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Mechanisms of sodium transport in bacteria

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In some bacteria, an Na^+ circuit is an important link between exergonic and endergonic membrane reactions. The physiological importance of Na^+ ion cycling is described in detail for three different bacteria. *Klebsiella pneumoniae* fermenting citrate pumps Na^+ outwards by oxaloacetate decarboxylase and uses the Na^+ ion gradient thus established for citrate uptake. Another possible function of the Na^+ gradient may be to drive the endergonic reduction of NAD^+ with ubiquinol as electron donor. In *Vibrio alginolyticus*, an Na^+ gradient is established by the NADH: ubiquinone oxidoreductase segment of the respiratory chain; the Na^+ gradient drives solute uptake, flagellar motion and possibly ATP synthesis. In *Propionigenium modestum*, ATP biosynthesis is entirely dependent on the Na^+ ion gradient established upon decarboxylation of methylmalonyl-CoA. The three Na^+-translocating enzymes, oxaloacetate decarboxylase of *Klebsiella pneumoniae*, NADH: ubiquinone oxidoreductase of *Vibrio alginolyticus* and ATPase (F_1F_0) of *Propionigenium modestum* have been isolated and studied with respect to structure and function. Oxaloacetate decarboxylase consists of a peripheral subunit (α), that catalyses the carboxyltransfer from oxaloacetate to enzyme-bound biotin. The subunits β and γ are firmly embedded in the membrane and catalyse the decarboxylation of the carboxybiotin enzyme, coupled to Na^+ transport. A two-step mechanism has also been demonstrated for the respiratory Na^+ pump. Semiquinone radicals are first formed with the electrons from NADH; subsequently, these radicals dismutate in an Na^+-dependent reaction to quinone and quinol. The ATPase of *P. modestum* is closely related in its structure to the F_1F_0 ATPase of *E. coli*, but uses Na^+ as the coupling ion. A specific role of protons in the ATP synthesis mechanism is therefore excluded.

1. Introduction

Bacteria are among the organisms of fastest growth and most active metabolism. This requires rapid exchange of matter between the environment and the bacterial interior. Effective substrate uptake systems exist therefore in the bacterial membranes. Many of these catalyse an active transport against the concentration gradient and utilize an electrochemical cation gradient as driving force. For a continuous operation of this machinery, the cation gradient must be regenerated by an additional transport system.

Protons are the most important coupling cations, but under certain conditions, Na^+ ions can replace H^+ in this function. Thus Na^+–solute co-transport systems are widely distributed among different bacterial species (Lanyi 1979). In many examples, the Na^+ ion gradient required to drive these transport systems is generated by an Na^+/H^+ antiporter. In these instances the Na^+-coupled transport systems are indirectly linked to the proton motive force. The Na^+ motive force may, however, also be generated by primary Na^+ pumps: certain decarboxylases of anaerobic bacteria catalyse the active extrusion of Na^+ ions from the

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cytoplasm (Dimroth 1987). Another primary Na^+ pump is the NADH: ubiquinone oxidoreductase of marine bacteria (Tokuda & Unemoto 1982; Ken-Dror *et al.* 1986) and of citrate fermenting *Klebsiella pneumoniae* (Dimroth & Thomer 1989). The Na^+ ion gradients established by these primary Na^+ pumps are not only used to drive solute uptake, but also for flagellar movement and for ATP synthesis (Dimroth 1987). In this article, I use selected examples to review recent studies on the physiological function of Na^+ ions in connecting exergonic and endergonic membrane reactions and on the mechanisms of the catalysts involved.

2. Selected examples for the physiological function of Na^+ ion cycling in bacteria

(*a*) Klebsiella pneumoniae *fermenting citrate*

Klebsiella pneumoniae and *Salmonella typhimurium* induce the enzymes of the citrate fermentation pathway citrate lyase and oxaloacetate decarboxylase during anaerobic growth on citrate (Dimroth 1987). The lyase cleaves citrate to acetate and oxaloacetate, and the latter is decarboxylated to pyruvate by oxaloacetate decarboxylase. Pyruvate is subsequently split to acetyl-CoA and formate by pyruvate formate lyase. Acetyl-CoA is converted to acetyl phosphate, from which ATP is formed by substrate-level phosphorylation. Thus 1 mol of ATP is synthesized in these cells per 1 mol of citrate fermented. Additional energy is conserved in the oxaloacetate decarboxylation step; the decarboxylase is a membrane-bound biotin-containing enzyme that functions as an Na^+ pump, transporting two mol Na^+ ions out of the cell per mol oxaloacetate decarboxylated (figure 1) (Dimroth 1982*b*, 1987).

The Na^+ transporting function of oxaloacetate decarboxylase was discovered in studies with inverted bacterial membrane vesicles (Dimroth 1982*b*). Sodium ions were rapidly accumulated by these vesicles upon oxaloacetate addition. In the presence of avidin, oxaloacetate decarboxylation and Na^+ transport were simultaneously inhibited. The transport of Na^+ ions was electrogenic creating a membrane potential ($\Delta\psi$) of about 65 mV and a Na^+ concentration gradient (ΔpNa^+) equivalent to 49 mV. The total sodium motive force ($\Delta\tilde{\mu}Na^+$), which, according to the chemiosmotic theory (Mitchell 1961) is the sum of the electrical and chemical potentials (equation 1) amounts to about 114 mV (Dimroth 1982*b*):

$$\Delta\tilde{\mu}Na^+ = \Delta\psi - [2.3\,RT/F]\Delta pNa^+. \qquad (1)$$

In the initial phase of the transport, 2 mol Na^+ ions were translocated per mol of oxaloacetate decarboxylated. The Na^+:oxaloacetate stoichiometry decreased continuously from 2 to 0, as the Na^+ ion gradient increased, and at thermodynamic equilibrium between the chemical and the transport reaction, the decarboxylation proceeded apparently uncoupled from Na^+ transport. This situation is unlikely to pertain under physiological conditions and we expect that the size of the Na^+ gradient in growing bacteria reflects an Na^+:oxaloacetate coupling ratio close to 2 (Dimroth 1987).

