energy-coupling site III in *P. denitrificans*. It has been shown previously that membrane vesicles derived from *P. denitrificans* can actively accumulate amino acids (White *et al.* 1974), lactate (Nichols and Hamilton, 1976), sulfate (Burnell *et al.*, 1975a), and phosphate (Burnell *et al.*, 1975b) in a respiration-dependent manner. If site III is functional, the oxidation of ascorbate-reduced TMPD results in accumulation of substrate. This was observed to be the case in autotrophic cells (with ascorbate + PMS) (Burnell *et al.*, 1975a) and in heterotrophic cells (Nichols and Hamilton, 1976; Pik and Lawford, 1979). Pik and Lawford (1979) chose to measure the respiration-coupled transport of dicarboxylate anions. They prepared membrane vesicles by osmotic lysis of spheroplasts of *P. denitrificans* grown aerobically in continuous culture under conditions of carbon limitation with succinate as sole carbon and energy source, and they demonstrated that these membrane vesicles could accumulate [^{14}C]succinate and L-[^{14}C]malate in an uncoupler-sensitive respiration-dependent manner. The oxidation of ubiquinol-1 or of ascorbate + TMPD in the presence of antimycin A both supported accumulative uptake of succinate or L-malate; this uptake was abolished in the presence of the protonophore carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). Pik and Lawford (1979) concluded that the energization of the dicarboxylate transport system by the oxidation of ascorbate (+TMPD) in the presence of antimycin A is evidence that cells of *P. denitrificans* that contain cytochrome a_3 (aerobic heterotrophic growth) do possess a functional energy-coupling site III.

Mutants such as the cytochrome *c*-deficient mutants of *P. denitrificans* isolated by Willison and John (1979) should be valuable tools for assessing, either by the method of growth yield or by measuring proton translocation, whether or not site III is present. C. W. Jones *et al.* (1978) studied oxidative phosphorylation and respiration-linked proton translocation in bacteria containing different terminal oxidases. They demonstrated that species that lacked cytochrome *c* had $\leftarrow H^+/O$ ratios lower than 4, whereas values of nearly 6 were found when cytochrome *c* donated its electrons to either cytochrome oxidase aa_3 or cytochrome *o* (Fig. 1).

It should be noted that the above-mentioned controversies on the occurrence of classical "sites" may be only apparent. A "site" may be present but not functioning under a given set of conditions. This may depend on the partitioning of the electron flow in the electron transport chain at the branch point (Figs. 1 and 2). As mentioned above, lack of cytochrome *c* led to a decrease in $\leftarrow H^+/O$ ratios. On the other hand,

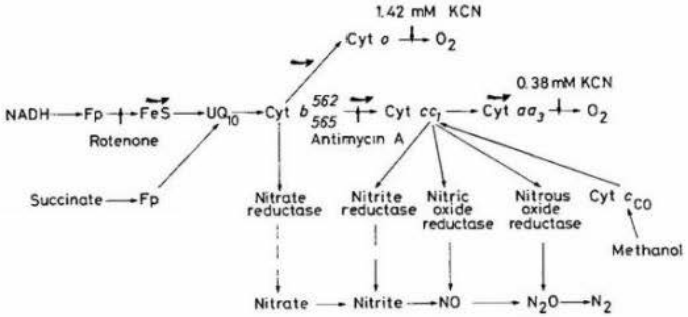

FIG. 2. The anaerobic respiratory chain of *Paracoccus denitrificans* including the denitrifying steps. The phosphorylation sites are indicated by the curved arrows; UQ, ubiquinone. Adapted from Stouthamer (1980).

cytochrome *c* when present in large amounts as in methanol-grown cells (van Verseveld and Stouthamer, 1978a) may channel a greater portion of the electron flow to cytochrome aa_3 and enable phosphorylating site III to be active (see Section IV,B,1).

4. *Energy Transduction by Proton Pumping*

Data reported in preceding sections dealt mainly with the experimental demonstration of respiration-linked ATP synthesis in *P. denitrificans*, not with mechanisms by which ATP synthesis is coupled to respiration. Models proposed so far are based on extensive studies with mitochondria and chloroplasts. It may be anticipated that data obtained at the molecular level with the mitochondrion-like *P. denitrificans* will contribute to the elucidation of how electron transport is coupled to proton motive activity in mitochondria and other respiratory systems. It seems, therefore, appropriate to review briefly the nature of the alternative schemes presently under discussion.

a. Redox Loop versus Proton Pump. Following the suggestions of Mitchell (1966), who proposed that alternative hydrogen carriers and electron carriers in an electron-transport chain could act to cause proton-motive activity, this "redox-loop" mechanism had apparently received much experimental support (Mitchell, 1976a; Haddock and Jones, 1977; Trebst, 1974; Hauska and Trebst, 1977; Crofts and Wood, 1978; Witt, 1979; Wraight *et al.*, 1978) by the mid-1970s, together with an elegant extension into the so-called "Q-cycle" (Mitchell, 1976b) to account for certain experimental findings that were inconsistent with the original

scheme. As Tiemann *et al.* (1979) have pointed out, a distinctive feature of this type of mechanism is that it is a neutral species (an H atom chemically bound in a redox species) that actually crosses the hydrophobic region of the membrane. In the alternative "proton pump" type of mechanism it is a charged species, the proton (or, properly, perhaps, the hydronium ion), that crosses the membrane. Additionally, in the pump mechanism it is proteins, not low-molecular-weight quinonoid compounds, that are responsible for carrying the proton across the membrane. In the terminology of Mitchell (1977), this "pump" kind of proton-motive activity would tend to be "an exclusively indirect type of chemiosmotic mechanism." Since until recently those favoring a pump type of mechanism have been very much in the minority, and there has been some confusion as to what is meant by a pump, we shall briefly review the requirements that must be satisfied for the presence of a proton pump.

b. Requirements for a Proton Pump. Following the principles of energy transduction by membranous systems elaborated by Devault (1971, 1976; cf. Blumenfeld, 1978), the major requirements of a proton pump have been summarized recently by Wikström and Krab (1979a) (cf. Wikström, 1980). In his perspicacious review, Walz (1979) also identified the main necessary features of such a pump, although, since he concentrated on thermodynamic aspects, he found himself loath to speculate upon molecular mechanisms. Papa (1976) had earlier reviewed evidence that he interpreted as favoring a pump in the "site II" region of the mitochondrial electron-transport chain. However, Papa (1976) did not make explicit the requirement that protons must be taken up on one side of the membrane and released on the other side to give a true proton pumping activity, not merely picked up and released on a single side as in his "membrane Bohr" mechanism. This terminology, the "membrane Bohr effect" seems to date from the paper of Chance *et al.* (1970), who observed a rapid proton binding only indirectly coupled to electron transfer in bacterial chromatophores. This terminology, which compares certain membrane-bound redox proteins with the well known change in affinity of hemoglobin for protons on binding oxygen (Wyman, 1968), is most unfortunate, because in a true Bohr mechanism such protons are purely scalar; i.e., they return to the aqueous phase whence they came upon reoxidation of the protein's electroactive center and cannot be pumped across the membrane. Thus the electron-transporting proton pump has the following requirements (see also Kell *et al.*, 1980) (Fig. 3).

1. The protein must contain acid-base groups whose pK is directly coupled to the redox state of the electron-transferring center.

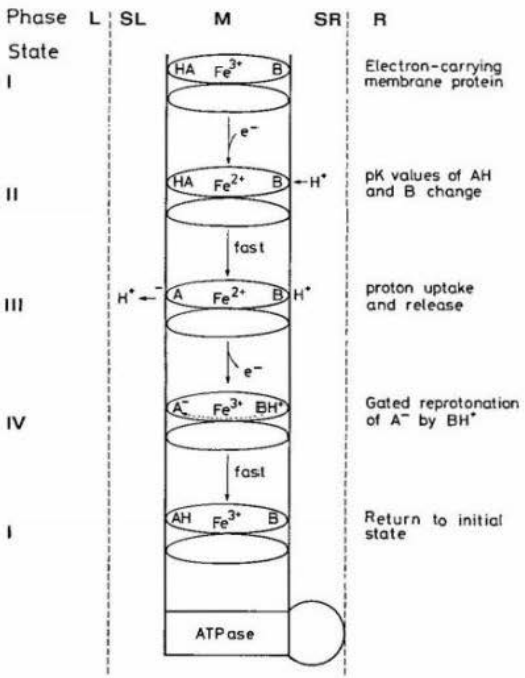

FIG. 3. The operation of a transmembrane, proton-motive electron-transport chain. The figure shows the four identifiable states during the operation of a proton-motive protein of the type introduced here. States I and III are the more stable states, so that the model may be rationalized as a two-state model. From Kell *et al.* (1980).

2. Such groups should have access to different sides of the membranes.
3. There should be a gated, proton-permeable channel (Nagle and Morowitz, 1978) through the protein to allow reprotonation of one group by the other.

The type of model outlined here is by no means unique, but we believe that it contains the most useful general features of the family of models that it represents. A special case, favored for cytochrome oxidase by Wikström and co-workers (Wikström and Krab, 1979a; Wikström, 1980), has a single reorienting group (or "protode"), which can have access to different faces of the membrane during the cycle of proton-motive activity. Although this type of mechanism may seem more attractive for the heme-containing proton pumps (Wikström, 1980), it is extremely difficult with present techniques to distinguish the two models mechanisti-

cally, although one possible experimental method has been outlined (Kell *et al.*, 1980). The "cubic" scheme of Wikström and Krab (1979a) is perhaps more complete than the present model in the sense that it includes branched pathways between the various states of such an energy-coupling machine; it is our view, however, that an essentially linear scheme of the type depicted in Fig. 3, with the additional *caveat* that the degree of coupling within the pump may not always be complete, is sufficient to describe the likely major energetic states assumed by such a proteinaceous proton pump during its cycle of proton-motive activity. In this sense there is a parallel with Albery's "scheme of squares," which describes all possible pathways for electron and proton transfer by electroactive species at electrodes, but reduces usually to one or more major pathways in real cases (Albery and Hitchman, 1971). However, we would stress that the present model is in no sense conceptually different from that described by Wikström (Wikström and Krab, 1979a; Wikström, 1980), since both are subject to the thermodynamic and geometrical restraints mandated for this type of system.

As has been pointed out elsewhere (Kell *et al.*, 1980; Wikström, 1980), this type of mechanism is likely to be a general one and applies to other proton-motive enzymes such as permease molecules, ATP synthetase, and bacteriorhodopsin. Since in at least two cases intermolecular cross-linking agents do not inhibit proton-motive activity (Packer *et al.*, 1977; Bonaventura *et al.*, 1978), and in the case of electron transport proteins and bacteriorhodopsin, activity is observed at liquid nitrogen temperature, rotation is almost certainly *not* a part of the protein's proton-motive cycle. We may now ask what other features this type of "pump" mechanism has that might be expected to allow a distinction between it and the redox loop–Q-cycle type of mechanism.

Two main features may be used to distinguish experimentally the pump and loop–Q-cycle types of mechanism, in addition to the obvious point that a single protein reconstituted such that it can pump protons in the absence of any artificial loops will naturally provide powerful evidence in favor of the pump type of mechanism. These features are the stoichiometry of respiration-driven proton translocation (measured under "level flow" conditions) and the effect of membrane energization on the redox potential or "electron affinity" (Walz, 1979) of the carrier.

c. Proton Pumping in P. denitrificans. Stouthamer (1980), in a thought-provoking article that nicely illustrates the utility of bacteria for answering questions concerned with mitochondria, has recently reviewed the stoichiometries of respiration-driven proton translocation observed by various laboratories for *P. denitrificans* and for mitochondria and has

concluded on the additional basis of cognate growth yield studies that under heterotrophic conditions *P. denitrificans* and mitochondria probably pump between 3 and 4 protons per so-called "site" (cf. Pozzan *et al.*, 1979a,b; Villalobo and Lehninger, 1979) and that this number is equal for each site. This is in contrast to the view of Hinkle, Mitchell, Papa, and Wikström (cf. Table II), who suggest that the third site may pump fewer protons than the second site. Whatever the outcome of this lively current debate, one may note that the stoichiometry of respiration-driven proton translocation is certainly greater than the 2 per site required in a loop type of mechanism (see Section II,B,3). The first workers to apply the oxygen-pulse method to *P. denitrificans* (Scholes and Mitchell, 1970b) suggested that an "extra" site, the so-called loop 0, might be involved in causing an observed H^+/O stoichiometry of greater than 2 per classical site. Later workers using this organism have largely eschewed this analysis (see Stouthamer, 1980; Lawford, 1979, for recent reviews), although in many organisms other than *P. denitrificans* (Jones, 1977; Haddock and Jones, 1977) a consistent value for the $\leftarrow H^+/O$ ratio of 2 per site has been obtained. One possible resolution of this discrepancy is given in Section II,B,3. At present, then, it must be concluded that, although stoichiometries of themselves do not provide a great deal of information about molecular mechanisms, they do seem to exclude the redox loop–Q-cycle type of mechanism in *P. denitrificans*.

Since the redox poise of a proton pump will be affected by the imposition across it of a transmembrane proton gradient (Dutton and Wilson, 1974; Läuger, 1979; Walz, 1979; Kell *et al.*, 1980), it is of interest to ask whether or not such an imposition affects the redox poise of any of the carriers in the respiratory chain of mitochondria or of *P. denitrificans*. As it turns out, a unifying view is possible: at each so-called "site" of oxidative phosphorylation, one redox center is affected by the imposition of a proton-motive force by ATP hydrolysis (in mitochondria). The respiratory components involved are pyridine nucleotide transhydrogenase (Rydström, 1977), Fe-S center N-2 (Ohnishi, 1976), cytochrome b_{566} (cytochrome b_T) (Dutton and Wilson, 1974), and cytochrome aa_3 (the latter two components have been shown to interact) (Wikström *et al.*, 1976). Of special interest is the demonstration in sulfate-limited cells of *P. denitrificans* (Meijer *et al.*, 1977b) that loss of Fe-S center N-2 *alone* was accompanied by loss of free-energy-conserving capacity at site I; however, see Lawford (1978) and Table IV. The only reasonable interpretation of this observation is that Fe-S center N-2 is a proton pump. Later work, studying rotenone-inhibited, chemostat-grown cells (Meijer *et al.*, 1978), further indicated that loss of Fe-S center N-2, and the presence of rotenone sensitivity and free-energy-conserving capacity at site I were all

intimately correlated. A similar analysis has not been performed at any other site as yet, but these approaches nicely illustrate again the utility of this organism for studying current problems of energy coupling. Finally, it has been demonstrated that under appropriate conditions proton-motive activity linked to the operation of site III could be observed (Kell *et al.*, 1978; Porte and Vignais, 1980). The demonstration of a two-subunit cytochrome oxidase in *Paracoccus* (Ludwig and Schatz, 1980) should thus allow an elegant analysis of energy transduction at site III in this organism.

C. Additional Mitochondrial-Type Features of *P. denitrificans*

From the foregoing data it appears that *P. denitrificans* displays some analogies with the mitochondrion additional to those already outlined by John and Whatley (1977a).

1. The Redox Components

One question presently under particularly active discussion in the field of mitochondrial bioenergetics is whether or not cytochrome oxidase represents a coupling site and, if it does, functions as a proton pump (Moyle and Mitchell, 1978; Wikström and Krab, 1978). *Paracoccus denitrificans* may offer a means to investigate such a problem since the cytochrome oxidase of *P. denitrificans* shows similarities with the mammalian enzyme (*a*) from a functional point of view; (*b*) in its low temperature kinetic behavior with O_2 and CO (Henry *et al.*, 1979); and (*c*) in its possible function as a proton pump.

Although the occurrence of phosphorylation site III has been questioned in aerobic, heterotrophically grown cells, it seems likely that it is present when the cells are grown autotrophically (van Verseveld and Stouthamer, 1978a; Porte and Vignais, 1980).

Another point that has emerged is the similarity in the nature and content of iron–sulfur centers present in the aerobic respiratory chain of *P. denitrificans* and of mitochondria (Meijer *et al.*, 1977b).

2. Production of Superoxide Anions

As in mitochondria, the plasma membrane of *P. denitrificans* is able to generate superoxide anions in the presence of O_2 whatever the growth conditions (Henry and Vignais, 1980). In mitochondria, production of superoxide radicals occurs in the ubiquinone–cytochrome *b* region of the

respiratory chain, before the antimycin-sensitive site (Loschen *et al.*, 1974; Cadenas *et al.*, 1977; Flohé *et al.*, 1977). It is assumed that a similar situation occurs in *P. denitrificans*, although the system may be complicated by the presence of branched respiratory pathways. It is inferred that the CO-reacting low potential *b*-type cytochrome may well be the source of the superoxide radicals in anaerobically grown cells. In mitochondria, Loschen *et al.* (1974) observed an enhancement of $O_2^{\cdot-}$ production when the redox potential of cytochrome b_{566} was lowered to near 0 mV. This cytochrome also binds CO and CN^- and is autoxidizable. Since the couple O_2/O_2^- has an E'_0 value of -0.33 V (Wood, 1974; Ilan *et al.*, 1976), the low-potential cytochrome *b* found in anaerobically grown cells of *P. denitrificans* meets the thermodynamic requirements for the monoelectronic reduction of O_2 into $O_2^{\cdot-}$ (Henry and Vignais, 1980).