What is the physiological function of the Na^+ ion gradient established by oxaloacetate decarboxylase? It has been shown that citrate fermenting *Klebsiella pneumoniae* cells express an Na^+-dependent citrate transport system. Uptake and degradation of citrate by whole cells was Na^+-dependent. The transport was severely inhibited by carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone, which could indicate that the membrane potential contributed to its driving

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

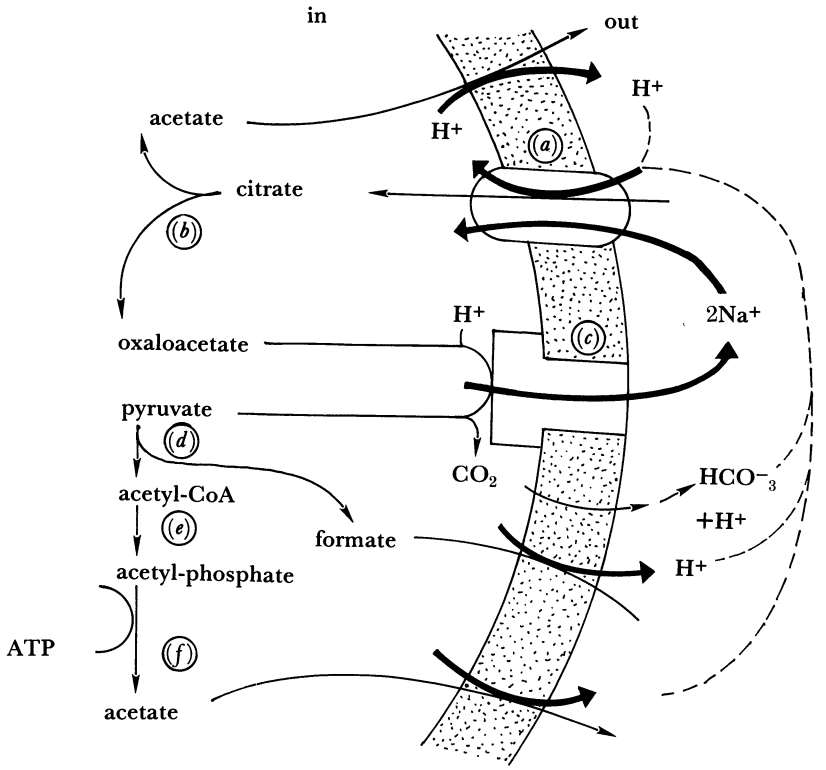

FIGURE 1. The citrate fermentation pathway of *Klebsiella pneumoniae* with an Na^+ circuit as a possible coupling mechanism of citrate uptake and oxaloacetate decarboxylation and a proton circuit as a possible coupling device between citrate uptake and end product extrusion. (*a*) Citrate uptake system; (*b*) citrate lyase; (*c*) oxaloacetate decarboxylase; (*d*) pyruvate formate lyase; (*e*) phosphotransacetylase; (*f*) acetate kinase.

force (Dimroth & Thomer 1986). However, recent experiments performed with reconstituted proteoliposomes indicated an electroneutral citrate transport mechanism (P. Dimroth, unpublished results). This implies that cations containing together three positive charges are taken up with citrate. As oxaloacetate decarboxylase pumps two Na^+ ions out of the cell, the Na^+ cycle is completed, if citrate uptake is supported with two Na^+ ions. The third cation could be a proton, which could recycle by an extrusion of the metabolic end products acetate, formate or carbonate together with protons (figure 1) (Dimroth & Thomer 1986).

This citrate transport mechanism appears to be ingeniously designed for the citrate-fermenting *K. pneumoniae* cells, because the growth substrate is actively accumulated without ATP consumption. This is essential for these cells, as only 1 mol ATP is synthesized per mol of citrate fermented (figure 1). This ATP yield would just be sufficient to recycle the protons and metal ions taken up with citrate, assuming the following stoichiometries: $(H^+ + Me^+)$: citrate = 3; $(H^+ + Me^+)$: ATP = 3.

Citrate fermenting *Klebsiella pneumoniae* cells also contain an Na^+/H^+ antiporter and an H^+ translocating ATPase (Dimroth & Thomer 1986). Thus, theoretically, some ATP could be synthesized by the decarboxylation of oxaloacetate via the cycling of Na^+ and H^+ ions. Another system of interest in the membrane of these cells is a NADH: ubiquinone oxidoreductase functioning as an Na^+ pump (Dimroth & Thomer 1989). NADH oxidation by inverted bacterial membrane vesicles was specifically enhanced by Na^+ ions, and these cations were accumulated inside the vesicles upon NADH oxidation. The transport and the chemical

reaction were simultaneously inhibited by low concentrations of heptyl hydroxyquinoline N-oxide (HQNO). A Triton X-100 extract of the bacterial membranes catalysed the stoichiometric reduction of ubiquinone-1 with NADH. This was Na^+-dependent and HQNO-sensitive. The site of Na^+ action therefore appears to be at the NADH: ubiquinone oxidoreductase step of the respiratory chain.

The significance of the respiratory Na^+ pump for these cells is not at all clear. One possible explanation could be the formation of NADH by Na^+-driven reversed electron flow from ubiquinol, which would be formed by formate oxidation. The oxaloacetate decarboxylase derived Na^+ gradient could thus have an additional function in these cells. The formation of NADH for biosyntheses may be a problem during citrate fermentation in *K. pneumoniae*, as the oxidative branch of the tricarboxylic acid cycle is blocked by repression of 2-oxoglutarate dehydrogenase (O'Brien 1975).

(*b*) Vibrio alginolyticus

Vibrio alginolyticus is a facultative anaerobic marine bacterium that requires Na^+ ions for growth and other physiological functions. In these bacteria, the respiratory Na^+ pump described above for *K. pneumoniae*, was discovered (Tokuda & Unemoto 1982). On aerobic growth, *Vibrio* cells establish an electrochemical Na^+ gradient by virtue of the Na^+-motive NADH: ubiquinone oxidoreductase segment of the respiratory chain. This enzyme has a pH optimum in the alkaline pH range (i.e. greater than 8) and is specifically inhibited by HQNO. The Na^+ ion gradient generated by this pump may have several different physiological functions (figure 2): the bacteria are non-motile in the absence of Na^+ and acquire mobility in its presence, suggesting the operation of an Na^+-dependent flagellar motor in *V. alginolyticus* (Dibrov *et al.* 1986*a*); several transport systems were shown to be Na^+-dependent (Tokuda & Unemoto 1982); the ATP level of bacterial cells increased after application of an Na^+ pulse, which could indicate the operation of an Na^+-motive ATP synthase (Dibrov *et al.* 1986*b*).

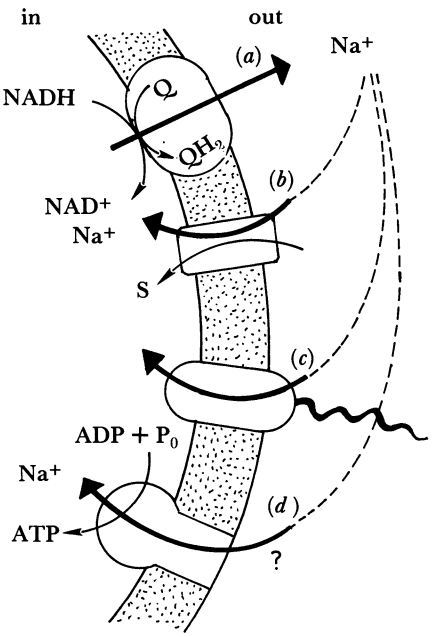

FIGURE 2. The Na^+ cycle in *Vibrio alginolyticus* as a possible link between (*a*) respiratory Na^+ pump, (*b*) Na^+-dependent transport, (*c*) flagellar motor and (*d*) ATP synthesis.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(*c*) Propionigenium modestum

Propionigenium modestum is a strictly anaerobic bacterium that can grow from the fermentation of succinate to propionate and CO_2. The pathway of succinate fermentation, shown in figure 3, (Hilpert *et al.* 1984) involves the intermediates succinyl-CoA, (R)- and (S)-methylmalonyl-CoA and propionyl-CoA. The only step in this pathway, that is sufficiently exergonic to be used for energy conservation, is the decarboxylation of (S)-methylmalonyl-CoA ($\Delta G^{0'} \approx -27$ kJ mol^{-1}). The decarboxylase is a membrane-bound Na^+ pump, related to oxaloacetate decarboxylase of *K. pneumoniae* and thus converts the free energy of the decarboxylation reaction into an electrochemical Na^+ gradient (Hilpert *et al.* 1984). This Na^+ gradient serves *P. modestum* as the only energy source for ATP synthesis. The bacteria contain an Na^+-stimulated ATPase in their membrane that uses the Na^+ gradient established by the decarboxylase directly for ATP synthesis (Laubinger & Dimroth 1987, 1988).

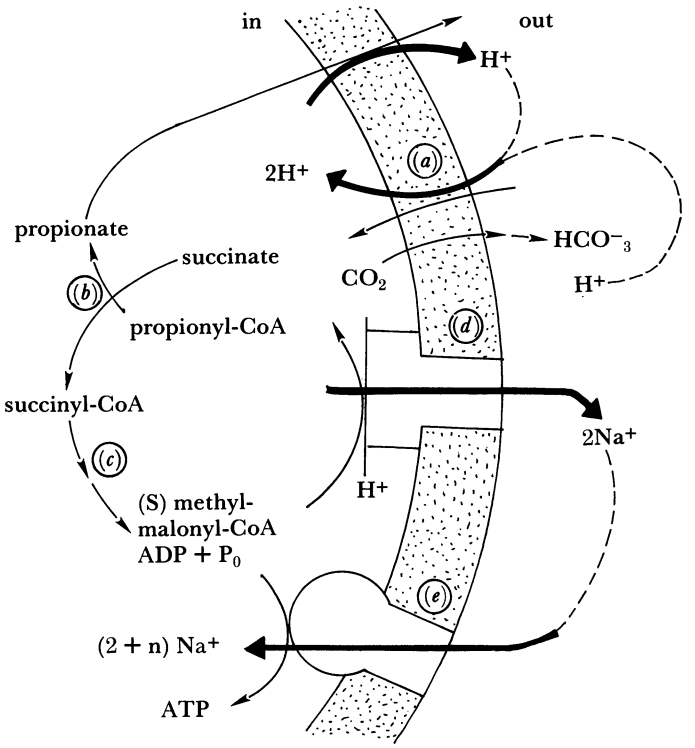

FIGURE 3. Energy metabolism of *Propionigenium modestum* with a Na^+ cycle coupling the exergonic decarboxylation of (S)-methylmalonyl-CoA to endergonic ATP synthesis. A hypothetical proton circuit could couple succinate uptake with the extrusion of propionate and CO_2. (*a*) Succinate uptake system; (*b*) succinate propionyl-CoA: CoA transferase; (*c*) methylmalonyl-CoA mutase and methylmalonyl-CoA epimerase; (*d*) methylmalonyl-CoA decarboxylase; (*e*) ATPase.

The stoichiometric relations are of special interest in this mechanism of decarboxylation phosphorylation: two mol Na^+ ions are pumped out of the cell per decarboxylation of 1 mol methylmalonyl-CoA at $\Delta G^{0'} \approx 1$–27 kJ mol^{-1} (Dimroth & Hilpert 1984). As the energy requirement for ATP synthesis from ADP and inorganic phosphate is about 50 kJ mol^{-1} under physiological conditions (Thauer *et al.* 1977) it is expected that 4 mol Na^+ ions pass through the ATPase into the cells to meet the energetic requirements of ATP synthesis. These considerations

have shed some light on the minimum quantum of energy that must be acquired by an organism per mole of substrate metabolized (Hilpert *et al.* 1984). It is not an equivalent sufficient to synthesize 1 mol of ATP, but only that fraction contained in 1 mol of the electrochemical cation gradient that energizes ATP synthesis (an amount equivalent to $\frac{1}{4}$ ATP, if the H^+ (Na^+): ATP stoichiometry is 4). *Propionigenium modestum* is therefore able to grow on the decarboxylation of succinate to propionate at a $\Delta G^{0'}$ of -21 kJ mol^{-1}, which is not sufficient for ATP biosynthesis. In view of the low energy output of the fermentation reaction, it is not conceivable that the Na^+ gradient established by methylmalonyl-CoA decarboxylase could drive succinate uptake in addition to ATP synthesis. We assume therefore that succinate enters the cells in symport with protons and that these protons recycle by extrusion with the metabolic end products propionate and carbonate. Na^+ and H^+ ions could thus both cycle through the membrane of *P. modestum*; the Na^+ ions in connecting exergonic and endergonic chemical reactions and the protons in connecting substrate uptake and product release (figure 3).