3. *Two Superoxide Dismutases*

Another feature that suggests a close relationship between *P. denitrificans* and mitochondria is the presence in this bacterium of two superoxide dismutases (SOD). One SOD is of the manganese type (Terech and Vignais, 1981) as found in mitochondria from mammals (Weisiger and Fridovich, 1973; Salin *et al.*, 1978) or from unicellular eukaryotes (Henry *et al.*, 1980). Although the mitochondrial Mn-SOD is a tetramer and the *P. denitrificans* enzyme is a dimer, both are made up of identical subunits of molecular weight 23,500 and have a very similar amino acid composition (Terech and Vignais, 1981).

The second enzyme with SOD activity is a distinct protein with an apparent molecular weight of 33,000, and cyanide sensitivity, a feature of copper-containing enzyme (P. M. Vignais, A. Terech, and C. M. Meyer, unpublished results). A cyanide-sensitive, Cu/Zn-SOD has also been reported to be associated with mammalian mitochondria (Tyler, 1975; Peeters-Joris *et al.*, 1975) and with mitochondria from *Neurospora crassa* (Henry *et al.*, 1980). It is localized in the intermembrane space and has a molecular weight of about 31,000 similar to the cytosolic Cu/Zn-SOD. Although the Cu/Zn-dismutase in the mitochondrial intermembrane space might have originated from the cytosol, it is striking that, like mitochondria, *P. denitrificans* contains both a Mn-SOD and a Cu/Zn-SOD.

III. Anaerobic Respiration on Nitrate and Nitrogen Oxides

Paracoccus denitrificans can carry out anaerobic respiration by reducing nitrate dissimilatively. The capacity of *P. denitrificans* to reduce nitrate to dinitrogen makes it a true denitrifying bacterium that is distinct

from nitrate-respiring bacteria, such as *Escherichia coli,* for which the reduction of nitrate ends with nitrite. Denitrifiers, which are aerobic bacteria, in contrast to sulfate and carbon dioxide reducers, which are obligate anaerobes, contribute to significant losses of nitrate into the earth's atmosphere as N_2.

About 15 genera have been reported to contain denitrifying species. The greatest number of denitrifiers is found in the genus *Pseudomonas* (Payne, 1973). Studies concerning the denitrifying systems of *P. denitrificans* can illustrate the trend of research on denitrification. Comprehensive review articles by Nason (1962) and Payne (1973) and other shorter reviews (Payne, 1976; Payne and Balderston, 1978; Zumft and Cárdenas, 1979) discuss the rapid developments in this field of research.

The biochemical reduction of nitrate to dinitrogen is currently thought to involve the following sequence (Payne, 1973; Pichinoty, 1973) (Table V).

TABLE V

REDOX CONVERSIONS OF INORGANIC NITROGEN COMPOUNDS DURING NITRATE RESPIRATION, DENITRIFICATION, AND NITRATE FERMENTATION[a]

Organism and type of reaction	ne^- and formal valence change	E_0' pH 7.0[b] (mV)	$\Delta G_0'$[b] (kJ/mol)
Nitrate respiration			
Escherichia coli			
$NO_3^- + [H_2] \rightarrow NO_2^- + H_2O$	$2e^-$, +5/+3	+420	−161.1
Denitrification			
Pseudomonas aeruginosa			
$2\,NO_3^- + 2\,H^+ + 5\,[H_2] \rightarrow N_2(g) + 6\,H_2O$	$10e^-$, +5/0	+749	−1121.2
Corynebacterium nephridii			
$2\,NO_3^- + 2\,H^+ + 4\,[H_2] \rightarrow N_2O(g) + 5\,H_2O$	$8e^-$, +5/+1	+599	−781.9
Alcaligenes odorans			
$2\,NO_2^- + 2\,H^+ + 3\,[H_2] \rightarrow N_2(g) + 4\,H_2O$	$6e^-$, +3/0	+970	−799.9
Other possible reactions			
$NO_2^- + \frac{1}{2}\,[H_2] + H^+ \rightarrow NO(g) + H_2O$	$1e^-$, +3/+2	+374	−76.2
$2\,NO(g) + [H_2] \rightarrow N_2O(g) + H_2O$	$2e^-$, +2/+1	+1177	−306.3
$2\,NO_2^- + 2\,H^+ + 2\,[H_2] \rightarrow N_2O(g) + 3\,H_2O$	$4e^-$, +3/+1	+777	−459.2
$N_2O(g) + [H_2] \rightarrow N_2(g) + H_2O$	$2e^-$, +1/0	+1352	−339.5
Nitrate fermentation			
Clostridium perfringens			
$NO_3^- + 2\,H^+ + 4\,[H_2] \rightarrow NH_4^+ + 3\,H_2O$	$8e^-$, +5/−3	+350	−590.8

[a] From Zumft and Cárdenas (1979).

[b] $\Delta G_0'$ are calculated versus the pair $H_2/2H^+$; E_0', pH 7.0 = −420 mV; E_0' refers to the nitrogenous redox pairs.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

The intermediary products, nitrite and nitrous oxide, can also serve as terminal electron acceptors and sustain growth of *P. denitrificans* in the absence of O_2. The indicated reductive steps are the result of the operation of four complex enzyme systems as illustrated in Fig. 2. Among these systems only nitrate reductase and nitrite reductase have been fully characterized so far. Reports on the enzyme systems involved in nitric oxide and nitrous oxide reduction have recently appeared (see below).

A. Anaerobic Electron Transport with Nitrogenous Oxides as Electron Acceptors

Although it is an aerobic bacterium, *P. denitrificans* is able to grow anaerobically provided that inorganic nitrogenous oxides (NO_3^-, NO_2^-, or N_2O) are supplied as oxidants in the growth medium (Verhoeven *et al.*, 1954; Kluyver, 1956; Pichinoty, 1973; Payne, 1973).

When growth of *Paracoccus* is carried out anaerobically on nitrate as added terminal electron acceptor, several alterations affect the aerobic respiratory chain, resulting mainly in an increased synthesis of electron carriers, such as *b*- and *c*-type cytochromes, and in the induction of certain new components (Scholes and Smith, 1968; Newton, 1969).

Only slight differences exist in the rate of oxygen uptake by whole cells or membrane vesicles obtained from both anaerobically and aerobically grown cultures of *P. denitrificans*. Membrane preparations from nitrate-grown cells exhibit high rates of NADH and succinate oxidation comparable to those observed with cells grown with O_2 (Lam and Nicholas, 1969a). Other substrates, such as NADPH, formate, lactate, and malate can also serve as electron donors when O_2 is used as the terminal acceptor of electrons (Lam and Nicholas, 1969a).

Succinate and NADH oxidase activities in cells grown on nitrate are sensitive to both antimycin A and KCN (Scholes and Smith, 1968; Lam and Nicholas, 1969a). Lam and Nicholas reported that 100 μM KCN inhibited NADH or succinate oxidation by only 50%. This concentration of KCN is one order of magnitude greater than is required for 50% inhibition in aerobically grown cells. In agreement with this observation it was shown that KCN inhibits NADH oxidation in membrane preparations in a biphasic manner, 100% inhibition being reached above 1 mM KCN (Henry, 1980). With the same preparation of membrane particles, only 70% inhibition is observed with antimycin A. These results suggest that part of the electron flow is diverted to O_2 by a second, alternative route, which is either less accessible or less sensitive to these inhibitors.

Although cytochrome oxidase activity, measured polarographically with ascorbate and cytochrome *c* as substrates is sometimes up to threefold higher in extracts from anaerobically grown cells as compared with aerobic cultures of *P. denitrificans* (Lam and Nicholas, 1969a), this does not necessarily imply an increased synthesis of the aa_3 components. It was demonstrated that anaerobic growth results in induction or enhanced synthesis of cytochrome *o*, a possible terminal oxidase, and of nitrite reductase with cytochrome oxidase activity (Lam and Nicholas, 1969c). These aspects of the anaerobic electron-transport chain will be discussed further in this section.

B. Redox Components of the Anaerobic Respiratory Chain

During anaerobic respiration, as discussed above, *Paracoccus* converts nitrate into nitrogen gas as the final product. The reduction of nitrate to N_2 is a multistep process, and each step is catalyzed by specific redox components, some of which are soluble whereas others are membrane bound. In addition to nitrate and nitrite reductases, the denitrifying respiratory chain of *P. denitrificans* consists of iron–sulfur proteins, UQ_{10}, *b*-, *c*-, *o*- and/or *a*-type cytochromes (Scholes and Smith, 1968).

1. Cytochrome Oxidases

It has been mentioned that heterotrophic growth on nitrate generally results in a sharp decrease in the cytochrome aa_3 content in whole cells or membrane preparations of *P. denitrificans*. However, Lam and Nicholas (1969a) observed a greater cytochrome oxidase activity in anaerobically grown cells than in aerobically grown cells. Thus other components of the respiratory chain must participate in cytochrome oxidase activity in anaerobically grown cells.

Anaerobic growth on nitrate or nitrite involves the synthesis of cytochrome *o* (Scholes and Smith, 1968) and of nitrite reductase, which displays a cytochrome oxidase activity (Lam and Nicholas, 1969c). In several bacterial systems cytochrome *o* acts as the sole terminal oxidase and is able to support high respiration rates. However, the capability of the CO-binding *b*-type cytochrome of anaerobically grown *P. denitrificans* to behave as an oxidase has not yet been investigated.

Potentiometric titrations of the *b*-type cytochromes, either in the absence or in the presence of CO, indicates that four components titrate with half-reduction potentials of +245, +145, 0, and −195 mV, respectively. Only the low-potential component reacts with CO, resulting in a positive shift in its midpoint potential of about 60 mV (Henry, 1980;

Henry and Wilson, 1981). This component also appears to be reduced very slowly by NADH and succinate and to bind CO very weakly unless the membrane preparation is reduced by dithionite. A similar situation has been reported in mitochondria from *Tetrahymena pyriformis* (Kilpatrick and Erecinska, 1977). To our knowledge, there are no other reports on the half-reduction potentials of the cytochromes in membrane preparations from anaerobically grown cells of *P. denitrificans*. It is puzzling that such a low-potential component, poorly reducible by physiological substrates, is able to support the high respiration rates observed during aerobic respiration in anaerobically grown cells with NADH as a substrate. Furthermore, stopped-flow experiments (M.-F. Henry, unpublished) indicate that the *b*-type cytochromes are not oxidized rapidly enough to account for the observed rate of NADH oxidation.

Lawford *et al.* (1976) have reached a similar conclusion by rapid kinetic experiments performed on membranes of aerobically grown cells of *P. denitrificans* where both cytochrome oxidase aa_3 and *o* were simultaneously present. Further work is thus necessary to ascribe a definite role to cytochrome *o in vivo* and to determine the components that act as cytochrome oxidase.

2. *Nitrate Reductase*

a. Molecular Properties. The first enzyme involved in denitrification, nitrate reductase (EC 1.7.99.4), is a membrane-bound component linked to the constitutive aerobic respiratory chain (cf. Stouthamer, 1976b, for review); it contains Mo at its active site, iron, and labile sulfide, but no flavin or cytochrome (Lam and Nicholas, 1969b). Cytochromes of the *b* type are directly involved in electron transfer to nitrate (John and Whatley, 1970).

The respiratory nitrate reductase A of *P. denitrificans* (Pichinoty, 1964; Forget and Pichinoty, 1965) was isolated by Lam and Nicholas (1969b) and Forget (1971). The purified enzyme has a molecular weight of 160,000 and an isoelectric point at pH 4.2; it is thought to contain 8 Fe and equal amounts of labile sulfide per mole (Forget, 1971). Isotope labeling with ^{99}Mo during purification of the enzyme indicated accumulation of the radioactivity in the fraction characterized by the highest enzyme activity (Lam and Nicholas, 1969b). Further experiments (Forget, 1971) showed that purified nitrate reductase contains approximately 0.4 g-atom of Mo per mole of enzyme.

In addition to the normal EPR signals of iron–sulfur centers, N-1 to N-4, found in aerobically grown cells, membranes from *P. denitrificans* grown on nitrate as oxidant, exhibit at 15°K resonance lines at $g = 2.058$, 1.952,

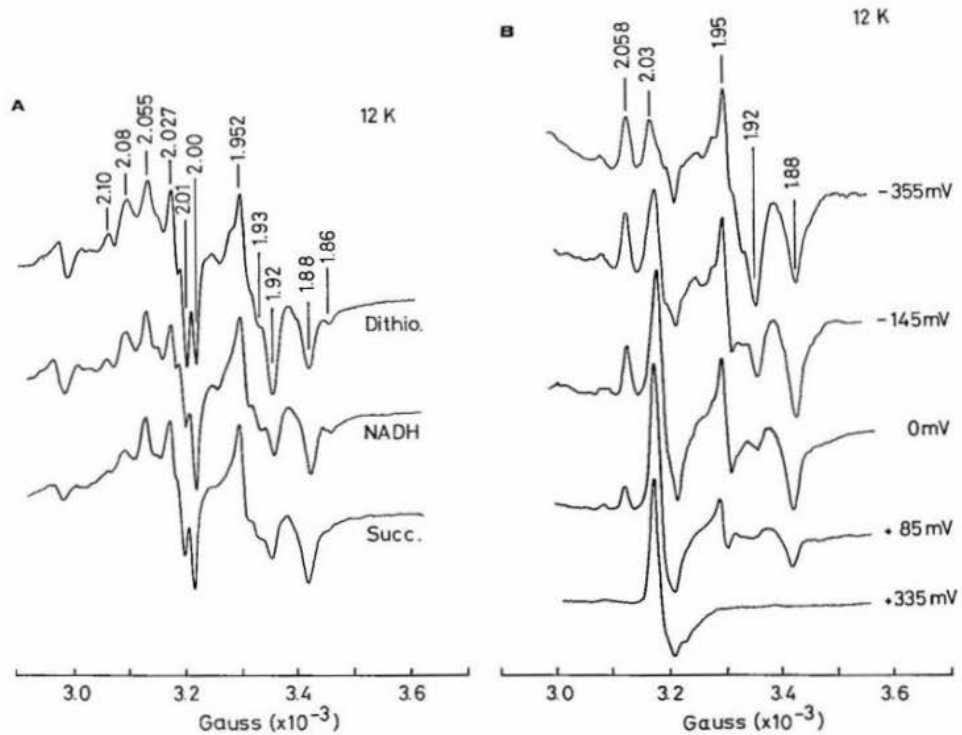

FIG. 4. Electron paramagnetic resonance (EPR) spectra of membrane particles prepared from anaerobically grown cells of *Paracoccus denitrificans*. (A) Membrane particles were reduced with 2.5 m*M* NADH, 20 m*M* succinate, or a few crystals of solid dithionite and were quickly frozen in an isopentane–cyclohexane freezing mixture (81°K). EPR conditions: microwave frequency, 9.115 GHz; time constant, 0.25 second; modulation amplitude, 10 G; microwave power, 1 mW. Protein concentration was about 45 mg/ml. (B) The membrane particles were poised at various redox potentials under an atmosphere of argon. A freshly prepared solution of dithionite was used as reductant, and ferricyanide served as oxidant. Other conditions as in (A).

and 1.88 (Meijer *et al.*, 1979b). The g values are close to those found by Forget and Dervartanian (1972) with purified nitrate reductase. These signals are visible at 12°K whether the membrane preparation is reduced enzymically with NADH or succinate or chemically with dithionite (Fig. 4A). The dependence of the EPR signals associated with the membrane-bound nitrate reductase on the redox potential is shown in Fig. 4B. We would tentatively assign the following midpoint potentials to the observed resonance lines: complex signal at $g = 1.95$, -210 mV; $g = 1.88$, -120 mV. (M.-F. Henry and T. Ohnishi, unpublished results).

Contrary to the observations on membranes derived from aerobically, heterotrophically grown cells (Meijer *et al.*, 1977b), sulfate limitation neither altered the EPR spectra of complex I nor affected rotenone sensitivity or NADH oxidase or NADH nitrate oxidoreductase activities (Meijer *et al.*, 1979b).

The purified enzyme reacts with various reduced viologen dyes, FMN, and FAD as electron donors in nitrate reduction. Nitrate, chlorate, and even bromate act as substrates, although in intact cells the enzyme appears to be inaccessible to chlorate (John, 1977; Alefounder and Ferguson, 1980).

b. Synthesis and Regulation. Oxygen has been shown both to repress the synthesis and inhibit the activity of intracellular nitrate reductase in growing cultures of *P. denitrificans* (Lam and Nicholas, 1969a; Sapshead and Wimpenny, 1972; John, 1977). During succinate oxidation, when both nitrate and oxygen are available in the suspending medium of anaerobically grown cells of *P. denitrificans*, the cells preferentially use the aerobic pathway and start to reduce oxygen; nitrate is reduced only when oxygen is exhausted (John, 1977). However, a similar experiment conducted with membrane vesicles indicates that membranes reduce oxygen and nitrate simultaneously. No complete explanation has yet been found for this discrepancy between the ability of whole cells and particles to reduce nitrate under aerobic conditions (John, 1977), but Alefounder and Ferguson (1980) suggested that O_2 might inhibit a putative permease necessary to effect the entry of NO_3^- to the cell.