3. Catalytic mechanisms of Na^+ translocating enzymes

(*a*) *Oxaloacetate decarboxylase*

Oxaloacetate decarboxylases of *Klebsiella pneumoniae* or *Salmonella typhimurium* are membrane-bound biotin-containing enzymes that are specifically activated by Na^+ ions and pump these cations through the membrane (Dimroth 1982*b*, 1982*c*, 1987). Related enzymes have been found in other anaerobic bacteria; methylmalonyl-CoA decarboxylase occurs in lactate fermenting *Veillonella alcalescens* (Hilpert & Dimroth 1983) and in succinate fermenting *Propionigenium modestum* (see above) (Hilpert *et al.* 1984), and glutaconyl-CoA decarboxylase in the glutamate fermenting bacteria *Acidaminococcus fermentans*, *Peptostreptococcus asaccharolyticus* and *Clostridium symbiosum* (Buckel & Semmler 1983). The biotin content of these enzymes was exploited for their purification by affinity chromatography on avidin-Sepharose (Dimroth 1982*a*, 1987).

The decarboxylases are also related with respect to composition and function of their subunits (Dimroth & Thomer 1983; Hilpert & Dimroth 1983; Buckel & Liedtke 1986; Hoffmann *et al.* 1989). They all contain a peripheral membrane-bound subunit with relative molecular mass (M_r) 60000–65000 that catalyses the transfer of the carboxyl group from the substrate to the prosthetic biotin group on the enzyme (carboxyltransferase; equation 2);

$$R{-}COO^- + \text{biotin}{-}\text{protein} \rightleftharpoons RH + {}^-OOC{-}\text{biotin}{-}\text{protein}. \qquad (2)$$

In addition, all decarboxylases contain a more firmly membrane-bound subunit of M_r 30000–35000 that catalyses the Na^+-dependent decarboxylation of the carboxybiotin-protein (lyase; equation 3), which is believed to be coupled to Na^+ translocation;

$$^-OOC{-}\text{biotin}{-}\text{protein} + H^+ + 2\ Na^+_{in} \rightleftharpoons CO_2 + \text{biotin}{-}\text{protein} + 2\ Na^+_{out}. \qquad (3)$$

Oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase contain an additional integral membrane protein of M_r 12000 and 14000 respectively, that may be related within these two enzymes. A protein of this size is not found, however, in glutaconyl-CoA decarboxylase. Considerable variation is also evident with respect to the biotin-binding subunit: the prosthetic group is located on a separate peptide of M_r 18500 and 120000 in methylmalonyl-CoA decarboxylase and glutaconyl-CoA decarboxylase, respectively; in

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

oxaloacetate decarboxylase the biotin binding domain is covalently attached to the carboxyltransferase subunit (α-chain, M_r 65000) (Dimroth 1987).

Investigations of oxaloacetate decarboxylase by electron microscopy (Däkena *et al.* 1988) revealed a cleft in the α-subunit, with the prosthetic biotin group located at its bottom in close proximity to the β- and γ-subunits. The existence of two different domains in the α-chain of oxaloacetate decarboxylase was also deduced from the results of limited proteolysis (Dimroth & Thomer 1983) as well as from sequencing studies (Schwarz *et al.* 1988). Trypsin cleaved the α-chain into an *N*-terminal domain of M_r 53000 and a C-terminal biotin-domain of M_r 12000, thereby completely inactivating the enzyme. The sequence of the N-terminal domain was strikingly homologous to that of the 5 *S* subunit of transcarboxylase from *Propionibacterium shermanii*, that catalyses exactly the same carboxyl-transfer reaction as the α-chain of oxaloacetate decarboxylase (cf. equation 3). The sequence of the C-terminal domain was strikingly homologous to the 1.2 *S* biotin subunit of transcarboxylase and other biotin-containing peptides (Schwarz *et al.* 1988). A remarkable sequence that is unique in the α-chain of oxaloacetate decarboxylase is found between residues −59 and −28 upstream of the biotin-binding lysine residue. This sequence is: ala—ala—ala—pro—ala—pro—ala—pro—ala—pro—ala—pro—ala—ser—ala—pro—ala—ala—ala—ala—pro—ala. Such an extended area of mostly alanine and proline residues may provide a point of flexibility within the protein structure to allow the flip-flop movement of the biotin group between the two catalytic centres on the α- and β-subunit, respectively. It is interesting in this context that the dihydrolipoamide acetyltransferase subunit of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli* contains a sequence rich in alanine and proline residues a similar distance from the dihydrolipoamide prosthetic group (Stephens *et al.* 1983). This part of the sequence is highly mobile, as shown by nuclear magnetic resonance (NMR) spectroscopic techniques (Radford *et al.* 1987).

The structural relation between the α-subunit of oxaloacetate decarboxylase and the 5 *S* and 1.2 *S* subunits of transcarboxylase is interesting from an evolutionary point of view. It suggests that enzyme complexes with very distinct overall functions such as the Na^+ transporting oxaloacetate decarboxylase and the transcarboxylase have developed by an assembly of subunits that derived in part from a common ancestral gene.

Valuable information on the function of the individual subunits was obtained by following the reversible dissociation of the enzyme complex (Dimroth & Thomer 1988). The decarboxylase dissociates upon freezing and thawing in the presence of chaotropic salts, e.g. LiCl or $LiClO_4$, and reassembles on dilution, as shown by the disappearance and recovery respectively, of catalytic activity. The results of figure 4 indicate that the pH is of central importance for the dissociation and reconstitution of the enzyme complex. Whereas an acidic pH promotes dissociation, a more alkaline pH promotes reconstitution. Plots of initial rate of either dissociation or reconstitution against pH gave sigmoidal curves with inflection points around pH 6.5. The sharp decrease in dissociation and increase in reconstitution above pH 6.5 indicates that a single ionizable group with a pK of about 6.5 (probably contributed by a histidine residue) is involved in these processes. If this group is positively charged, the enzyme complex is destabilized and dissociation takes place; the uncharged form of this residue appears to be required for the reconstitution of the decarboxylase from its subunits.

After dissociation, the α-chain could be separated from the β- and γ-subunits by chromatography on avidin–Sepharose in the denaturing solvent (Dimroth & Thomer 1988).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

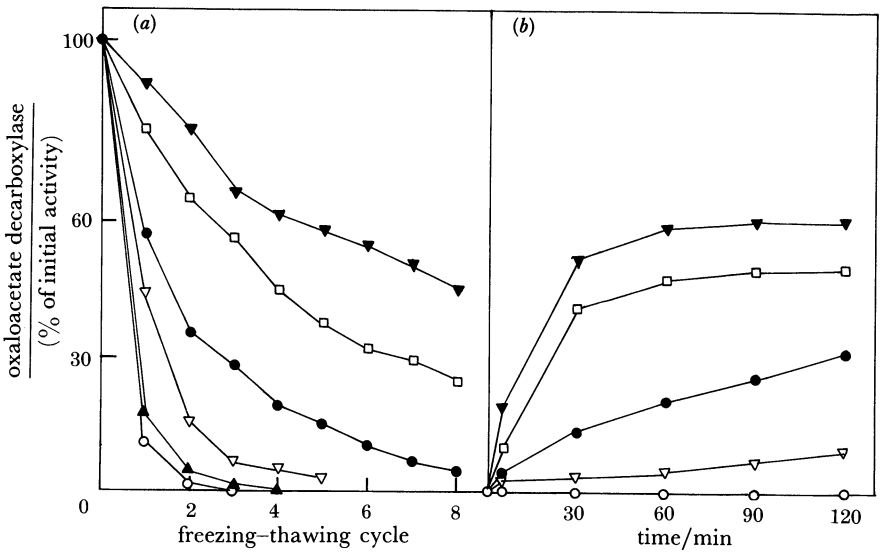

FIGURE 4. Reversible dissociation of oxaloacetate decarboxylase. (*a*) The decarboxylase (85 μg) in 75 μl 40 mM Mes/Tris buffer of the respective pH, containing 0.8 M LiCl, was frozen in liquid nitrogen and thawed at 25 °C, followed by the determination of enzymic activity; 100% activity corresponds to 3.5 U. (*b*) Decarboxylase, completely inactivated by 4 cycles of freezing and thawing at pH 5.5, was diluted 1:9 with 50 mM Mes/Tris buffer of the pH indicated and incubated at 25 °C. Enzymic activity recovered after various times is recorded as percentage of the initial activity (before dissociation); (○), pH 5.0; (▲), pH 5.5; (▽), pH 6.4; (●), pH 6.5; (□), pH 7.0; (▼), pH 7.5.

Subsequently, the enzyme complex has been reconstituted from the isolated α- and β- plus γ-subunits. The reconstituted enzyme recovered all functions of the native decarboxylase, including that of Na^+ transport. The Na^+ transport function was also recovered by a sequential reconstitution; after incorporation of the β- and γ-subunits into proteoliposomes, the reconstitution was completed by adding the α-subunit. Interestingly, the Na^+ conductance of the proteoliposomes was low and was not increased if only the β- and γ-subunits were incorporated (P. Dimroth, unpublished observations). These membrane-bound subunits therefore appear not to constitute an open Na^+ channel analogous to the proton conducting properties suggested for the F_0 portion of the F_1F_0 ATPases (Schneider & Altendorf 1987). A hypothetical model summarizing the data discussed above on the structure and function of the oxaloacetate decarboxylase subunits is shown in figure 5.

(*b*) *NADH: ubiquinone oxidoreductase*

The NADH: ubiquinone oxidoreductases of *Vibrio alginolyticus* (Tokuda & Unemoto 1984) and of citrate fermenting *Klebsiella pneumoniae* (Dimroth & Thomer 1989) have been demonstrated to function as electrogenic Na^+ pumps, and the reductase of the marine bacterium Ba_1 is likely to have an analogous function (Ken-Dror *et al.* 1986). The enzyme of *V. alginolyticus* has been purified and studied in most detail (Hayashi & Unemoto 1984, 1987). It is composed of three different subunits α, β and γ, with apparent relative molecular masses of 52000, 46000 and 32000, respectively. The relative molecular mass of the complex is 254000 and the subunit stoichiometry appears to be $\alpha_2:\beta_2:\gamma_2$. The α-subunit contains FMN, whereas the β-subunit contains FAD. The isolated β-subunit catalysed NADH oxidation in the presence of ubiquinone-1, but not its reduction to ubiquinol-1 and has therefore been termed as NADH

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

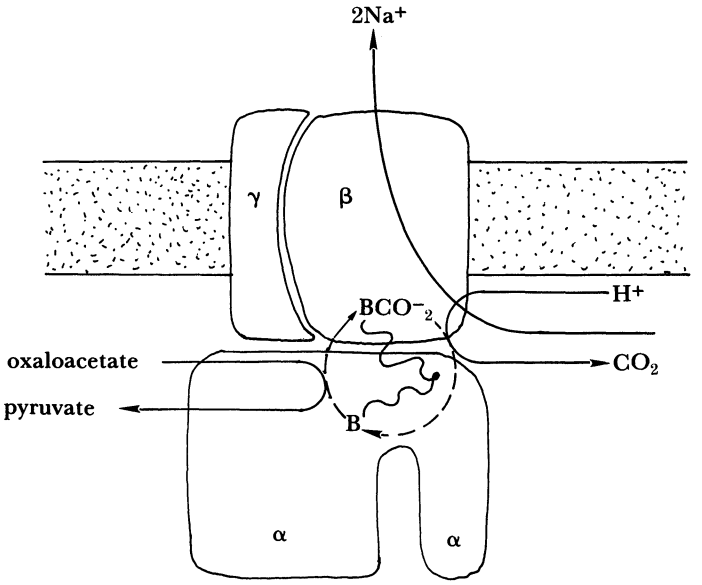

FIGURE 5. A hypothetical model linking structure and function of the oxaloacetate decarboxylase subunits.

dehydrogenase. The electrons apparently pass through semiquinone radicals to oxygen-yielding superoxide radicals, which have been assayed by the reduction of acetylated cytochrome *c*. The NADH dehydrogenase activity was completely independent of Na^+ ions. The reduction to ubiquinol is accomplished by a dismutation of the ubisemiquinone radicals to ubiquinone and ubiquinol and is apparently catalysed by the α-subunit. In the presence of all three subunits, NADH caused an Na^+-dependent and HQNO-sensitive reaction sequence (Hayashi & Unemoto 1984). A hypothetical mechanism is shown in figure 6.