Lam and Nicholas (1969a) and Sapshead and Wimpenny (1972) studied the effects of both oxygen and nitrate on cytochrome formation in *P. denitrificans* and drew different conclusions as to the effects of oxygen and nitrate. According to Lam and Nicholas (1969a), no nitrate reductase is formed under anaerobic conditions unless NO_3^- is supplied to the growth medium. These authors therefore concluded that nitrate reductase formation is due to a specific induction rather than to a derepression due to oxygen removal from the culture medium. Sapshead and Wimpenny (1972) found that cells grown under air limitation or under oxygen limitation with either NH_4^+ or NO_3^-, all possess cytochrome oxidase aa_3 and little or no nitrate reductase. They found that cytochrome oxidase aa_3 disappeared only under full anaerobiosis. Fully anaerobic cells also contained more *b*-, *c*-, and *cd*- (nitrite reductase)-type cytochromes than cultures from oxygen-limited growth. Therefore, these authors concluded that the predominant effect of oxygen resides on the regulation of the synthesis of redox carriers in *P. denitrificans*. However, Lam and Nicholas (1969a) also noted that although nitrate and nitrite reductases are

induced by their substrates, their synthesis is also repressed by O_2. These results taken together with several observations on other denitrifiers (Payne, 1973; Pichinoty, 1973) strongly suggest that oxygen rather than nitrate exerts a regulatory effect on the amount and the type of the components of the anaerobic electron transport chain.

Calder *et al.* (1980) have designed a system allowing the induction of nitrate reductase in whole cell suspensions of *P. denitrificans*. Cells showing little nitrate reductase activity are incubated with either nitrate, nitrite, or azide. Among the three components tested, azide, a competitive inhibitor of the enzyme (Forget, 1974), proved to be the best inducer. Three to four times more nitrate reductase activity is found with azide than with nitrate or nitrite. The cells incubated with azide exhibit an approximately 50-fold increase in activity over control cells. Formation of cytochromes *b* paralleled the appearance of nitrate reductase.

Similar experiments were conducted on a mutant strain M-1 apparently lacking nitrate reductase activity. This mutant is unable to carry out anaerobic growth on nitrate; however, it can be cultured either aerobically or anaerobically in a medium supplied with nitrite. Under any of the incubation conditions tested (NO_3^-, NO_2^-, or N_3^-), nitrate reductase activity was undetectable. Nevertheless, the mutant synthesized cytochromes at levels comparable to the wild-type strain, indicating that nitrate reductase does not need to be active for its coregulation with cytochrome *b*.

Sodium dodecyl sulfate (SDS) gel electrophoresis has revealed the presence of a polypeptide (MW 150,000) corresponding to nitrate reductase in the wild-type strain and in the mutant as well. In the mutant the protein can be detected even in the absence of inducer. Nitrate and nitrite do not affect formation of the polypeptide in the mutant, but azide depresses its synthesis.

It is known that nitrate reductase is a Mo-containing iron–sulfur protein (Lam and Nicholas, 1969b; Forget, 1971) and that in *E. coli* tungsten acts as a Mo antagonist in nitrate reductase formation in Mo-defective mutants (Scott and De Moss, 1976). Therefore, Burke *et al.* (1980) studied the effects of Mo and W on nitrate reductase formation. Cells of *P. denitrificans* incubated in the presence of both azide and sodium molybdate form high levels of nitrate reductase whose activity is five times greater than in cells incubated in a Mo-free medium. Tungsten is ineffective in replacing Mo. However, the cells incubated with tungsten and the inducer still contain the polypeptide of MW 150,000, as detected by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The polypeptide is present in the mutant strain under all conditions tested. Labeling experiments with ^{55}Fe and ^{99}Mo indicate incorporation of ^{55}Fe into both

the wild-type and the mutant protein, whereas only the wild-type protein incorporates ^{99}Mo. As pointed out by Burke *et al.* (1980), the ability of mutant strains of *P. denitrificans* to accumulate demolybdoprotein may constitute a convenient tool for reconstitution studies and incorporation of molybdenum cofactors in nitrate reductase.

c. Topography. The orientation of nitrate reductase on the membrane appears now to be established. The problem was initially studied by Garland *et al.* (1975) for the nitrate reductase of *E. coli*. These authors used azide, a potent competitive inhibitor of nitrate reductase (Forget, 1974), to localize the binding site of the enzyme. The rationale was to link the ability of azide to enter the cell with the rate and degree of inhibition of electron transport to nitrate. Azide is a weak acid with pK 3.34; it diffuses across membranes in the protonated form much more rapidly than in the ionized form. By lowering the pH of the external medium to a value close to the pK of azide, one should observe an increased inhibition if the binding site is on the cytoplasmic face of the membrane.

Indeed, R. W. Jones *et al.* (1978) reported that the concentration of azide required in the suspension of *E. coli* cells was diminished by 40- to 50-fold when the extracellular pH was lowered from 8.0 to 6.0 and concluded, in contrast to Garland *et al.* (1975), that the azide-sensitive site is intracellular.

Similarly, for *P. denitrificans*, the nitrate binding site of the respiratory nitrate reductase has been proposed to lie on the internal (cytoplasmic) face of the plasma membrane by John (1977) and by Kristjansson *et al.* (1978). The nitrate reductase from *P. denitrificans* once isolated can reduce chlorate as effectively as nitrate (Forget, 1971). John (1977) observed that intact cells do not reduce chlorate unless Triton X-100 is added, whereas inside-out membrane vesicles of *P. denitrificans* reduce chlorate and nitrate at similar rates. In consequence, John (1977) suggested that nitrate gains access to its binding site from the cytoplasmic face of the plasma membrane and that a nitrate carrier (unable to transport chlorate) permits nitrate to cross the membrane. A more extended analysis of this question has very recently been given by Alefounder and Ferguson (1980).

The evidence of Kristjansson *et al.* (1978) is more indirect. It is based on the stoichiometry of proton and nitrate uptake in oxidant pulse experiments. In *Paracoccus denitrificans, Pseudomonas denitrificans* (Kristjansson *et al.*, 1978), and *E. coli* (Kristjansson and Hollocher, 1979) the small transient alkalinization observed in the absence of valinomycin during nitrate pulse studies was attributed to a proton uptake linked to nitrate uptake. From this type of evidence, these authors concluded that the substrate site must lie on the inner face of the membrane.

More direct evidence for this topography comes from the work of MacGregor and Christopher (1978), who examined the nitrate reductase proteins that were accessible to a surface labeling probe after labeling the inner and outer surfaces of the cytoplasmic membrane of *E. coli*. For surface labeling they used transglutaminase to couple dansyl cadaverine to membrane proteins. None of the three subunits (A, B, C) was labeled when nitrate reductase was isolated from dansyl cadaverine-labeled spheroplasts. When nitrate reductase was isolated from labeled French press vesicles (assumed to be inverted vesicles), subunit A was labeled and labeling was enhanced by the presence of nitrate during labeling. It was concluded that subunit A, which contains the active site of the enzyme (MacGregor, 1975), is exposed only on the inner surface of the membrane.

Other experiments based on lactoperoxidase-catalyzed radioiodination have shown that nitrate reductase spans the membrane of *E. coli* (Boxer and Clegg, 1975).

3. *Nitrite Reductase*

a. Nature of Respiratory Nitrite Reductases. The nitrite reductase of *Paracoccus* is a soluble multiheme enzyme called cytochrome cd_1 (Lemberg and Barrett, 1973) (EC 1.9.3.2); it is totally repressed in aerated cultures even in the presence of nitrate (Newton, 1969; Lam and Nicholas, 1969c). The enzyme was reported to be present in *Pseudomonas aeruginosa* (Yamanaka and Okunuki, 1963), *Alcaligenes faecalis* (Iwasaki and Matsubara, 1971), *Thiobacillus denitrificans* (Sawhney and Nicholas, 1978), and *Pseudomonas perfectomarinus* (Zumft *et al.*, 1979).

Another type of respiratory nitrite reductase containing two copper atoms per mole and no iron (EC 1.7.99.3) is present in *Achromobacter cycloclastes* (Iwasaki and Matsubara, 1972; Iwasaki *et al.*, 1975), *Ps. denitrificans* (*Alcaligenes sp.*) (Iwasaki *et al.*, 1963), and (EC 1.7.2.1) in *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* (Sawada *et al.*, 1978).

Other nitrite reductases, such as the cytochrome-*c*-containing system of *Achromobacter fischeri* (Prakash and Sadana, 1972) or the nitrite reductase (EC 1.6.6.4) of *E. coli* described by Coleman *et al.* (1978), which consists of a flavoprotein and contains no heme, are able to reduce nitrite and hydroxylamine to ammonia and are therefore assimilatory rather than respiratory enzymes.

b. Physicochemical and Catalytic Properties of Cytochrome cd_1. The *Pseudomonas* enzyme, often called *Pseudomonas* cytochrome oxidase, has been studied in detail and has been shown to consist of a

protein of MW 120,000 with two identical subunits, each of which contains one *c*-type and one *d*-type heme (Gudat *et al.*, 1973; Kuronen *et al.*, 1975). The enzyme can transfer electrons from reduced cytochrome *c* and artificial donors to nitrite or oxygen as electron acceptor [ferrocytochrome *c*:nitrite (or oxygen) oxidoreductase, EC 1.9.3.2]. In the cytochrome cd_1 molecule, heme *c* is reduced first and d_1 is subsequently reduced. Electrons are then transferred to the final electron acceptor, oxygen or nitrite (Wharton *et al.*, 1973).

In vivo the primary role of the *Paracoccus* enzyme is nitrite reduction since it is synthesized only when denitrification is taking place and is repressed in the presence of oxygen (Newton, 1969; Lam and Nicholas, 1969c). Although the reduction of O_2 by nitrite reductase probably has no physiological importance (Yamanaka, 1964), the *Paracoccus* cytochrome cd_1 does have cytochrome oxidase activity, but its affinity for O_2 ($K_m \sim 27$ μM) is much lower than that of cytochrome *c* oxidase ($K_m < 0.1$ μM) (Lam and Nicholas, 1969c). Timkovich and Robinson (1979) demonstrated that water is the product of reduction of O_2 by showing that the rate of ferrocytochrome oxidation by cytochrome cd_1 is four times the rate of O_2 reduction. In other words cytochrome cd_1 acts like cytochrome oxidase by transferring directly four electrons to O_2. These authors observed no effect of superoxide dismutase, catalase, and peroxidase on the rate of ferrocytochrome *c* oxidation, consistent with the view that superoxide anion or hydrogen peroxide are not formed as intermediates in O_2 reduction.

Recently Robinson *et al.* (1979) studied the kinetics of reoxidation of ferrocytochrome *c* by nitrite and molecular oxygen in order to investigate whether the kinetic behavior of cytochrome cd_1 is analogous to that of cytochrome aa_3. Indeed cytochrome cd_1 has similarities with mitochondrial cytochrome oxidase (similarities between heme *d* and *a*, specificity for cytochrome *c* as electron donor and O_2 as electron acceptor). Robinson *et al.*, (1979) have proposed an integrated rate law for the oxidation of ferrocytochromes *c* by the nitrite reductase of *P. denitrificans* using either nitrite or molecular oxygen as electron acceptor. For this purpose they have used synthetic deazaflavin as an *in situ* photoreductant of cytochrome *c*. Either cytochrome c_{550} from *Paracoccus* or horse cytochrome *c*, which reacts with cytochrome cd_1 with 77% efficiency, were used as electron donors. It was concluded that the kinetics of ferrocytochrome *c* oxidation are more complex with cytochrome cd_1 than with mitochondrial cytochrome oxidase. This is not unexpected since NO, the presumed product of nitrite reduction by one-electron transfer, can bind to both hemes (at acidic pH between 4 and 7) or to ferroheme d_1 (at pH 8 and 9) or, in other words can exert product inhibition (Silvestrini

et al., 1979). The high affinity of NO for heme components renders NO highly toxic for the cell and, as pointed out by Robinson *et al.* (1979), it is probable that cytochrome cd_1 is regulated by strict control mechanisms.

In *Pseudomonas* species, the electron donor to nitrite reductase may be a blue copper protein, azurin. In *Paracoccus* a blue chromophore was observed by Newton (1967) during one of the steps of nitrite reductase purification although its nature was not established. It is only recently that Martinkus *et al.* (1980) purified this blue component and identified it as an azurin or type I Cu protein. This protein isolated from cells grown anaerobically to the late stationary phase exhibits a broad absorption band at 595 nm in the oxidized form. The purified enzyme has a molecular weight of 13,790 and a half-reduction potential of +230 mV and contains one Cu atom per mole (Martinkus *et al.*, 1980). The pure protein was shown to have a five times greater electron transport activity with membrane fragments from aerobically grown cells than with isolated nitrite reductase. The authors therefore could not ascribe a predominant role for azurin as a direct physiological electron donor for nitrite reductase, nor could they assign it a role in aerobic respiration.

c. The Intermediate Products NO and N_2O. Whereas the assimilatory nitrite reductase [the iron flavoprotein NAD(P)H nitrite reductase] transfers six electrons to nitrite and reduces it directly to ammonia (cf. Losada, 1975–1976), the respiratory nitrite reductase is thought to produce first nitric oxide, by a one-electron transfer. Whether nitric oxide is produced as a free intermediate during the reduction of nitrite to nitrous oxide is controversial.

Release of NO by soil denitrifying bacteria has been reported to occur at acid pH (Garcia, 1973). Whole cells of *Corynebacterium nephridii* incubated at a physiological pH produce nitric (and nitrous) oxide in the presence of an elevated concentration of nitrite (Renner and Becker, 1970). A soluble fraction from cells of *Pseudomonas perfectomarinus* reduces nitrite stoichiometrically to nitric oxide (Payne *et al.*, 1971), and formation of a heme–NO complex could be demonstrated by EPR measurements when a nitrite reductase preparation from *Ps. perfectomarinus* was incubated with NADH, flavins, cytochrome *c*, and nitrite (Cox *et al.*, 1971). Nitric oxide was also shown to be formed during nitrite reduction by the isolated nitrite reductase from *Ps. aeruginosa* (Yamanaka *et al.*, 1961; Gudat *et al.*, 1973), *Ps. perfectomarinus* (Cox and Payne, 1973), and *A. faecalis* (Matsubara and Iwasaki, 1971).

More recently, Rowe *et al.* (1977) reported the presence of an NO-binding protein in crude extracts (5000 *g* supernatant) from *Ps. aeruginosa*. This protein was identified spectrophotometrically from the

appearance of absorption maxima (542 and 573 nm) in difference spectra of NO-treated versus untreated samples. The authors observed a decrease in absorbance at 573 nm upon prolonged incubation with NO. The absorbance decrease was accelerated by addition of malate and accompanied by the formation of N_2O. Rowe *et al.* concluded that the identified NO-binding protein fulfills a physiological role in the reduction of nitrite to N_2O. Zumft *et al.* (1979) have repeated the same experiments with extracts from *Ps. perfectomarinus*. The difference spectrum of a NO- (or nitrite-)treated cell-free extract of *Ps. perfectomarinus* versus the untreated extract was similar to that of *Ps. aeruginosa* (Rowe *et al.*, 1977). Zumft *et al.* (1979) identified this spectrum as cytochrome cd_1. They pointed out that in the untreated extract cytochrome cd_1 was being reduced by endogenous substrates and then reoxidized upon addition of NO or nitrite, which act as electron acceptors. In other words, the observed difference spectrum was actually that of oxidized heme *c* (with "troughs" at 551 and 522 nm) and oxidized heme *d* (with "troughs" at 467 and 660 nm) against reduced cd_1. Appearance of a peak at 572 nm accompanying oxidation of cytochrome cd_1 from *Thiobacillus denitrificans* by nitrite has been observed by Sawhney and Nicholas (1978).

Using purified cytochrome cd_1 from *Ps. perfectomarinus*, Zumft *et al.* (1979) demonstrated that nitrite was reduced to NO as the main product with ascorbate plus phenazine methosulfate (PMS) as electron-donating system. That NO is really an intermediate in the reduction of nitrite into N_2 has been questioned by St. John and Hollocher (1977). These authors observed that while both $^{15}NO_2^-$ and ^{14}NO were used as terminal electron acceptors by whole cells of *Ps. denitrificans* insufficient amounts of $^{14}N^{15}N$ were formed to prove that NO is an intermediate. However, Firestone *et al.* (1979) in label exchange studies observed that $[^{13}N]NO_2^-$ rapidly exchanged with unlabeled NO during denitrification in *Pseudomonas aureofaciens* and *Pseudomonas chlororaphis*. These results suggest that NO is an intermediate, or is in rapid equilibrium with an intermediate, in the sequence of reactions.