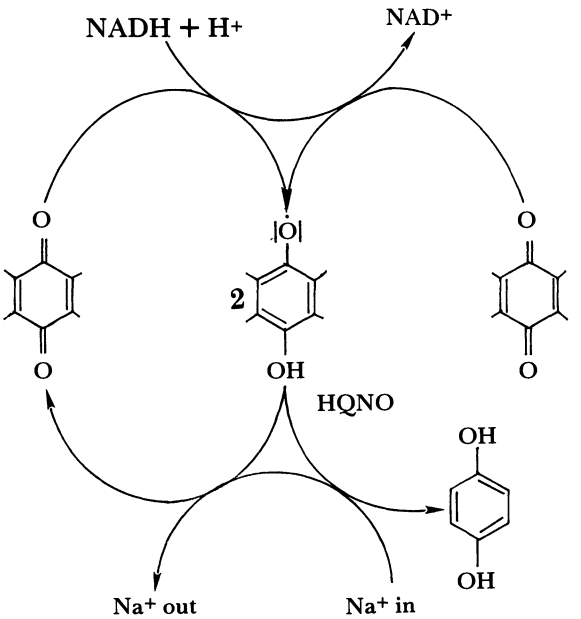

FIGURE 6. Hypothetical mechanism of the respiratory Na^+ pump of marine bacteria and of *Klebsiella pneumoniae*.

Evidence that the Na^+-dependent dismutation of the semiquinone represents the actual Na^+ pump has been obtained from experiments with proteoliposomes containing the purified NADH: ubiquinone oxidoreductase ($\alpha_2:\beta_2:\gamma_2$) (Hayashi & Unemoto 1987). A membrane potential, indicative of Na^+ translocation, was established on electron transfer from NADH to ubiquinone-1 yielding ubiquinol-1 and decreased successively after the complete reduction of the quinone. No membrane potential was obtained however, in the presence of menadione, which accepts electrons of NADH from some intermediate of the electron transfer chain, before the Na^+- and HQNO-sensitive reaction step. The functional relation among the Na^+-translocating NADH: ubiquinone oxidoreductases from different bacteria appears to extend to the catalytic mechanism. In *Klebsiella pneumoniae* (Dimroth & Thomer 1989) and the bacterium Ba_1 (Ken-Dror *et al.* 1986), electron transfer from NADH to ubiquinone yielding ubiquinol involves the intermediate formation of ubisemiquinone radicals, and the Na^+-dependent and HQNO-sensitive step is the dismutation of the semiquinone radicals to quinol and quinone.

(*c*) *ATPase of* Propionigenium modestum

As described above, the ATPase of *P. modestum* uses Na^+ instead of H^+ as a coupling ion for ATP synthesis. In spite of this unusual property, the enzyme has a typical F_1F_0 structure (Laubinger & Dimroth 1987, 1988). The F_1 portion, which contains the ATP hydrolysing activity, has been dissociated from the membrane-bound F_0 moiety by incubation with EDTA at low ionic strength and pH 8.0 and purified to homogeneity. The enzyme consists of five different subunits, α, β, γ, δ and ε, with similar molecular masses and staining intensities on SDS gels as the corresponding five F_1 ATPase subunits of *E. coli*. The purified F_1 ATPase was not stimulated by Na^+ ions in contrast to the membrane-bound enzyme (F_1F_0). Upon reconstruction of the enzyme complex from purified F_1 and F_1-depleted membranes, the activation by Na^+ ions was recovered, thus indicating that the Na^+ binding site is located on the F_0 moiety, not on F_1 (Laubinger & Dimroth 1987).

Following solubilization of F_1F_0 by Triton X-100, the enzyme complex was purified. It contains, in addition to the five F_1 subunits, three subunits (a, b, c) that correspond in molecular mass to the three F_0 subunits of the *E. coli* ATPase. The enzyme complex was activated about tenfold by Na^+ ions (5 mM). It exhibited the usual inhibition characteristics of prokaryotic F_1F_0 ATPases: dicyclohexyl carbodiimide (DCCD), venturicidin, tributyltin chloride and azide were powerful inhibitors, whereas vanadate was not inhibitory. Analogous to H^+ translocating ATPases, DCCD was specifically bound to subunit *c* of the *P. modestum* enzyme. This subunit forms unusually stable aggregates of probably six polypeptide chains, which resist dissociation by SDS even at 100 °C; 121 °C is required to dissociate the complex (Laubinger & Dimroth 1988).

The close relation between the ATPases of *P. modestum* and *E. coli* was further substantiated by immunological and sequencing studies. Antibodies raised against the β-subunit of the ATPase of *E. coli* or *Sulfolobus acidocaldarius* cross-reacted with the β-subunit of the *P. modestum* ATPase (P. Dimroth, unpublished results). The sequence homology between the ATPases of *P. modestum* and *E. coli* was 69% for the β-subunit (Amann *et al.* 1989) and 21% for the 41 N-terminal residues of subunit c (P. Dimroth, unpublished results).

The function of the *P. modestum* ATPase as a primary Na^+ pump (the reversal of the physiological reaction) was firmly secured by reconstitution experiments. Sodium ions were pumped into proteoliposomes containing the ATPase in response to ATP hydrolysis. The

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

transport and the chemical reaction were simultaneously inhibited by DCCD. The transport was electrogenic, and the transport rate increased about fourfold after dissipation of the rate-determining membrane potential with either valinomycin or the uncoupler carbonylcyanide *m*-chloro phenylhydrazone (CCCP) (Laubinger & Dimroth 1988). Stimulation rather than inhibition of Na^+ transport by the uncoupler firmly substantiates the functioning of the ATPase as a primary Na^+ pump and excludes the possibility of an Na^+ transport by the combined action of an H^+-translocating ATPase and an Na^+/H^+ antiporter. Amiloride, an inhibitor of such an antiporter, was without effect on the Na^+ transport and thus further confirms the conclusion that the ATPase acts as a primary Na^+ pump.

It is interesting that at unphysiologically low Na^+ concentrations (i.e. less than 1 mM) the ATPase of *P. modestum* pumped protons (Dimroth & Laubinger 1987; Laubinger & Dimroth 1989). The rate of proton pumping increased at slightly acidic pH values (optimum at pH 6), but did not exceed 10% of the Na^+ transport rate at neutral pH. Interestingly, proton pumping decreased from its maximum value to zero, as the Na^+ concentration increased from 0 to 1 mM, whereas the Na^+ transport rate increased from 0 to about 80% of its maximum value simultaneously. We concluded from these and other data that the ATPase of *P. modestum* is a proton pump at low Na^+ concentrations and switches to a Na^+ pump, if the Na^+ concentration increases. Under physiological conditions (Na^+ concentration inside the cells > 20 mM), the ATPase will exclusively act as an Na^+ pump. It should be noted that the V_{max} of the ATPase for Na^+ is ten-times higher than for H^+, but the affinity for protons exceeds that for Na^+ by three orders of magnitude.

Recent experiments performed in our laboratory increase our understanding of the relation between the Na^+-translocating ATPase of *P. modestum* and H^+-translocating ATPases. Enzyme hybrids, constructed with the F_0 moiety of the *P. modestum* enzyme and the F_1 ATPase of either *E. coli* or the thermophilic bacterium PS3 were highly efficient Na^+ pumps, whereas the F_1F_0 ATPase of *E. coli* is unable to pump Na^+ ions. The F_0 part thus clearly defines the specificity for the coupling cation, and the ion translocation through F_0 apparently triggers ATP synthesis within the F_1 moiety independent of whether the translocated cation is Na^+ or H^+. The change in cation specificity in F_0 could be caused by relatively minor alterations at the cation binding site, not significantly affecting the recognition site for F_1, as suggested from the formation of functional hybrids described above.

These findings have consequences for possible mechanisms of ATPases in general. Thus any proposal in which H^+ ions play a specific role that could not be substituted by Na^+ must be dismissed. One such proposal by Mitchell (1974) assumes a direct participation of the vectorial protons in the chemistry of ATP synthesis at the catalytic site. The protons are supposed to flow down the electrochemical gradient through F_0F_1 into the catalytic site, where they attack one of the oxygen atoms of phosphate, forming H_2O and $H_2PO_3^+$, which can directly react with ADP to form ATP. As this mechanism is obviously restricted to protons as coupling ions, it is not favoured by the detection of an Na^+-coupled ATPase. However, the alternative suggestion of Boyer (1975) that the energy available from the electrochemical gradient is used to promote substrate binding and product release by a distinct set of conformational changes is in accord with either H^+ or Na^+-coupled ATP synthesis.

A specific proton conduction mechanism, suggested for ATPases, is that of a 'proton wire', in which a hydrogen-bonded chain of protonatable groups adds H^+ on one side of the membrane and delivers an H^+ on the other side (Nagle & Morowitz 1978). The use of Na^+ or H^+ as

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

coupling cations by the *P. modestum* ATPase suggests a common conduction mechanism for these cations and argues therefore against 'proton wires'. Recently, an interesting proposal was made by Boyer (1988), that the cations may interact with the enzyme through coordination complexes with appropriately positioned oxygen or nitrogen atoms, or both. From analogy with coordination complexes formed by crown ethers, either Na^+ or H_3O^+ could be complexed by the protein. The translocation would be completed by a conformational change, which exposes the cation binding region to solute on the other side of the membrane. This mechanism is an attractive model for the conduction of Na^+ or H^+ by the same route and is thus fully in accord with our experimental data.

An alternative coupling with Na^+ or H^+ is not unique for the *P. modestum* ATPase. The Na^+/K^+ ATPase of pig kidney cells likewise switches from Na^+ to H^+ pumping, if Na^+ ions are absent (Hara & Nakao 1986). Other examples of membrane proteins that may have a common conduction mechanism for protons and metal ions (Na^+ or Li^+) are the melibiose carrier of *E. coli* (Tsuchiya & Wilson 1978) and the alanine carrier of the thermophilic bacterium PS3 (Hirata *et al.* 1984). The melibiose carrier accepts Na^+, H^+ or Li^+ as the co-transported cation, depending in part on the sugar transported. Numerous mutants have been isolated in which the sugar or cation specificity, or both, were affected (Kawakami *et al.* 1988; Botfield & Wilson 1988). All these mutations consisted of single amino-acid substitutions clustered mainly in three areas of the porter, which could thus reflect the cation binding region. In several such mutants, the ability to couple melibiose transport to H^+ was lost, and either Na^+ or Li^+ could be used as coupling ions, or an absolute requirement for Na^+ was developed. For the ATPase, these observations may suggest that only minor changes were required during evolution to change the specificity from H^+ to Na^+ coupling. In addition, the silent H^+-translocating activity of the *P. modestum* ATPase may be a remnant of the evolutionary development of an Na^+ translocating ATPase from an H^+ translocating ATPase.

References

Amann, R., Ludwing, W., Laubinger, W., Dimroth, P. & Schleifer, K. H. 1988 Cloning and sequencing of the gene encoding the beta subunit of the sodium ion translocating ATP synthase of *Propionigenium modestum*. *FEMS Microbiol. Lett.* **56**, 253–260.

Botfield, M. C. & Wilson, T. H. 1988 Mutations in the melibiose carrier of *E. coli*. *J. biol. Chem.* **263**, 12909–12915.

Boyer, P. D. 1975 A model for conformational coupling of membrane potential and proton translocation to ATP synthesis and to active transport. *FEBS Lett.* **58**, 1–6.

Boyer, P. D. 1988 Bioenergetic coupling to protonmotive force. *Trends biochem. Sci.* **13**, 5–7.

Buckel, W. & Liedtke, H. 1986 Specific cleavage of the sodium pump glutaconyl-CoA decarboxylase by primary alcohols. *Eur. J. Biochem.* **156**, 251–257.

Buckel, W. & Semmler, R. 1983 Purification, characterization and reconstitution of glutaconyl-CoA decarboxylase. *Eur. J. Biochem.* **136**, 427–434.