Zumft and Cárdenas (1979) speculated that *in vivo* NO_2^- might be directly reduced to N_2O without release of free NO. They pointed out that reduction of nitrite to NO involves the transfer of only one electron whereas an even number of electrons are transferred in the reactions of nitrate respiration and denitrification. Furthermore, reduction of nitrite to N_2O would correspond to a four-electron transfer analogous to the four-electron reduction of oxygen by nitrite reductase (Timkovich and Robinson, 1979). Zumft and Vega (1979) have isolated from *Ps. perfectomarinus* a membrane fraction which reduced nitrite to nitrous oxide stoichiometri-

cally without nitric oxide as free intermediate. They tentatively assigned this activity to tightly bound cytochrome cd_1 (representing only a small portion of the total cytochrome cd_1 of the cell). The membrane system had a specific requirement for FMN with NAD(P)H as electron donors. Other electron donors were ascorbate-reduced cytochrome c_{551} or PMS.

d. Localization of Nitrite Reductase in the Cell. Conflicting results have been obtained for the localization of nitrite reductase. Saraste and Kuronen (1978) prepared ferritin-conjugated antibodies to cytochrome cd_1 from *Ps. aeruginosa* and observed by electron microscopy these antibodies bound to *Pseudomonas* spheroplasts, cell ghosts, and plasmolyzed cells. In all cases the label appeared to bind to the inner surface of the cytoplasmic membrane whereas no labeling was observed when membranes or spheroplasts were prepared from nir mutants of *Ps. aeruginosa* which have no nitrite reductase activity. Similarly to Saraste and Kuronen (1978), Kristjansson *et al.* (1978) placed the nitrite reducing site of *P. denitrificans* in the cytoplasm or on the inner aspect of the cytoplasmic membrane. These authors performed oxidant pulse experiments on *P. denitrificans* cells grown anaerobically on nitrate with nitrate or nitrite as electron acceptors. Upon addition of nitrate (or nitrite) they observed a transient alkalinization with a $\rightarrow H^+$/anion ratio of -1 (maximally), which they attributed to proton-coupled anion transport inside the cell. From this stoichiometry ($\rightarrow H^+$/anion ~ -1) and from the fact that following nitrate addition they observed a temporary drop in pH between two transitory increases in pH, they concluded that nitrate and nitrite are taken up (and nitrite released with one proton), indicating that both nitrite and nitrate are reduced inside the cell. This conclusion is also based on the interpretation of the effect of FCCP. Oxidation of endogenous substrates by nitrite was shown to produce a rapid proton efflux from the cell, whereas in the presence of FCCP addition of nitrite resulted in a rapid alkalinization of the medium. Kristjansson *et al.* (1978) attributed the effect of FCCP to inhibition of nitrite (or nitrate) reduction only. On the other hand, Meijer *et al.* (1979b) from similar results considered that the effect of FCCP resulted mainly from its uncoupling action and that the alkalinization of the medium was due to the consumption of scalar protons accompanying the reduction of NO_2^- to N_2. To avoid interference with protons ejected outward when endogenous substrates are oxidized by nitrite, Meijer *et al.* (1979a) used the couple ascorbate + TMPD as electron donor to cytochrome *c* and nitrite reductase. Pulses of small (nonuncoupling) amounts of nitrite (5 nmol) produced a rapid alkalinization of the suspending medium. When aerobically grown cells that lacked nitrite reductase (Newton, 1969; Lam and Nicholas, 1969c) were used, no

pH change was observed. The speed of proton disappearance suggested to these authors that protons were consumed on the outer face of the cytoplasmic membrane. Furthermore, it was calculated from reaction (1) (A standing for ascorbate)

$$1.5\ AH^- + NO_2^- + 2.5\ H^+ \rightarrow 1.5\ A + 0.5\ N_2 + 2\ H_2O \qquad (1)$$

that the stoichiometry of proton uptake for complete reduction of NO_2^- to nitrogen gas, with ascorbate donating two electrons and one proton at neutral pH, should be 2.5 g-ion of H^+ per mole of nitrite consumed. This is what was found experimentally. Therefore Meijer *et al.* (1979a) concluded that the observed proton uptake resulted from the consumption of scalar protons and that the nitrite reductase of *P. denitrificans* is periplasmic (Fig. 5).

It should be noted that in the studies of Kristjansson *et al.* (1978) and Meijer *et al.* (1979a) only proton movement (or consumption) was measured. If the concomitant consumption of nitrate and nitrite had been measured, the conclusions of the authors would have been more firmly established.

Wood (1978) reported that the nitrite reductase of *Ps. aeruginosa* is in the periplasmic space. This author measured the distribution of marker enzymes together with nitrite reductase in different cellular fractions obtained after lysozyme–EDTA treatment and osmotic shock of the cells.

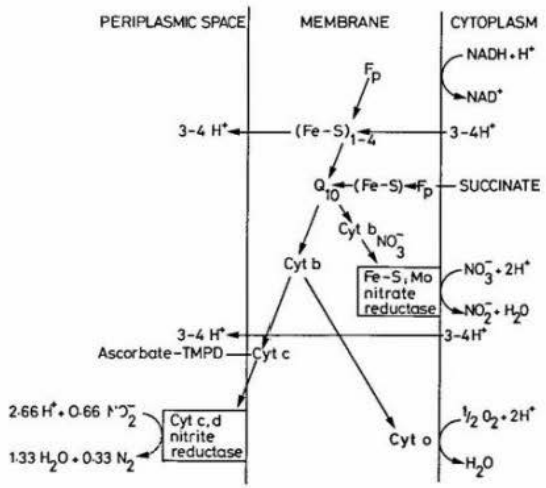

FIG. 5. Localization of the site of reduction of nitrite on the plasma membrane of *Paracoccus denitrificans*. The figure also indicates the stoichiometry of respiration-driven proton translocation for anaerobically grown *P. denitrificans*. From Meijer *et al.* (1979a).

Nearly all of the nitrite reductase and azurin and most of cytochrome c_{551} were recovered in the periplasmic fraction, where only 1% of the isocitrate dehydrogenase (a marker for the cytoplasm) was found. Although this result is in agreement with our own data (see below) it should be noted that Wood (1978) did not measure nitrite reductase activity but assessed the presence of cytochrome cd_1 from the absorbance of the pyridine hemochrome of heme d_1 (at 620 *minus* 680 nm). Heme d_1 is not bound covalently to the protein and may be released, for example, at acidic pH. The same results, as reported by Wood (1978), might have been obtained if during the incubation of spheroplasts (30 minutes at 30°C) which preceded centrifugation, heme d_1 was detached from cytochrome cd_1 and released from the cells.

Similarly, M.-F. Henry and J. Doussière (1981) concluded that nitrite reductase interacts with the outer face of the cytoplasmic membrane and the binding site of nitrate to the membrane-bound nitrate reductase lies on the inner side of the cell. The evidence on which these conclusions are based was obtained with particles prepared from cells grown anaerobically on succinate and nitrate and harvested at the stationary phase. At that stage cells contain nitrite reductase, which remains in the 100,000 *g* supernatant fraction after centrifugation of a sonicated cell homogenate. Sonicated particles are apparently 100% inverted since they have a high ATPase activity, which is not increased by permeabilizing treatments (1% toluene or bee venom) (Porte, 1979). These particles oxidize NADH with nitrate as electron acceptor. This oxidation is accompanied by an alkalinization of the medium; it was found experimentally that 1.4 H^+ were consumed per mole of nitrate reduced (to nitrite) (Fig. 6a). When nitrite was added, instead of nitrate, the particles could not oxidize NADH even in the presence of the 100,000 *g* supernatant containing nitrite reductase (Fig. 6b). Oxidation of NADH by nitrite was observed, however, when PMS was added to mediate electron transfer from the membranes to nitrite reductase (Fig. 6c). A H^+/NO_2^- ratio of 3.9 was measured. These protons consumed in the bulk medium are the scalar protons involved in the reduction of NO_2^- to N_2 according to reaction (2).

$$NO_2^- + 4\,H^+ + 3\,e^- \rightarrow 0.5\,N_2 + 2\,H_2O \qquad (2)$$

This type of experiment with inverted vesicles shows that, while nitrate has direct access to its binding site on the membrane, nitrite reductase has not. Therefore the two binding sites are on opposite sides of the cytoplasmic membrane. The site on nitrate reductase to which nitrate binds is on the inner face, and the membrane component reacting with nitrite reductase lies on the outer face.

These data were corroborated by the following experiments. Cells

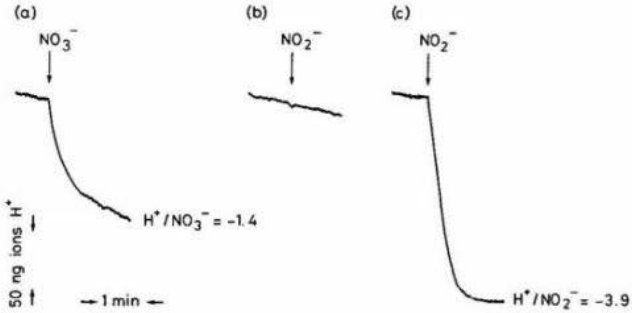

FIG. 6. Alkalinization of the bulk medium following nitrogen oxide pulses in membrane particles from *Paracoccus denitrificans*. Membrane particles were prepared by sonication of anaerobically grown cells harvested at the stationary phase of growth. The reaction chamber contained 2.75 ml of 0.3 *M* sucrose and 0.1 *M* KCl, 1.3 μg of valinomycin and 3 μg of oligomycin per milliliter, and 0.5 ml of membrane particles at a concentration of 13 mg/ml. The suspension was kept anaerobic under a stream of argon. (a) NADH (2 m*M*) was present in the medium. At the arrow KNO_3 (50 nmol) was introduced. (b) In addition to NADH (2 m*M*), the medium also contained the 100,000 *g* supernatant obtained after centrifugation of the homogenate. The reaction was initiated with KNO_2 (30 nmol). (c) Similar conditions as in (b) except that phenazine methosulfate (25 μM) was present in the medium. KNO_2 (50 nmol) started the reaction. From M.-F. Henry and J. Doussière (1981).

grown on succinate and nitrate and harvested at the stationary phase were used to prepare spheroplasts. With such spheroplasts oxidation of endogenous substrates yielded $\leftarrow H^+/O = 5$ and $\leftarrow H^+/NO_3 = 3.9$ (Table VI). Reduction of NO_2^- was marked by a transient alkalinization of the suspending medium but resulted in a $H^+/NO_2 \sim 0$. When the uncoupler FCCP (5 μM) was added before nitrite, the alkalinization of the medium remained stable and accounted for a proton consumption of 3.5 H^+ per mole of nitrite (Fig. 7). It is concluded that FCCP, by abolishing the vectorial protons linked to the oxidation of endogenous substrates, enabled measurement of only the scalar protons involved in reaction (2). In the absence of uncoupler, the protons ejected outward almost compensate for the uptake of scalar protons, so that a $\rightarrow H^+/NO_2^- \sim 0$ is obtained. Finally, Alefounder and Ferguson (1980) have shown that it is possible to prepare $\geq 99\%$ intact spheroplasts from *P. denitrificans* that lack NO_2^- reductase activity, providing powerful evidence that this enzyme is on the outer aspect of the cytoplasmic membrane (or in the periplasmic space) *in vivo*.

It has already been reported by Lawford *et al.* (1976) that the stoichiometry of respiration-driven proton translocation varies during the growth cycle. Oxidation of endogenous substrates yielded $\leftarrow H^+/O$ ratios of 5.8–6 in the early exponential phase and this dropped to between 4 and

TABLE VI

STOICHIOMETRIES OF PROTON TRANSLOCATION FOR CELLS OF *Paracoccus denitrificans* GROWN ANAEROBICALLY

Growth of cells								
Substrate	Terminal electron acceptor	Type of cells	Substrate	$\leftarrow H^+/O$	$\leftarrow H^+/NO_2^-$	$\leftarrow H^+/NO_3^-$	$\leftarrow H^+/N_2O$	References
Succinate limited	NO_3^-	Chemostat	Endogenous	4.5	—	—	—	van Verseveld *et al.*, 1977
			Succinate + rotenone	3.4	—	—	—	van Verseveld *et al.*, 1977
Sulfate limited	NO_3^-	Chemostat	Endogenous	3.4	—	—	—	van Verseveld *et al.*, 1977
			Succinate + rotenone	3.4	—	—	—	van Verseveld *et al.*, 1977
Succinate	NO_2^-	NO_2^--limited	Endogenous	3.5	—	—	—	van Verseveld *et al.*, 1977
Succinate	NO_3^-	Log phase	Endogenous	7.5	6.9	—	—	Meijer *et al.*, 1979a
			Succinate	4.3	2.5	—	—	Meijer *et al.*, 1979a
	NO_3^-	Chemostat	Endogenous	7.8	7.1	—	—	Meijer *et al.*, 1979a
			Succinate	4.5	2.6	—	—	Meijer *et al.*, 1979a
	NO_3^-	Mid-log phase	Endogenous	7.5	3.7	4.3	4.5	Kristjansson *et al.*, 1978
Succinate	NO_3^-	Early exponential phase (spheroplasts)	Endogenous	12	—	8.4	—	M.-F. Henry and J. Doussière (1981)
			Endogenous + antimycin	8.4	—	8.2	—	M.-F. Henry and J. Doussière (1981)
			Endogenous + antimycin + azide	8.1	—	0	—	M.-F. Henry and J. Doussière (1981)
	NO_3^-	Mid-log phase (spheroplasts)	Endogenous	9.1	—	—	—	M.-F. Henry and J. Doussière (1981)
			Endogenous + rotenone	6.4	—	—	—	M.-F. Henry and J. Doussière (1981)
			Endogenous + rotenone + antimycin	6.1	—	—	—	M.-F. Henry and J. Doussière (1981)
	NO_3^-	Stationary phase (spheroplasts)	Endogenous	4.5–5	~0	3.5–3.9	—	M.-F. Henry and J. Doussière (1981)
			Endogenous + FCCP	~0	−3.9	−0.4	—	M.-F. Henry and J. Doussière (1981)

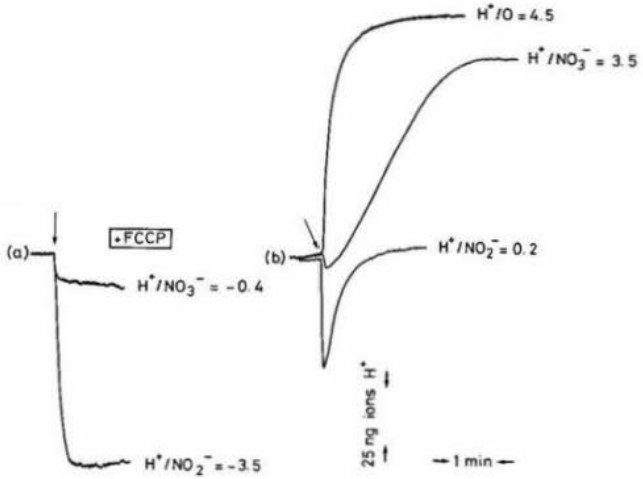

FIG. 7. Outward proton ejection in spheroplasts from anaerobically grown *Paracoccus denitrificans*. The cells were harvested at the stationary phase and spheroplasts were prepared according to Sim and Vignais (1978). One hundred fifty microliters of spheroplast suspension (50 mg/ml) were added to the medium described in Fig. 6. (a) Either KNO_3 or KNO_2 (30 nmol) was added at the arrow. Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) was present at a concentration of 5 μM. (b) Proton release in the outer medium upon addition of either O_2 (20 ng-atoms in air-saturated KCl solution), KNO_2 (25 nmol) or KNO_3 (25 nmol in the presence of 25 nmol of KNO_2). No difference in the stoichiometry of $\leftarrow H^+/NO_3^-$ was oberved when nitrite was omitted, although the rate of protons translocated increased from 0.3 ng-ion of H^+ per second in the absence of nitrite to 1 ng-ion of H^+ per second in the presence of nitrite. From M.-F. Henry and J. Doussière (1981).

5 in the late log phase and then to 3 in the stationary phase. They interpreted these data to show that in cells harvested in the early phase of growth a proton translocating transhydrogenase (EC 1.6.1.1) and a proton translocating NADH dehydrogenase are active and at the end of the growth cycle these two activities are modified so that there is no longer any associated proton translocation.

With cells grown anaerobically on succinate and nitrate, the $\leftarrow H^+/O$ ratio linked to the oxidation of endogenous substrate dropped from 12 (early exponential phase) to 9 (mid log phase) and to 4.5 (stationary phase) (Table VI). Cells harvested at the early phase of growth do not contain nitrite reductase, and nitrate is converted merely to nitrite. At the stationary phase the $\leftarrow H^+/NO_3^-$ ratios obtained (3.5) were not changed by the presence of antimycin; this means that electrons are transferred from cytochrome *b* to nitrate reductase before the antimycin-inhibited site (Table VI).

e. Nitrate and Nitrite Transport. If nitrate is reduced inside the cell and nitrite is reduced in the periplasmic space, the two anions have to cross the membrane in opposite directions. Kristjansson *et al.* (1978) observed a transient alkalinization with a $\rightarrow H^+/\text{anion} = -1$ upon addition of nitrite or nitrate to cells of *P. denitrificans*. They assumed that this alkalinization reflected proton uptake accompanying inward transport of nitrate or nitrite.

In agreement with the postulate of a H^+/NO_3^- symport (Kristjansson and Hollocher, 1979), M.-F. Henry and J. Doussière (1981) observed a proton uptake (instead of a proton ejection) with a stoichiometry of $\rightarrow H^+/NO_3^- = -0.4$ when spheroplasts were pulsed with nitrate in the presence of FCCP (Fig. 7a). In these conditions the scalar protons consumed inside the spheroplast for the reduction of nitrate to nitrite are not detected in the bulk phase, and the vectorial protons linked to nitrate reduction are not observed; only the protons linked to NO_3^- transport are seen.

With a nitrite pulse an immediate consumption of four protons is recorded, indicating that only the scalar protons of reaction (2) are measured (Fig. 7a). It appears therefore that a H^+/NO_3^- symport importing nitrate and a H^+/NO_2^- symport exporting nitrite may be functioning in the membrane. Under steady-state conditions, the coupling of these two transporters results in an overall electroneutral exchange of NO_3^- against NO_2^- as a NO_3^-/NO_2^- antiporter would do (cf. also Kristjansson and Hollocher, 1979; Alefounder and Ferguson, 1980).

The rate of proton release in oxidant pulse experiments (Fig. 7b) indicates that such transporters may be functioning. Upon addition of nitrate alone, the rate of proton ejection (0.3 ng-ions of H^+ per second) was slow compared to the rate observed after an oxygen pulse (9 ng-ions of H^+ per second) and seemed to be diffusion-limited. When nitrite was added prior to nitrate, the rate of proton ejection increased more than threefold. It is shown in Fig. 7b that, in the absence of the uncoupler FCCP, reduction of nitrite yields an H^+ balance of practically zero; nitrite reduction does not contribute, therefore, to the acidification of the medium resulting from nitrate reduction. In the presence of nitrite the rate of proton ejection during nitrate reduction is enhanced; it is attributed to the increase in the rate of nitrate entry into the spheroplast. These results are in agreement with the assumption that nitrate entry into the cell is facilitated by exchange against nitrite.

4. Nitric Oxide Reductase

Very little is known about the enzyme nitric oxide reductase, and no data are available for this enzyme in *P. denitrificans*. Indeed some

confusion exists even about the exact reaction catalyzed by this enzyme. According to Payne (1976) nitric oxide reductase is the enzyme that catalyzes the conversion of nitric oxide into nitrous oxide. According to other authors nitric oxide reductase is the enzyme (EC 1.7.99.2) that reduces NO to nitrogen gas. The distinction between the two enzymic activities was not clear in early publications. The metalloprotein purified from the soluble fraction of *Ps. aeruginosa* (Fewson and Nicholas, 1960) probably catalyzes the reduction of NO to N_2 (i.e., EC 1.7.99.2). Nitric oxide reductase has been identified as a soluble, cytochrome-*c*-containing, system in *Ps. perfectomarinus* (Cox and Payne, 1973) and as cytochrome cd_1 in *A. faecalis* (Matsubara and Iwasaki, 1972).

In *A. faecalis,* nitric oxide reductase activity was detected in a particulate fraction (Matsubara and Iwasaki, 1972) that reduced NO to N_2O when ascorbate–PMS was the electron donor. The membrane-bound NO reductase and the NO-reducing activity of cytochrome cd_1 of *A. faecalis* had the same pH optimum (5.5), and both were inhibited by cyanide, azide, or diethyldithiocarbamate, but the NO-reducing activity of the particulate enzyme was inhibited by more than 20% of NO in the gas phase, and that of cd_1 was not saturated even at 60% of NO in the gas phase (Matsubara and Iwasaki, 1972).

Owing to NO toxicity, it has not been possible to grow bacteria with NO as added terminal electron acceptor as was done with nitrate, nitrite, or nitrous oxide (Pichinoty, 1973; Payne, 1973). It was not possible therefore to demonstrate that an organism can gain energy by growth on NO. The membrane-bound cytochrome cd_1 described in *A. faecalis* (Matsubara and Iwasaki, 1972) and in *Ps. perfectomarinus* (Zumft and Vega, 1979) may be expected to couple respiration to phosphorylation. The soluble cytochrome cd_1 may have the physiological function of removing excess of nitrite known to be toxic for the cell (Vogt, 1965), perhaps in part by its uncoupling effect (Meijer *et al.*, 1979b), and, if it has a nitric oxide reductase activity, to act as scavenger of NO.

5. *Nitrous Oxide Reductase*

Nitrous oxide reductase reduces N_2O to N_2. It is a membrane-bound enzyme involving *c*-type cytochromes in N_2O reduction (Matsubara, 1975; Boogerd *et al.*, 1980). The synthesis of N_2O reductase activity is quickly derepressed by lowering the oxygen tension of suspensions of *Ps. perfectomarinus* even in the absence of nitrogen oxides (Payne *et al.* 1971; Payne and Balderston, 1978). The stoichiometric reduction of N_2O to N_2 with formate as electron donor produces energy for growth of *Vibrio succinogenes* (Yoshinari, 1980).

The biochemistry of nitrous oxide reductase should be greatly enhanced by the discovery of a specific inhibitor, namely acetylene, that inhibits the reduction of N_2O in *Ps. perfectomarinus* (Balderston *et al.*, 1976), other *Pseudomonas* species (Yoshinari and Knowles, 1976) and denitrifying bacteria including *P. denitrificans* (Payne and Balderston, 1978). Sulfide (0.3 m*M*) has also been shown to inhibit strongly N_2O reduction in *Ps. fluorescens* resting cells and in other denitrifiers (*A. faecalis, Flavobacterium, Ps. aeruginosa*) (Sørensen *et al.*, 1980). However, H_2S may not be as specific as acetylene, since reduction of NO is also partially inhibited.

Isolation and purification of nitrous oxide reductase has been hampered by a rapid loss of activity upon cell breakage (Payne *et al.*, 1971; Cox and Payne, 1973; Matsubara, 1975; Garcia, 1977; Kristjansson and Hollocher, 1980).

In *P. denitrificans*, N_2O has been shown to support anaerobic respiration, growth (Pichinoty, 1973; Pichinoty *et al.*, 1977), respiratory-linked proton translocation (Kristjansson *et al.*, 1978), and the generation of a proton-motive force (McCarthy *et al.*, 1981). The oxidation of the cationic methyl or benzyl viologen radicals upon reduction of N_2O to N_2 by cell-free extracts has been proposed as a convenient spectrophotometric assay of N_2O reductase activity (Kristjansson and Hollocher, 1980). The K_m for N_2O was about 5 μM and the apparent K_m for reduced benzyl and methyl viologen was 0.9 and 0.5 μM, respectively. N_2O reductase was inhibited by acetylene (K_i = 28 μM), CO (K_i = 3.5 μM), azide (K_i = 0.35 μM), and cyanide (K_i = 0.045 μM). All these compounds were noncompetitive inhibitors of N_2O reduction. It should be noted that for this study Kristjansson and Hollocher (1980) used lysed spheroplasts, not membrane-free supernatant; therefore it is not clear whether N_2O reductase activity was really soluble as claimed by the authors.

C. Energy Yield of Denitrification

Phosphorylation coupled to nitrate reduction (to nitrite) was demonstrated in *Pseudomonas denitrificans, Ps. aeruginosa* and *Paracoccus* (*Micrococcus*) *denitrificans* (Yamanaka *et al.*, 1962; Ohnishi, 1963; Naik and Nicholas, 1966).

Using membrane particles of *Paracoccus denitrificans*, John and Whatley (1970) observed that oxidative phosphorylation coupled to O_2 uptake is about 70% more efficient than that coupled to nitrate reduction. With NADH as electron donor they obtained a P/O ratio of 1.5 and a P/NO_3^- ratio of 0.9.

Koike and Hattori (1975a) used growth yield as an index of the efficiency of anaerobic respiration on nitrate with *Pseudomonas denitrificans* which, like *Paracoccus denitrificans,* lacks the capacity for fermentation. On glutamate as the sole source of energy and carbon, the maximum growth yield (corrected for so-called maintenance energy) on nitrate was about 60% of that on oxygen ($Y^{max}_{nitrate} = 0.58\ Y^{max}_{O_2}$) both in batch cultures and in chemostat cultures under glutamate-limited conditions, conditions where glutamate is completely oxidized. For Koike and Hattori (1975a) this lower yield obtained under anaerobiosis indicated that respiration on nitrate is associated with a smaller number of phosphorylating sites than aerobic respiration.

In subsequent experiments with electron acceptor-limited continuous cultures, Koike and Hattori (1975b) examined which steps of the denitrification sequence are coupled to oxidative phosphorylation. [Under electron acceptor-limited conditions, the electron flux through the denitrifying respiratory systems controls the overall process of bacterial growth (Senez, 1962).] For this purpose *Pseudomonas denitrificans* was grown with nitrate, nitrite, or nitrous oxide as terminal electron acceptor. The molar growth yields were 28.6, 16.9, and 8.8 g/mol with nitrate, nitrite, and nitrous oxide, respectively. As pointed out by Koike and Hattori (1975b), these yields are proportional to the oxidation number of nitrogen in nitrate (+5), nitrite (+3), or N_2O (+1) (Table V). It is therefore concluded that there is no difference in the extent of coupling or in the number of phosphorylation sites associated with the reduction of nitrate to nitrite, nitrite to nitrous oxide, and nitrous oxide to nitrogen. Since NO_2^- and N_2O are reduced by a *c*-type cytochrome (see above), it seems actually that a smaller number of energy-transducing sites must be used for respiration on nitrogen oxides compared to O_2 respiration.

Van Verseveld *et al.* (1977) reached similar conclusions. They calculated P/2*e* ratios for anaerobic chemostat cultures of *Paracoccus denitrificans* using nitrate or nitrite as electron acceptor and succinate as electron donor. The complete dissimilation of succinate corresponds to the oxidation of 5 NADH and 2 FADH. With nitrate, the P/2*e* values were 0.5 and 0.7 for the sulfate- and the succinate-limited cultures, respectively. The nitrite-limited culture yielded a P/2 *e* of 0.9. Assuming two coupling sites, the authors calculated that the theoretical P/2*e* ratio for the oxidation of succinate should be 1.31 and 1.71 with nitrate and nitrite, respectively. Assuming that only site II was functioning, they calculated theoretical P/2 *e* values of 0.60 (for nitrate reduction) and 1.00 (for nitrite reduction). With these types of cells, van Verseveld *et al.* (1977) determined the $\leftarrow H^+/O$ ratios for the oxidation of endogenous substrates. They

observed that only 3 or 4 protons were released by these anaerobically grown cells instead of 7 or 8 yielded by aerobic cells (Table VI). Taking into account the conclusions of Meijer *et al.* (1977a) that the $\leftarrow H^+$/site ratio during electron transport is 3–4 and that sulfate limitation induces the loss of the iron–sulfur protein in the site I region, van Verseveld *et al.* (1977) concluded that only site II is present under sulfate and nitrite limitation and that regulation of energy recovery is effected by loss of site I.

M.-F. Henry and J. Doussière (1981) obtained an $\leftarrow H^+/O$ ratio of 12 with cells grown on succinate and nitrate and harvested at the early exponential phase (Table VI). Such an $\leftarrow H^+/O$ ratio may be attributed to either four sites with 3 H^+, assuming that transhydrogenase is more active at the beginning of the logarithmic phase of growth (P. Mitchell, personal communication) or three sites with 4 H^+ per site.

According to the chemiosmotic theory (Mitchell, 1966, 1976a) respiration-linked energy transduction involves the generation of a membrane potential. More recently McCarthy *et al.* (1981) have shown that NO_2^- reduction is indeed coupled with the generation of a membrane potential (negative inside) in washed cell suspensions of *Paracoccus denitrificans*. Using membrane vesicles derived from anaerobically grown cells, Kell *et al.* (1978) were able to observe the generation of a proton-motive force linked to the oxidation of reduced TMPD, indicating that the third energy coupling site may still be present in anaerobically grown cells.

IV. Respiration in Autotrophically Grown Cells

Paracoccus denitrificans is a facultative chemolithotrophic hydrogen bacterium able to grow autotrophically on $H_2 + O_2 + CO_2$ (Kluyver and Verhoeven, 1954b). Under these conditions CO_2 is assimilated via the Calvin cycle (Kornberg *et al.*, 1960). *Paracoccus denitrificans* has been shown also to grow aerobically on methanol or formate (and for some strains on methylamine) as sole carbon and energy sources (Cox and Quayle, 1975; Pichinoty *et al.*, 1977). These C_1 compounds are oxidized to CO_2 and then also assimilated via the ribulose-bisphosphate cycle (Cox and Quayle, 1975). The D-ribulose-1,5-bisphosphate carboxylase, which is not detectable in cells of *P. denitrificans* grown heterotrophically on acetate (Kornberg *et al.*, 1960) has been isolated from cells grown autotrophically on hydrogen (Bowien, 1977) and on methanol (Shively *et al.*, 1978).

Three active energy-conserving sites have been identified in cells grown autotrophically on $H_2 + CO_2 + O_2$ (Knobloch *et al.*, 1971; Porte and

Vignais, 1980) or on methanol (van Verseveld and Stouthamer, 1978a,b), but only two were observed in cells grown on formate (van Verseveld and Stouthamer, 1978b, 1980).

A. Electron Transport in Autotrophic Cells

1. The Branched Respiratory Chain

Cells of *P. denitrificans* grown autotrophically on methanol (van Verseveld and Stouthamer, 1978a) or on molecular hydrogen (Porte and Vignais, 1980) contain a branched respiratory chain where cytochrome aa_3 and cytochrome *o* act as terminal oxidases.

Membrane vesicles prepared from H_2-grown cells actively oxidize H_2, NADH, ascorbate, and to a lesser extent succinate (Knobloch *et al.*, 1971). H_2 oxidation is insensitive to rotenone (Knobloch *et al.*, 1971; Sim and Vignais, 1978). Porte and Vignais (1980) have shown that, with 100 nmol of rotenone per milligram of protein, H_2 oxidation remained unaltered whereas NADH oxidation was completely inhibited, indicating that the electron flow from H_2 enters the respiratory chain after NADH dehydrogenase or the rotenone-sensitive site.

The presence of a branched respiratory chain with two terminal oxidases in cell-free preparations from H_2-grown cells is indicated by the partial inhibition of NADH and succinate oxidase activities by antimycin A and by the biphasic titration curve obtained with KCN (Porte and Vignais, 1980). With KCN two sites are titrated that differ in their K_i. The first one, with a K_i of approximately 10 μM presumably corresponds to inhibition of cytochrome oxidase aa_3; the second site titrated (K_i = 1 mM) probably indicates the second oxidase, i.e., cytochrome *o*, which is much less sensitive to cyanide inhibition.

Curiously enough, H_2 oxidation proceeds only through the second electron transfer pathway to O_2 as indicated by its total insensitivity to the low concentrations of antimycin and cyanide, sufficient to block NADH and succinate oxidation via the cytochrome oxidase aa_3 pathway (Porte and Vignais, 1980). The substrates whose reducing equivalents enter the respiratory chain before the antimycin-sensitive site follow the two pathways to O_2.

Taken together these data suggest that the branching point of the two electron-transfer pathways may occur at the level of the *b*-type cytochromes, a proposal further substantiated by the observation that H_2 induces an extra reduction of cytochrome *b*-562 compared to the level reached in the NADH or succinate aerobic steady state.

On the basis of inhibitor and oxidative phosphorylation studies, Knobloch *et al.* (1971) also suggested the occurrence of a branched respiratory chain in their cell-free preparations of H_2-grown cells. However, these authors proposed the entry of the electrons from H_2 at the level of NADH dehydrogenase, cytochromes *b* and *c*, the branching point of the two respiratory pathways occurring at the cytochrome *c* level.

The scheme presented by Porte and Vignais (1980) for cells grown on molecular hydrogen (Fig. 1) is in agreement with the branched respiratory chain proposed by van Verseveld and Stouthamer (1978a) for methanol-adapted cells and by Willison and John (1979) for heterotrophically grown cells.

The oxidation of endogenous substrates mainly proceeds through the alternative, cytochrome *o* branch, that less sensitive to cyanide (K_i = 400 μM; 90% inhibition at 1.42 mM), whereas both *c*- and *a*-type cytochromes are involved in methanol oxidation (K_i for KCN = 115 μM) (van Verseveld and Stouthamer, 1978a).

2. *The Redox Components of the Respiratory Chain*

When growth of *P. denitrificans* proceeds autotrophically on H_2, methanol, or formate new dehydrogenases are induced, namely, hydrogenase, methanol dehydrogenase, formaldehyde dehydrogenase, and formate dehydrogenase. These components are normally not synthesized in the absence of the appropriate substrates. Formaldehyde and formate dehydrogenases are both NAD-linked (Cox and Quayle, 1975).

In addition to these dehydrogenases, some new redox carriers are also synthesized and are linked to the normal respiratory chain. A CO-binding *c*-type cytochrome is closely involved in methanol oxidation and is thought to act as electron acceptor for methanol in *P. denitrificans* (van Verseveld and Stouthamer, 1978a).

Besides cytochrome oxidase aa_3, autotrophically grown cells also possess a second oxidase, namely cytochrome *o* (van Verseveld and Stouthamer, 1978a; Porte and Vignais, 1980). Depending on the growth substrate (methanol or H_2) the electrons flow preferentially through one or another route of the branched respiratory system. When growth is carried out autotrophically on methanol, there is spectral evidence that the cells form more *a*-type cytochrome than *b*- and *o*-type, while under aerobic heterotrophic growth the reverse is true (van Verseveld and Stouthamer, 1978a). Some kinetic experiments confirm the former observation and indicate that methanol oxidation proceeds only through a respiratory limb involving *c*- and *a*-type cytochromes, whereas *b*-type pigments are not implicated.

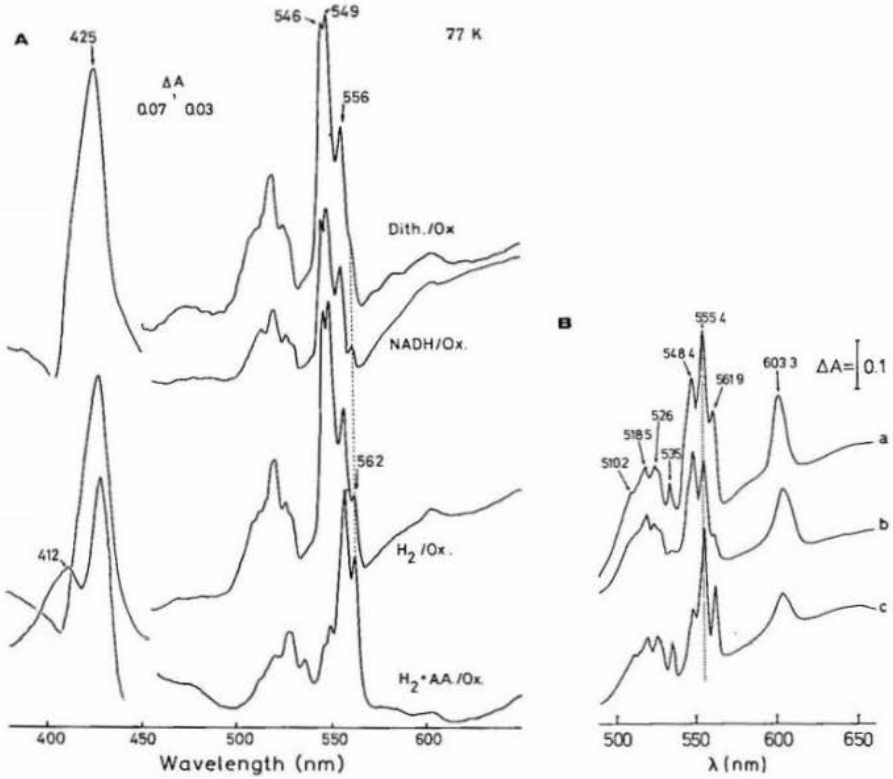

FIG. 8. Low-temperature absorption difference spectra of membrane particles from *Paracoccus denitrificans*. (A) Membranes derived from cells grown autotrophically on H_2:CO_2:O_2. Additions were the following: 2.5 m*M* NADH; 0.4 m*M* H_2; antimycin A, 10 nmol/ml. The oxidized state was reached by addition of H_2O_2 + catalase. Protein concentration was 4.7 mg/ml. (B) Membrane particles prepared from aerobically grown cells. Additions were the following: 5 m*M* NADH; antimycin A, 2.5 nmol/ml; proteins, 12.6 mg/ml. From Henry and Vignais (1979).

In contrast, cells of *P. denitrificans* grown on $H_2 : CO_2 : O_2$ possess much less cytochrome aa_3 (Fig. 8A) (Porte and Vignais, 1980) than do aerobic heterotrophic cells (Fig. 8B). However, these autotrophically grown cells contain a CO-reacting pigment (cytochrome *o*) that is H_2 reducible (M.-F. Henry, unpublished results) and is believed to act as the sole oxidase when H_2 is oxidized. Cytochrome aa_3 accepts electrons from substrates such as NADH and succinate, and ascorbate-TMPD (Porte and Vignais, 1980). Cells of *P. denitrificans* grown on H_2 also contain higher levels of a cytochrome *b*-562 whose reduction is enhanced when H_2 is the electron donor. This cytochrome may well be involved in the branching

point of the two electron-transfer pathways to O_2 or may be a component of the H_2–cytochrome o–O_2 limb.

B. Energy Conservation in Autotrophically Grown Cells

1. Autotrophic Growth on Methanol

Cox and Quayle (1975) have shown that *P. denitrificans* is capable of autotrophic growth on methanol as only carbon and energy source. Methanol is oxidized stepwise via formaldehyde and formate to CO_2 (Bamforth and Quayle, 1978). Van Verseveld and Stouthamer (1978a) demonstrated that methanol oxidation is insensitive to antimycin A and involves electron transport via cytochrome c and an a-type cytochrome to oxygen (Fig. 2).

In cells grown on methanol, methanol oxidation in the presence of rotenone and antimycin A yielded an $\leftarrow H^+/O$ ratio of 3.5; as seen in Fig. 2 this corresponds to the cytochrome c–O_2 span of the respiratory chain. An $\leftarrow H^+/O$ ratio of 5–6 was obtained for succinate oxidation, whereas cells grown heterotrophically (on succinate) yielded an $\leftarrow H^+/O$ of only 3–4 when oxidizing succinate. It was concluded that oxidation of succinate included two sites (II and III) in autotrophic cells and one site in heterotrophic cells (van Verseveld and Stouthamer, 1978a).

Van Verseveld and Stouthamer (1978b) determined the efficiency of oxidative phosphorylation from the growth yields of aerobic cultures in chemostat cultures of *P. denitrificans* on methanol. During oxidation of methanol (or formate) to CO_2, ATP is generated by oxidative phosphorylation only, and not by substrate-level phosphorylation. The rate of ATP synthesis (q_{ATP}) can then be equated to the rate of O_2 consumption (q_{O_2}) multiplied by twice the P/O ratio ($q_{ATP} = q_{O_2} \times 2$ P/O). The rate of ATP synthesis is in principle linked to the growth yield (Y) (grams of biomass per mole of substrate) and the specific growth rate (μ) by the following relation where m_e is the maintenance coefficient (moles per gram dry weight per hour).

$$q_{ATP} = \mu/Y_{ATP} + m_e = \mu/Y_{ATP}$$

The overall P/O ratio (2.05) calculated with the matching Y_{ATP}^{max} (maximum growth yield on ATP) value of 3.8 corresponded to three coupling sites present (three being used for NADH oxidation and one for methanol dehydrogenation). This value of 3.8 was much lower than the theoretical Y_{ATP}^{max} value of 6.5 calculated by Harder and van Dijken (1976) for autotrophic growth of organisms that use the ribulose-bisphosphate cycle

for CO_2 fixation. Because of the large difference between experimental and theoretical Y_{ATP}^{max} it was proposed by van Verseveld and Stouthamer (1978b) that energy-requiring processes other than the formation of new cell material were present under the growth conditions used. Alternatively, methanol might have affected the efficiency of proton-coupled energy transduction per se.

When *P. denitrificans* was grown heterotrophically on mannitol and methanol, experimental growth parameters determined during two-(carbon) substrate-limited conditions fit with the presence of three sites of oxidative phosphorylation, whereas cells grown only on mannitol appeared to possess two sites only.

2. *Autotrophic Growth on Formate*

With formate-grown cells, van Verseveld and Stouthamer (1978a) determined the experimental $\leftarrow H^+/O$ ratios for the oxidation of endogenous substrates and of succinate of 6.3 and 3.5, respectively. Taking a value of 3 for the $\leftarrow H^+$/site ratio (Meijer *et al.*, 1977a; van Verseveld and Stouthamer, 1978a), van Verseveld and Stouthamer (1978a) concluded that an $\leftarrow H^+/O$ of 6.3 is in accordance with the presence of two sites in formate-grown cells (sites I and II).

In another study, the same authors (van Verseveld and Stouthamer, 1978b) estimated the number of coupling sites from the determination of growth yields. Assuming the presence of three sites, the calculated Y_{ATP}^{max} was 1.8 for growth with formate. This is considerably lower than the theoretical value of 6.5 calculated by Harder and van Dijken (1976) and than the Y_{ATP}^{max} obtained in the same study for growth with methanol (Y_{ATP}^{max} = 3.8 assuming three sites for NADH oxidation, or 5.1 assuming two sites for NADH oxidation and one site for methanol dehydrogenase oxidation). When the presence of two sites was assumed (P/O for NADH = 2), the calculated Y_{ATP}^{max} value for formate was 2.6, which is still much lower than that for growth on methanol. These differences were attributed to the nature of the two substrates. Methanol is an uncharged molecule that can presumably freely enter the cell, whereas formate crosses the membrane as the free acid and thus moves in effectively with a cotransported proton (Garland *et al.*, 1975). The ATP equivalence of one proton is 0.5 mol ATP if the transfer of 2 g-ions of proton leads to the synthesis of 1 mol of ATP, or 0.33 mol of ATP if 3 g-ions of protons are consumed per mole of ATP synthesized. Taking into account the energy required for formate transport, and assuming two sites present in the respiratory chain, the P/O ratio will be either 1.5 (with a H^+/site ratio of 2) or 1.67 (with a H^+/site ratio of 3). Matching experimental Y_{ATP}^{max} at both these P/O

ratios gave Y_{ATP}^{max} of 3.1 and 3.5, respectively; these values are very close to the Y_{ATP}^{max} value (3.8) calculated for methanol-grown cells in the possession of three sites. The authors (van Verseveld and Stouthamer, 1978b) concluded that formate-grown cells contain two active energy-conserving sites (probably sites I and II) and that the amount of ATP necessary to synthesize 1 g of biomass is the same during autotrophic growth on methanol or on formate when the ATP cost of formate transport is taken into account.

The same conclusion, the occurrence of only the first two energy-conserving sites in heterotrophic cells, was reached from growth experiments on mannitol and formate [two-(carbon)substrate] limitation (van Verseveld and Stouthamer, 1980). It should be noted that for the above-mentioned calculations it was assumed that formate was oxidized via a soluble NAD-linked formate dehydrogenase, as occurs in *Pseudomonas oxalaticus OX1* (Dijkhuizen *et al.*, 1977). It has recently been shown (Dijkhuizen *et al.*, 1979) that *Ps. oxalaticus OX1* also contains a membrane-bound formate dehydrogenase able to transfer electrons into the respiratory chain at the level of ubiquinone or cytochrome *b*, as does the membrane-bound formate dehydrogenase *E. coli* (Enoch and Lester, 1975; De Moss, 1977). In this case it is sites II and III that should be involved in energy conservation as concluded by Dijkhuizen *et al.* (1977), rather than sites I and II.

3. *Autotrophic Growth on Molecular Hydrogen*

The energy transduction linked to the respiration of cells grown autotrophically on molecular hydrogen was estimated by Porte and Vignais (1980) from the stoichiometry of respiration-driven proton translocation in spheroplasts. The advantage of spheroplasts over membrane vesicles is that all spheroplasts are right-side-in, and therefore protons are ejected outward during respiration; thus the pH change can be recorded in the suspending medium. The advantage of spheroplasts over whole cells is that the cytoplasmic membrane is more directly accessible to inhibitors from the bulk phase; thus, unlike the case with whole cells (Scholes and Mitchell, 1970b), valinomycin, which minimizes the membrane potential built up by proton translocation by its K^+ ionophoric activity, can be used at relatively low concentrations (2 μg per milligram of protein) and has an immediate effect upon addition to spheroplasts.

Although it was not possible to deplete the spheroplasts completely from endogenous substrates, the use of relatively small amounts of spheroplasts in the incubation medium allowed the measurement of proton translocation linked to the oxidation of externally added substrates

free from interference by endogenous substrates. Under these conditions the number of translocated protons associated with the oxidation of H_2 and succinate was 6–7 and was unaffected by rotenone, but the $\leftarrow H^+/O$ values obtained for the oxidation of endogenous substrates by spheroplasts averaged 9–10 and 6–7 in the presence of rotenone (Table IV) (Porte and Vignais, 1980). Assuming that three energy-conserving sites are involved during the oxidation of endogenous substrates and two during succinate oxidation, Porte and Vignais (1980) concluded that the $\leftarrow H^+$/site ratio is 3 in agreement with the studies of Meijer *et al.* (1977a), Lawford (1978, 1979), and van Verseveld and Stouthamer (1978a), which indicated an average value of 3–4 for the $\leftarrow H^+$/site ratio. With a $\leftarrow H^+$/site ratio of 3, the $\leftarrow H^+/O$ values of 6–7 for the H_2 oxidation indicated that two energy-transducing sites are involved in the oxidation of H_2 by O_2, and the $\leftarrow H^+/O$ ratio of 2.7 with ascorbate-TMPD indicated the presence of one site.

Measurement of ATP synthesis in membrane vesicles confirmed that phosphorylation was coupled to H_2 oxidation. However, such determinations, which necessitated the use of inverted vesicles, gave P/O values too low to allow any conclusions to be made on the number of coupling sites.

The sites of energy conservation proposed by Porte and Vignais (1980) for autotrophically grown cells are indicated on Fig. 1.

V. Hydrogenase

A. General Background on Hydrogenase

The existence of a class of enzymes using molecular hydrogen, H_2, as substrate was discovered some 50 years ago (Stephenson and Stickland, 1931). Since that time, hydrogenases, which catalyze the reaction

$$H_2 \rightleftharpoons 2\,H^+ + 2\,e^- \qquad (3)$$

have been found in a wide variety of bacteria and blue-green algae (see earlier reviews by Gray and Gest, 1965; Mortenson and Chen, 1974; Schlegel and Schneider, 1978). Hydrogenases are also present in certain eukaryotes, e.g., *Tritrichomonas foetus,* which is a parasite of the urogenital tract of cattle (Müller and Lindmark, 1978), and in certain eukaryotic algae (Kessler, 1978). Hydrogenase activity is independent of ATP and is inhibited by CO (Peck *et al.*, 1956), the distinguishing features between hydrogen production catalyzed by hydrogenase and that catalyzed by nitrogenase. The latter enzyme requires a source of ATP, and hydrogen production catalyzed by nitrogenase is not inhibited by CO (Winter and Burris, 1968).

The physiological and ecological roles of hydrogenases are diverse. In organisms that can use protons as terminal electron acceptor, molecular hydrogen is produced. Such organisms include obligate anaerobic bacteria, for example, species of *Clostridium* where pyruvate is degraded to H_2 and CO_2, and facultative anaerobes, such as *E. coli*, which produce H_2 when grown on glucose in the absence of an electron acceptor other than protons. Species of *Desulfovibrio*, when grown on pyruvate in the absence of sulfate as electron acceptor also produce H_2 (Vosjan, 1975). Hydrogenases in *Desulfovibrio* species can also catalyze anaerobic oxidation of hydrogen gas coupled with sulfate reduction (Badziong *et al.*, 1978). *Escherichia coli* can derive energy for anaerobic growth by H_2-dependent reduction of fumarate (Bernhard and Gottschalk, 1978). Hydrogen uptake activity has been demonstrated in the methanogenic bacteria, and this is an important ecological role of hydrogenase as it results in methane production, one of the most abundant atmospheric trace gases, from H_2 and CO_2 (Schlegel and Schneider, 1978). Photosynthetic bacteria, e.g., *Rhodopseudomonas capsulata*, have hydrogen oxidizing activity (Kelley *et al.*, 1977; Paul *et al.*, 1979), as do blue-green algae (Bothe *et al.*, 1978; Tel-Or *et al.*, 1978). Aerobic nitrogen-fixing bacteria, e.g., *Azotobacter*, also have hydrogenase activity that catalyzes hydrogen uptake *in vivo* (van der Werf and Yates, 1978); and in many organisms, such as *Anabaena cylindrica* (Bothe *et al.*, 1978), *R. capsulata* (Kelley *et al.*, 1977; Jouanneau *et al.*, 1980), *Rhodospirillum rubrum* (reviewed by Meyer *et al.*, 1978), and *Rhizobium* (Dixon, 1978) hydrogen-cycling has been described where hydrogen is produced by nitrogenase and is re-used by hydrogenase-catalyzed H_2-uptake.

The group of bacteria to which *P. denitrificans* belongs, the aerobic chemoautotrophic, or hydrogen, bacteria, have the ability to grow autotrophically with H_2 as the sole source of reducing power; this group includes species of *Alcaligenes* and *Pseudomonas*. The designation of organisms as "proton-reducing" (hydrogen producers) or as "hydrogen-oxidizing" (hydrogen consumers) depends on growth conditions, as discussed above for *E. coli* and species of *Desulfovibrio*. It has even been suggested (Schlegel and Schneider, 1978) that hydrogen-dehydrogenase as found for example in the aerobic hydrogen bacteria, may also be able to catalyze proton reduction if no other suitable electron acceptor is present. There have been reports of "unidirectional" hydrogenases in *Clostridium pasteurianum* (Chen, 1978) and in *Anabaena cylindrica* (Hallenbeck and Benemann, 1978), which can catalyze only H_2 oxidation *in vitro*, although most hydrogenases catalyze both H_2 uptake and evolution *in vitro*, albeit at different rates.

There has been renewed interest in hydrogenase in the 1970s as a possible means of providing H_2 as a source of energy (Hall, 1976;

TABLE

MOLECULAR PROPERTIES OF

Property	Source of *Alcaligenes eutrophus*	*Nocardia opaca*	*Clostridium pasteurianum*	*C. pasteurianum*
Localization	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm
Sensitivity to oxygen	Stable	Fairly stable	Very Sensitive	Very sensitive
Electron donor/acceptor	NAD^+	NAD^+	Ferredoxin	Unknown
Molecular weight	200,000	—	60,000	32,000
No. of subunits	1 × 68,000 1 × 60,000	—	1 × 60,000	1 × 32,000
Partial specific volume ($ml\ g^{-1}$)	2 × 29,000	—	—	—
Absorption bands (nm)	380,420	—	Around 400	—
Flavin/molecule	2 FMN	[b]	None	None
Fe/molecule	12	—	12	—
Labile sulfide/molecule	12	—	12	—
Type of Fe-S center	4Fe-4S 2Fe-2S	—	4Fe–4S	—
References	Schneider and Schlegel, 1976, 1977, 1978; Schneider *et al.*, 1979	Aggag and Schlegel, 1974	Nakos and Mortenson, 1971; Chen and Mortenson, 1974	Chen and Blanchard, 1978

[a] Adapted from Schlegel and Schneider (1978).

[b] Enzymic activity, as measured by reduction of NAD^+ by H_2, is dependent on FMN.

Benemann *et al.*, 1977; Hallenbeck and Benemann, 1979); as a result, hydrogenases from a variety of sources have been investigated at the molecular level. Although absolute classification of hydrogenases is not yet possible, certain broad groups are beginning to emerge. Both soluble and membrane-bound hydrogenases have been identified. Since the methodology for studying soluble enzymes was established earlier more is known about the soluble hydrogenases. Soluble hydrogenases have been purified from *C. pasteurianum* (Chen and Mortenson, 1974; Chen and Blanchard, 1978), *D. vulgaris* strain Hildenborough (van der Westen *et al.*, 1978), *D. gigas* (Hatchikian *et al.*, 1978), and *Alcaligenes eutrophus* (Schneider and Schlegel, 1976). In the case of the soluble hydrogenases, in most instances, the electron donor or acceptor has been identified. The properties of the soluble hydrogenases are compared in Table VII.

The membrane-bound hydrogenases that have been investigated so far do not have an identified electron donor or acceptor, except in the case of *D. vulgaris* strain Miyazaki, although they form an integral part of the electron-transport chain of the organism in which they are found. There is no predilection for soluble or membrane-bound hydrogenases to partici-

VII

SOLUBLE HYDROGENASES[a]

hydrogenase				
		Desulfovibrio vulgoris		
Thiocapsa roseopersicina	*Anabaena cylindrica*	Strain NC1B 8303	Strain Hildenborough	*D. gigas*
Soluble fraction	Soluble fraction	Soluble fraction	Periplasm	Periplasm
Fairly stable	Slightly sensitive	Relatively stable	Slightly sensitive	—
Cytochrome c_3?	—	Cytochrome c_3?	Cytochrome c_3	Cytochrome c_3
66,000–68,000	230,000	45,000	50,000	89,000
1 × 47,000	—	1 × 45,000	1 × 50,000	1 × 62,000
1 × 25,000	—	—	—	1 × 26,000
—	—	0.74	—	0.73
Around 400	—	Around 410	Around 400	Around 400
—	—	—	—	—
4	—	—	12	12
4	—	—	12	12
—	—	—	—	4Fe-4S
Gogotov *et al.*, 1976, 1978	Hallenbeck and Benemann, 1978	Haschke and Campbell, 1971	van der Westen *et al.*, 1978	Hatchikian *et al.*, 1978

pate in H_2 uptake or H_2 production. In *Proteus mirabilis* grown under anaerobic conditions on a complex medium, the membrane-bound hydrogenase functions mainly in the direction of H_2 production (Schoenmaker *et al.*, 1979) whereas in *P. denitrificans* grown autotrophically (Kluyver and Verhoeven, 1954a,b), in *Rhodopseudomonas capsulata* (Colbeau *et al.*, 1978), and in *Chromatium* (Gitlitz and Krasna, 1975) the membrane-bound hydrogenases function *in vivo* primarily to catalyze hydrogen uptake. The characteristics of membrane-bound hydrogenases are shown in Table VIII.

All hydrogenases that have been studied so far are iron–sulfur proteins, and many, but by no means all, hydrogenases are oxygen sensitive, a feature that initially hindered progress in investigations of hydrogenase at the molecular level.

B. AEROBIC HYDROGEN BACTERIA

The aerobic hydrogen bacteria can be divided into three groups on the

TABLE

MOLECULAR PROPERTIES OF

Property	Source of *Paracoccus denitrificans*	*Alcaligenes eutrophus*	*Chromatium*	*Proteus mirabilis*
Sensitivity to O_2	Stable in membrane, sensitive when solubilized	Stable	Stable	Unknown
Electron donor/acceptor	Unknown	Unknown	Unknown	Unknown
Solubilization method	Triton X-100	Triton X-100 plus sodium deoxycholate	Triton X-100 or sodium deoxycholate	Amyl alcohol
Molecular weight in nonionic detergent	242,500 ± 8000 (*s* value 10.4)	—	—	(*s* value 8.3)
No. subunits in nonionic detergent	4 × 63,000	—	—	—
Molecular weight without nonionic detergent	466,000 ± 15,000 (*s* value 15.9)	98,000	100,000 (*s* value 5.16)	208,000 (*s* value 8.68)
No. subunits without nonionic detergent	7–8 × 63,000	1 × 67,000 1 × 31,000	2 × 50,000	2 × 63,000 2 × 33,000
Partial specific volume (ml g^{-1})	0.74			0.73
Absorption bands (nm)	Around 400	408	410	Around 410
Fe/molecule	—	6	4	24
Labile sulfide/molecule	—	6	4	24
Type of Fe-S center	—	—	—	—
References	Sim and Vignais, 1979; Sim and Sim, 1979	Schink and Schlegel, 1978a, 1979	Gitlitz and Krasna, 1975	Schoenmaker *et al.*, 1979

[a] Adapted from Schlegel and Schneider (1978).

[b] This protein may be a peripheral membrane protein because these authors report that the enzyme is more readily eluted from an acetone powder by buffer alone than by buffer and detergent. Most of the hydrogenase activity in this organism is found in the soluble cell fraction and the "membrane" enzyme may be soluble hydrogenase that is not washed off.

basis of the hydrogenases found (Schneider and Schlegel, 1977; Schink and Schlegel, 1978a).

1. Organisms of this group contain only a soluble hydrogenase capable of reducing NAD^+, i.e., hydrogen:NAD^+ oxidoreductase (EC 1.12.1.2). An example of such an organism is *Nocardia opaca*.
2. Some aerobic hydrogen bacteria have two hydrogenases, a soluble enzyme that reduces NAD^+ and a membrane-bound hydrogenase that does not reduce NAD^+ or $NADP^+$. *Alcaligenes eutrophus* falls into this category.
3. The major group of aerobic hydrogen bacteria has only one hydrogenase, and it is membrane-bound. The membrane-bound enzyme is

VIII

MEMBRANE-BOUND HYDROGENASES[a]

hydrogenase				
Rhodospirillum rubrum	*Escherichia coli*	*Thiocopsa roseopersicina*	*Desulfovibrio vulgaris* strain Miyazaki	*Rhizobium japonicum*
Relatively stable	$t_{1/2} \approx$ 12 hours at room temperature	Fairly stable	—	Sensitive when solubilized
Unknown Sodium deoxycholate and pancreatin	Unknown Sodium deoxycholate[b] and pancreatin	Cytochrome c_3? Acetone at −30°C	Cytochrome c_3 Trypsin	Unknown Triton X-100
—	—	—	—	—
—	—	—	—	—
67,000 ± 2000	113,000	68,000–73,000	89,000	63,000
1 × 65,000 ± 3000	2 × 56,000	1 × 25,000 1 × 47,000[c]	1 × 59,000 1 × 28,000	1 × 63,000
—	—	—	0.75†	—
—	400	—	Around 400	—
4	12	3	7–9	12 (tentative)
—	12	4	7–8	—
4Fe-4S	—	—	—	—
Adams and Hall, 1977, 1979a	Adams and Hall, 1979b	Serebriakova *et al.*, 1977; Gogotov *et al.*, 1978	Yagi *et al.*, 1976	Arp and Burris, 1979

[c] Gel electrophoresis in the presence of sodium dodecyl sulfate shows the presence of three bands corresponding to 68,000, 47,000, and 25,000 MW. It may therefore be that the two smaller polypeptides represent proteolytic fragments of the larger polypeptide chain.

unable to reduce NAD^+ or $NADP^+$, and it is to this group that *P. denitrificans* belongs.

In aerobic hydrogen bacteria, grown under autotrophic conditions, hydrogenase activity has a dual physiological role to play. It provides reducing power for CO_2 fixation, as NADH, and it also provides cellular energy more directly by feeding electrons into the electron-transport chain.

For those members of the aerobic hydrogen bacteria with both a soluble and a membrane-bound hydrogenase, the soluble enzyme reduces NAD^+ whereas the membrane-bound enzyme feeds the electron-transport chain directly. However, NADH can also provide electrons for oxidative phosphorylation. The situation is less clear for those aerobic hydrogen bacteria possessing only a membrane-bound hydrogenase. To investigate

how these organisms cope with having only one hydrogenase, Schink and Schlegel (1978b) looked at various hydrogenase-deficient mutants of *A. eutrophus*. They found that each type of hydrogenase alone could support autotrophic growth. However, the growth rate was decreased in mutants with only the membrane-bound hydrogenase. Such a finding is as would be expected if the membrane-bound enzyme is consuming some of the available energy for growth by providing NADH by reversed electron flow. This implies that there is a phosphorylation coupling site from NADH, and this has been described in the case of *A. eutrophus* (Ishaque and Aleem, 1970) and also *P. denitrificans* (Asano *et al.*, 1967). Membrane vesicles of *P. denitrificans* have been shown to catalyze ATP-dependent reduction of NAD^+ by succinate, indicating that energy-dependent reversal of electron transport to produce NADH can occur (Asano *et al.*, 1967). Reverse electron flow is therefore likely to be the mechanism whereby NAD^+ is reduced in *P. denitrificans* when grown autotrophically on H_2 and CO_2. There is good evidence that the membrane-bound hydrogenase of *P. denitrificans* interacts with the electron-transport chain at a point distinct from NADH. Rotenone, which inhibits oxidation of NADH in membranes of *P. denitrificans*, does not inhibit oxidation of H_2 with either ferricyanide or O_2 as electron acceptor (Knobloch *et al.*, 1971; Sim and Vignais, 1978; Porte and Vignais, 1980). The interaction of hydrogenase with the electron-transport chain of *P. denitrificans* has been discussed in Section IV,B,3.

C. Hydrogenase from *P. denitrificans*

1. Enzymic Characteristics

When *P. denitrificans* is grown autotrophically, either aerobically or anaerobically, hydrogenase activity is induced (Kluyver and Verhoeven, 1954a,b; Fewson and Nicholas, 1961). Very recently, Nokhal and Schlegel (1980) have proposed that the regulation of hydrogenase formation may serve as a distinguishing feature between different strains of *P. denitrificans*. During adaptation from growth on succinate to autotrophic growth it has been observed that the specific activity of hydrogenase reaches a maximum when the turbidity of the culture (OD_{550}) is 0.9–1.0 (E. Sim, unpublished). Adaptation to autotrophic growth is also accompanied by a change in the cytochrome content of membranes of *P. denitrificans* as shown in Fig. 8 (see Section IV,A,2).

As already discussed, the hydrogenase of *P. denitrificans* is associated with the cytoplasmic membrane. In crude preparations it does not reduce

NAD^+ but it will catalyze the reduction of a variety of artificial electron acceptors including benzyl viologen, methyl viologen, methylene blue, ferricyanide, and cytochrome *c*, but not spinach ferredoxin (Sim *et al.*, 1977; Sim and Vignais, 1978). The purified hydrogenase from *P. denitrificans* interacts with benzyl viologen and methyl viologen. The K_m values of hydrogenase in membrane preparations of *P. denitrificans* are shown in Table IX. The hydrogenase of *P. denitrificans* reduces 5 m*M* benzyl viologen more rapidly than 5 m*M* methyl viologen, and this is due mainly, to the difference in K_m of hydrogenase for the two substrates (Table IX). It may be, therefore, that the hydrogenase of *P. denitrificans* interacts more strongly with the more extensive aromatic structure of benzyl viologen than with methyl viologen. Although the same is true for the hydrogenase from *E. coli* (Table IX), this is not a general feature of hydrogenases. The soluble hydrogenase from *A. eutrophus* is more active toward methyl viologen than toward benzyl viologen (Schneider and Schlegel, 1976), and the membrane-bound hydrogenase from *A. eutrophus* will not reduce either viologen dye (Schink and Schlegel, 1979). The hydrogenase from *Rhizobium japonicum* shows no reactivity toward methyl viologen and only slight reactivity toward benzyl viologen (Arp and Burris, 1979). The enzymic characteristics of hydrogenases other than that of *P. denitrificans* are shown in Table IX. All data are for hydrogenase activity measured in the direction of hydrogen uptake except for the data on inhibitors where H_2 uptake and H_2 production have been combined. The comparisons serve to emphasize that hydrogenases are a heterogeneous group of enzymes.

Hydrogen gas dissolves in water to a concentration of 0.8 m*M*, at 20°C, under 1 atm. The measured K_m value for H_2 for the hydrogenase from *P. denitrificans* is 20 μM; this means that even in conditions of a low concentration of H_2 the enzyme can remain almost saturated with substrate. This may be important for the survival of the organism in its natural environment. The K_m for H_2 of both the soluble (37 μM) and the membrane-bound (32 μM) hydrogenases from *A. eutrophus* and from *E. coli* (26 μM) (Table IX) as well as the hydrogenase from *Methanobacterium thermoautotrophicum* (20 μM) (Fuchs *et al.*, 1978) are similar. However, the hydrogenase from *C. pasteurianum* has a much higher K_m for H_2 (225 μM) (Erbes and Burris, 1978). This may be due to the fact that the main function of the hydrogenase of *C. pasteurianum* is to evolve H_2, and therefore a high affinity of the enzyme for H_2 is not essential. Hydrogenase from *Rhizobium japonicum* and *Rhodopseudomonas capsulata* have a very low K_m for H_2 (1.4 μM and 0.25 μM, respectively) even though the bacteroid (Maier *et al.*, 1978) and the photosynthetic bacterium (Jouanneau *et al.*, 1980) have nitrogenase-catalyzed production of

TABLE

ENZYMIC PROPERTIES

Properties	Source of: *Paracoccus denitrificans* — Membrane-bound	*Paracoccus denitrificans* — Detergent solubilized	*Alcaligenes eutrophus* — Soluble enzyme	*Alcaligenes eutrophus* — Membrane-bound	*Alcaligenes eutrophus* — Solubilised enzyme
K_m (m*M*)					
Benzyl viologen	0.29	0.25	—	No reactivity	No reactivity
Methyl viologen	2.94	2.8	—	No reactivity	No reactivity
H_2	0.026	0.020	0.037	—	0.032
pH optimum	Around 9	Around 9	8.0	8.0	5.5
Form of Arrhenius plot	Straight line	Straight line	Straight line	"break" at 30°C	Straight line
Activation energy (kcal/mol)	11.0	9.8	14.3	—	6.25
Inhibited by high salt	+	+	+	—	+
Purification factor	—	100	68	—	—
Inhibitors (% inhibition)					
N-Ethylmaleimide 1 m*M*	5	37	—	—	—
Iodoacetamide, 10 m*M*	5	36	—	—	—
p-Chloromercuri-benzenesulfonic acid, 4 m*M*	43	80	—	—	—
p-Chloromercuri-benzoate, 1 m*M*	—	—	—	—	100
Dithiothreitol, 0.2 m*M*	0	24	—	—	—
$FeCl_3$, 1 m*M*	100	100	—	—	Slight inhibition
$CuSo_4$, 1 m*M*	95	95	—	—	—
$HgCl_2$, 1 m*M*	27	29	—	—	100
$CuCl_2$, 1 m*M*	—	—	—	—	100
References	Sim and Vignais, 1979; Sim *et al.*, 1978	Sim and Vignais, 1979; Sim *et al.*, 1978	Schneider and Schlegel, 1976	Schink and Schlegel, 1978a, 1979	Schink and Schlegel, 1978a, 1979

[a] Ferriycy. = ferricyanide; MB = methylene blue; BV = benzylviologen.

IX

OF HYDROGENASE

hydrogenase*

Escherichia coli	*Rhodospirillum rubrum*	*Rhizobium japonicum*	*Rhodopseudomonas capsulata*	*Proteus mirabilis*	*Chromatium*	*Clostridium pasteurianum*	*Desulfovibrio vulgaris* strain Hildenborough
0.07	—	Slight reactivity	0.11	9	—	—	3
3.48	—	No reactivity	3.70	—	—	—	0.2
0.026	—	0.0014, 0.0014	0.00025	—	—	0.225	—
8.5	8–9	5.7 (Ferricy. reduction 5.5 (MB reduction)	5.5 (MB reduction) 8.5 (BV reduction)	—	5.5 and 8.5 (MB reduction) 6 (HD exchange)	—	—
Straight line	—	Breaks at 25° and 55°C	Break at 13°C	—	—	—	—
—	—	9.2 (between 25°C and 55°C)	—	—	—	—	—
+	Tris + but not PO_4^{2-}	—	+	—	—	—	+
2140	490	200	—	1146	1788	—	380
0	No inhibition at 5 m*M*	—	27	—	—	—	—
		—	19	—	3	—	—
—	—	—	20	—	—	—	—
16	—	—		—	11	—	—
—	—	—	14	—	41	—	—
—	—	—	70	—	—	—	—
32	100 (5 m*M*)	—	100	—	100	—	—
100	100 (5 m*M*)	—	100	—	100	—	—
—	—	—	—	—	—	—	—
Adams and Hall, 1979b	Adams and Hall, 1977, 1979a	Arp and Burris, 1979	A. Colbeau (unpublished)	Schoenmaker *et al.*, 1979	Gitlitz and Krasna, 1975	Chen and Mortenson, 1974; Erbes and Burris, 1978	van der Westen *et al.*, 1978

H_2, which is recycled by the hydrogenase. The specific activity of *R. japonicum* hydrogenase has been found to be low (Arp and Burris, 1979) and the high affinity for H_2 may be a reflection of the necessity to sequester H_2 in competition with diffusion.

The hydrogenase activity from *P. denitrificans* shows a pH optimum for benzyl viologen reduction at pH 9. However, studies of pH optima of hydrogenase, as has been pointed out by Chen (1978), are open to many interpretations because protons are substrates of the reaction. In addition, many of the electron acceptors used to measure hydrogenase activity, e.g., methylene blue, have pH-dependent redox potentials. This is not the case for benzyl viologen, however. Different effects of pH on hydrogenase activity have been reported depending on the assay system used. For example, the hydrogenase from *Chromatium* has a pH optimum of 6 when hydrogen–deuterium exchange activity is measured, but shows a biphasic pH curve with peaks of activity at pH 5.5 and 8.5 when methylene blue reduction by H_2 is measured (Gitlitz and Krasna, 1975). Similarly, an optimum at acidic pH (around 5) was observed for the production of HD resulting from the hydrogen–deuterium exchange catalyzed by the hydrogenase of *P. denitrificans* (P. M. Vignais, M.-F. Henry, Y. Berlier, and P. A. Lespinat, unpublished observations). This indicates that the iron–sulfur cluster is well shielded in the membrane-bound hydrogenase to withstand such low pH values.

The hydrogenase of *P. denitrificans* is inhibited by high ionic strength (greater than 40 m*M* NACl) (Sim and Vignais, 1979). This is a general feature of hydrogenases, although it has been reported that the hydrogenase from *Megasphaera elsdenii* is stimulated by high ionic strength (Mayhew *et al.*, 1978).

The hydrogenase from *P. denitrificans* is inhibited by sulfhydryl reagents, especially after solubilization by detergent. Heavy-metal salts inhibit hydrogenase activity equally in both the membrane-bound and solubilized forms. The effects of these compounds on other hydrogenases are shown in Table IX. In general, heavy metals are extremely deleterious to hydrogenase activity. However, the effects of sulfhydryl reagents are more variable. It seems unlikely, therefore, that the effects of heavy-metal salts on hydrogenase activity are entirely due to their interaction with sulfhydryl groups, as has been documented for heavy-metal inhibition of other enzymes (Means and Feeney, 1971).

A general characteristic of hydrogenases is that they are thermostable. The hydrogenase activity of *Rhodospirillum rubrum*, for example, survives heating to 70°C for 10 minutes (Adams and Hall, 1979a). The hydrogenase of *P. denitrificans* shares this feature and retains activity after heating at 75°C for 10 minutes, although in impure preparations the

hydrogenase adheres to other denatured contaminating proteins (E. Sim, unpublished).

Despite the robust nature of hydrogenase activity on heating, hydrogenases show varying degrees of loss of activity during purification, and the oxygen sensitivity of hydrogenases has already been mentioned. The hydrogenase from *P. denitrificans* is perfectly stable in the intact cell under aerobic storage conditions at 4°C and at −20°C (Sim *et al.*, 1977). However, on solubilization, the enzyme becomes inactivated in air at 4°C. Hydrogenase activity is less sensitive to inactivation in air at 20°C and at −20°C. The ability of the hydrogenase to retain activity in air at room temperature has been explored in the purification of the enzyme. The cold-lability of the hydrogenase from *P. denitrificans* may be related to the oligomeric nature of the enzyme, which will be discussed in the next section. It has also been reported that the membrane-bound hydrogenase from *Alcaligenes eutrophus* is more stable at 18°C than at 4°C (Schink and Schlegel, 1979) and the enzyme has two nonidentical subunits. Other membrane-bound hydrogenases have also been observed to become oxygen-sensitive when they are solubilized, including the enzyme from *Azotobacter chroococcum* (van der Werf and Yates, 1978) and the hydrogenase from *Rhizobium japonicum* (Arp and Burris, 1979). There are exceptions, however, and the purified, solubilized hydrogenase from *Chromatium* is completely stable in air (Gitlitz and Krasna, 1975). Among the soluble hydrogenases, the enzyme from *Clostridium pasteurianum* is extremely sensitive to oxygen, but other soluble enzymes, e.g., from *D. vulgaris* strain Hildenborough and hydrogen dehydrogenase from *A. eutrophus* are fairly resistant to loss of activity in air. These results are summarized in Tables VII and VIII. The oxygen sensitivity of hydrogenases is presumably due to the presence in these proteins of iron–sulfur clusters. Synthetic models of these clusters are known to be extremely labile in air (Holm and Ibers, 1977). It has been found that the activity of the hydrogenase from *P. denitrificans* can be augmented if the enzyme is stored at −20°C or at 20°C in an atmosphere of H_2. This effect is not due to lack of oxygen, because an atmosphere of N_2 has no activating effect (Sim *et al.*, 1978).

An investigation of the effect of temperature on hydrogenase activity in *P. denitrificans* showed the Arrhenius plot of activity to be linear, and the same is true of the detergent-solubilized hydrogenase. It has been observed for several membrane-bound enzymic activities, e.g., 5′-nucleotidase from rat liver plasma membrane (Stanley and Luzio, 1978), that there are discontinuities in an Arrhenius plot, and these have been interpreted as corresponding to a change in the physical state of the lipid environment of the membrane-bound enzyme. Not all membrane en-

zymes show discontinuities in Arrhenius plots, however. For some membrane proteins, changes in the form of the Arrhenius plot have been observed on solubilization of the enzyme with detergent (Dipple *et al.*, 1978). No such change is found for the hydrogenase from *P. denitrificans* when it is solubilized by Triton X-100 (Sim and Vignais, 1979) under conditions in which no phospholipid could be detected in association with the hydrogenase (Sim and Sim, 1979).

The membrane-bound hydrogenase from *Alcaligenes eutrophus* shows a break in the Arrhenius plot at 30°C, but no discontinuities are found in an Arrhenius plot of the activity of the solubilized, purified, enzyme (Schink and Schlegel, 1979). It was not determined whether the purified hydrogenase from *A. eutrophus* was associated with lipid, but it has been observed that the activity of the purified membrane-derived enzyme is activated by the presence of negatively charged phospholipids but inactivated by neutral phospholipids (Fromherz and Ruhr, 1978).

The solubilized and purified hydrogenase from *R. japonicum* shows breaks in the Arrhenius plot at 25°C and 55°C, but no information is available on whether there is any lipid associated with the solubilized enzyme. However, as Drost-Hansen (1971) has pointed out, discontinuities in Arrhenius plots should be interpreted with caution, and may reflect changes in the structure of interfacial water molecules.

Measurement of the activation energy of hydrogenase from *P. denitrificans* gives values of 11.0 kcal/mol for the membrane-bound form of the enzyme and 9.8 kcal/mol for the solubilized form of the enzyme. Activation energies of other hydrogenases are all very similar (see Table IX).

2. *Structural Characteristics*

During the discussion of the enzymic characteristics of the hydrogenase from *P. denitrificans*, comparisons have been drawn between the membrane-bound form of the enzyme and the detergent-solubilized enzyme. The two forms of the enzyme have similar enzymic characteristics except that the membrane-bound form is protected against damage by oxygen and it is less sensitive to sulfhydryl reagents. However, the K_m values, pH profile, and activation energies of the membrane-bound and solubilized forms of the enzyme are extremely similar. It is therefore likely that the regions of the enzyme involved in substrate binding and catalysis are not disrupted on solubilization of hydrogenase from the membrane. The structural characteristics of the hydrogenase from *P. denitrificans* have been investigated in the presence and in the absence of Triton X-100. The enzyme consists of a tetramer of non-disulfide-linked subunits of MW

63,000 in the presence of detergent. When detergent is removed, the enzyme forms soluble aggregates consisting of two such tetramers. It seems likely that, on removal of detergent, two molecules of hydrogenase associate via the newly exposed hydrophobic surfaces to produce a larger molecule that is still water-soluble. Other amphipathic membrane proteins have been shown to form water-soluble aggregates on removal of detergent (Kuchel *et al.*, 1978; Simons *et al.*, 1978). However, there have been very few reports on the effect of detergents on the behavior of other membrane-derived hydrogenases. The hydrogenase from *Proteus mirabilis* was initially released from the membrane by organic solvent. The sedimentation coefficient of the solubilized enzyme with or without detergent remains unchanged (Schoenmaker *et al.*, 1979). There is evidence that the hydrogenase from *Chromatium* binds detergent (Kakuno *et al.*, 1977), and it is also likely that the membrane-derived hydrogenase from *A. eutrophus* binds detergent (Schink and Schlegel, 1978a). However, no further discussion of these observations has been reported.

It is possible to estimate the amount of detergent bound to a protein detergent complex (Meunier *et al.*, 1972). The basis for such an investigation is that the partial specific volume of Triton X-100 is 0.91 ml g^{-1}, whereas the partial specific volume of most proteins, including hydrogenases (see Tables VII and VIII), is 0.73 ml g^{-1}. The partial specific volume of the hydrogenase from *P. denitrificans* in the presence of Triton X-100 is 0.73 ml g^{-1}, and this is essentially unchanged on removal of detergent (Table VIII). Therefore it is likely that the hydrogenase from *P. denitrificans* binds much less than one micelle of detergent. For a protein of similar size, the adenyl cyclase from rat brain (MW 220,000), which binds approximately one micelle of Triton X-100 (MW 90,000), the partial specific volume is increased from 0.74 ml g^{-1} to 0.79 ml g^{-1} (Neer, 1978). It seems that the hydrophobic area of hydrogenase that is exposed on solubilization of the enzyme from the membrane and becomes associated with detergent is restricted to a small area. This is not due to the presence of phospholipids masking an extensive hydrophobic site on the surface of the solubilized hydrogenase, because experiments using ^{32}P-labeled phospholipids have demonstrated that the detergent-solubilized hydrogenase is not associated with phospholipids (Sim and Sim, 1979). There is a hydrophobic region on the solubilized hydrogenase, as determined from its aggregation properties when detergent is removed. The extent of the hydrophobic surface of the protein can be investigated unambiguously only by studying the binding of radioactive detergents to the pure protein (Makino *et al.*, 1973). Such a study is now possible for the hydrogenase from *P. denitrificans*, as it has been isolated in good yield (Sim and Vignais, 1979).

The frictional coefficient of the hydrogenase from *P. denitrificans* in the presence and in the absence of detergent is 1.2, which is indicative of an axial ratio greater than 2. Indeed, *E. coli* F_1-ATPase has a $f/f_0 = 1.10$; this has led to the conclusion that the axial ratio is 2 or 3 (Paradies and Schmidt, 1979). Also the MoFe protein of nitrogenase, which has a $f/f_0 = 1.08$ (Eady *et al.*, 1972), has been found by neutron scattering to have an axial ratio of about 2 (Meyer and Zaccaï, 1980).

In view of the similarities of the enzymic properties of the hydrogenase in the membrane and after solubilization, it is probable that the hydrogenase, as solubilized by detergent, exists as it is found in the membrane, i.e., as a tetramer. It should be borne in mind, however, that the enzymic properties that have been investigated in both forms of the enzyme are nonvectorial, whereas the action of hydrogenase in membranes is vectorial (see Section V,C,3). In this context, a multisubunit membrane protein is a good candidate for a transport protein. The anion transport protein from the human red cell membrane band 3, is multimeric in the presence of Triton X-100 and is thought to be multimeric within the membrane (see review by Guidotti, 1977).

3. *Orientation of the Hydrogenase*

The orientation of the hydrogenase in the cytoplasmic membrane of *P. denitrificans* has been investigated using functional properties of the enzyme. It is known that benzyl viologen interacts directly with the hydrogenase of *P. denitrificans* (Sim and Vignais, 1978). Intact cells and spheroplasts will catalyze the reduction of benzyl viologen by hydrogen (Sim and Vignais, 1978). The reduction of benzyl viologen is not increased when spheroplasts are rendered permeable by Triton X-100 under conditions where the reduction of dichlorophenolindophenol by NADH increases over eightfold (E. Sim, unpublished). It has been observed that the latter activity is found in the cytoplasm of *P. denitrificans* (Porte, 1979), not associated with the inner surface of the cytoplasmic membrane as previously suggested for membrane vesicles (Burnell *et al.*, 1975a); nevertheless, it serves as a measure of whether or not spheroplasts are intact. Although studies have indicated that those viologen dyes that in the oxidized state carry two positive charges do not cross the cytoplasmic membrane of *E. coli* (Jones and Garland, 1977), this may not be the case for *P. denitrificans*. We have observed that oxidized benzyl viologen (BV^{2+}) adheres strongly to cells of *P. denitrificans*, this results in ambiguities in determining whether BV^{2+} is excluded from the intracellular space (E. Sim, unpublished). Reduction of benzyl viologen by hydrogen also is catalyzed by inside-out vesicles of the cytoplasmic membrane

of *P. denitrificans* (Porte, 1979), although it has been established that hydrogenase interacts directly with benzyl viologen (Sim and Vignais, 1978). It may be that the dye can also be reduced indirectly in whole membrane preparations.

To overcome these problems a more direct approach to studying the orientation of the active site of hydrogenase from *P. denitrificans* has been used. In this investigation the release of protons from spheroplasts and from inside-out membrane vesicles has been studied (Porte *et al.*, 1979; Doussière *et al.*, 1980). When hydrogenase-catalyzed reduction of benzyl viologen is initiated, a release of protons inside spheroplasts and outside the inside-out membrane vesicles is observed. This indicates that protons are released from the inner surface of the cytoplasmic membrane of *P. denitrificans*, providing information on the functional orientation of the hydrogenase.

From structural studies, it is likely that the area of the hydrogenase that interacts with the hydrophobic region of the membrane is rather limited, as determined from the partial specific volume of the hydrogenase–detergent complex. There is likely to be a hydrophobic region, however, because the protein aggregates on removal of detergent. It is not possible to determine at present whether or not the hydrogenase spans the membrane. From the functional studies, it would appear that the active site is exposed at the inner surface of the membrane, since release of protons inside the cell has been demonstrated on reduction of benzyl viologen by H_2. The interaction of hydrogenase with benzyl viologen presents a confusing picture because the enzyme interacts with an apparently nonpenetrant dye both in intact cells and in inside-out membrane vesicles. If BV^{2+} is really nonpermeant and if electron transfer is taking place within the hydrogenase molecule as suggested to occur in other hydrogenases from hydrogen–deuterium exchange studies (Tamyia and Miller, 1963), it may be that BV^{2+} can accept electrons from H_2 by interacting either directly with the active site (e.g., with solubilized hydrogenase or with inside-out membrane vesicles) or indirectly (as in intact spheroplast). The most likely explanation however is that BV^{2+} does cross the cytoplasmic membrane of *P. denitrificans* under the conditions that have been used. Further studies, e.g., lactoperoxidase-catalyzed radioiodination and eventual protein sequence data, are required to provide additional insight into the precise topography of the hydrogenase. The purification procedure is available (Sim and Vignais, 1979), and so such studies are now possible. The number of membrane proteins for which extensive structural information is available is limited; thus the hydrogenase from *P. denitrificans* may represent a new class of intrinsic membrane protein.

The only other membrane hydrogenase that has been investigated from the point of view of its orientation in the membrane is the hydrogenase from *E. coli* (Jones, 1979a,b, 1980). In this organism the reduction of benzyl viologen by H_2 has been studied, and the release of protons has been investigated by measuring the quenching of atebrine fluorescence. In intact cells, BV^{2+} is not reduced, but the viologen dye is reduced in sonicated cell preparations, and protons are released into the interior of inverted vesicles of *E. coli* membranes, indicating that proton translocation is outward from intact cells.

Jones (1980) measured also the H_2-dependent reduction of BV^{2+} to its radical (BV^+) by spheroplasts of *E. coli* and observed that protons were liberated at the periplasmic aspect of the cytoplasmic membrane—a conclusion opposite to that of Porte *et al.* (1979) and Doussière *et al.* (1980) for the hydrogenase of *P. denitrificans*. In this latter case an outward proton ejection was observed, however, when spheroplasts of *P. denitrificans* were pulsed with small amounts of H_2-saturated water in the presence of electron acceptors of redox potential higher than that of benzyl viologen, such as cytochrome *c*, ferricyanide, or oxygen (Doussière *et al.*, 1980, and unpublished results). In his experiment, Jones (1980) incubated *E. coli* spheroplasts with benzyl viologen and dithionite, then aerated the suspension to reoxidize intracellular BV^+ and again added more dithionite to reduce external BV^{2+}. During the vigorous aeration step some dithionite was probably oxidized to bisulfite. Mayhew (1978) has shown that the redox potential of the couple dithionite/bisulfite varies with the pH of the medium and the concentration of the components. This author reported that, in the presence of methyl viologen and hydrogenase, dithionite can even be formed as a product of the reduction of bisulfite by H_2. Similarly it may be that bisulfite or some other oxidation product of dithionite able to interact with the membrane, but not benzyl viologen, was the true electron acceptor in the experiment of Jones (1980), and that the observed proton release resulted from the functioning of an artificial loop of the Mitchellian type. These differing results for the orientation of the hydrogenases from *E. coli* and *P. denitrificans* are not incompatible, however, since the hydrogenases in these bacteria have very different physiological roles.

It is evident that hydrogenases are a heterogeneous group of iron–sulfur proteins, and much more structural and functional information will be required before the interrelationships of these enzymes can be established. Several hydrogenases have a subunit of around 60,000 MW, including the hydrogenase from *P. denitrificans*. To determine whether or not this has any evolutionary significance will require more information on the molecular structure of the hydrogenases, including sequence determi-

nation. Since the electron-transport chain of *P. denitrificans* has been investigated very intensively, it is a good candidate for determining how the hydrogenase is coupled to the rest of the electron-transport chain. This may provide a model for the mechanism of association of other membrane-bound hydrogenases with the electron-transport chains of the organisms in which they are found.

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