Däkena, P., Rohde, M., Dimroth, P. & Mayer, F. 1988 Oxaloacetate decarboxylase from *Klebsiella pneumoniae*: size and shape of the enzyme, and localization of its prosthetic biotin group by electron microscopic affinity labeling. *FEMS Microbiol. Lett.* **55**, 35–40.

Dibrov, P. A., Kostyrko, V. A., Lazarova, R. L., Skulachev, V. P. & Smirnova, I. A. 1986*a* Na^+-dependent motility and modes of membrane energization in *Vibrio alginolyticus*. *Biochim. biophys. Acta* **850**, 449–457.

Dibrov, P. A., Lazarova, R. L., Skulachev, V. P. & Verkhovskaya, M. L. 1986*b* Na^+-coupled oxidative phosphorylation in *Vibrio alginolyticus* cells. *Biochim. biophys. Acta* **850**, 458–465.

Dimroth, P. 1982*a* Purification of the sodium transport enzyme oxaloacetate decarboxylase by affinity chromatography on avidin-Sepharose. *FEBS Lett.* **141**, 59–62.

Dimroth, P. 1982*b* The generation of an electrochemical gradient of sodium ions upon decarboxylation of oxaloacetate by the membrane-bound and Na^+-activated oxaloacetate decarboxylase from *Klebsiella aerogenes*. *Eur. J. Bichem.* **121**, 443–449.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Dimroth, P. 1982*c* The role of biotin and sodium in the decarboxylation of oxaloacetate by the membrane-bound oxaloacetate decarboxylase from *Klebsiella aerogenes*. *Eur. J. Biochem.* **121**, 435–441.
Dimroth, P. 1987 Sodium ion transport decarboxylases and other aspects of sodium ion cycling in bacteria. *Microbiol. Rev.* **51**, 320–340.
Dimroth, P. & Hilpert, W. 1984 Reversal of decarboxylation-dependent Na^+ pumps. *Biochemistry* **23**, 5360–5366.
Dimroth, P. & Laubinger, W. 1987 Decarboxylation phosphorylation, the third mode of ATP synthesis in living cells. *Biol. Chem. Hoppe-Seyler* **368**, 547–548.
Dimroth, P. & Thomer, A. 1983 Subunit composition of oxaloacetate decarboxylase and characterization of the α-chain as carboxyltransferase. *Eur. J. Biochem.* **137**, 107–112.
Dimroth, P. & Thomer, A. 1986 Citrate transport in *Klebsiella pneumoniae*. *Biol. Chem. Hoppe-Seyler* **367**, 813–823.
Dimroth, P. & Thomer, A. 1988 Dissociation of oxaloacetate decarboxylase and reconstitution of the active complex from the isolated subunits. *Eur. J. Biochem.* **175**, 175–180.
Dimroth, P. & Thomer, A. 1989 A primary respiratory Na^+ pump of an anaerobic bacterium: the Na^+-dependent NADH: quinone oxidoreductase of *Klebsiella pneumoniae*. *Arch. Microbiol.* **151**, 439–444.
Hara, Y. & Nakao, M. 1986 Proton transport catalyzed by the sodium pump. *J. biol. chem.* **261**, 12655–12658.
Hayashi, M. & Unemoto, T. 1984 Characterization of the Na^+-dependent respiratory chain NADH: quinone oxidoreductase of the marine bacterium *Vibrio alginolyticus*, in relation to the primary Na^+ pump. *Biochim biophys. Acta.* **767**, 470–478.
Hayashi, M. & Unemoto, T. 1987 Subunit components and their roles in the sodium transport NADH: quinone reductase of a marine bacterium, *Vibrio alginolyticus*. *Biochim. biophys. Acta* **390**, 47–54.
Hilpert, W. & Dimroth, P. 1983 Purification and characterization of a new sodium transport decarboxylase. Methylmalonyl-CoA decarboxylase from *Veillonella alcalescens*. *Eur. J. Biochem.* **132**, 579–587.
Hilpert, W., Schink, B. & Dimroth, P. 1984 Life by a new decarboxylation-dependent energy conservation mechanism with Na^+ as coupling ion. *EMBO J.* **3**, 1665–1670.
Hirata, H., Kambe, T. & Kagawa, Y. 1984 A purified alanine carrier driven by either proton or sodium ion gradient. *J. biol. Chem.* **259**, 10653–10656.
Hoffmann, A., Hilpert, W. & Dimroth, P. 1989 Carboxyltransferase activity of methylmalonyl-CoA decarboxylase. *Eur. J. Biochem.* **179**, 645–650.
Kawakami, T., Azikawa, Y., Ishikawa, T., Shimamoto, T., Tsuda, M. & Tsuchiya, T. 1988 Amino acid substitutions in the melibiose carrier. *J. biol. Chem.* **263**, 14276–14280.
Ken-Dror, S., Lanyi, J. K., Schobert, B., Silver, B. & Avi-Dor, Y. 1986 NADH: quinone oxidoreductase of bacterium Ba_1. *Archs Biochem. Biophys.* **244**, 766–772.
Lanyi, J. K. 1979 The role of Na^+ in transport processes of bacterial membranes. *Biochim. biophys. Acta* **559**, 377–397.
Laubinger, W. & Dimroth, P. 1987 Characterization of the Na^+-stimulated ATPase of *Propionigenium modestum* as an enzyme of the F_1F_0 type. *Eur. J. Biochem.* **168**, 475–480.
Laubinger, W. & Dimroth, P. 1988 Sodium ion translocating ATP synthase. *Biochemistry* **27**, 7531–7537.
Lambinger, W. & Dimroth, P. 1989 Proton pumping by the Na^+-ATPase. *Biochemistry* **28**, 7194–7198.
Mitchell, P. 1961 Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. *Nature, Lond.* **191**, 144–148.
Mitchell, P. 1974 A chemiosmotic molecular mechanism for proton-translocating adenosine triphosphatases. *FEBS Lett.* **43**, 189–194.
Nagle, J. F. & Morowitz, H. J. 1978 Molecular mechanisms of proton transport in membranes. *Proc. natn. Acad. Sci. U.S.A.* **75**, 298–302.
O'Brien, R. W. 1975 Metabolism of citrate by *Klebsiella aerogenes*. *J. Bact.* **122**, 468–473.
Radford, S. E., Laue, E. D., Perham, R. N., Miles, J. S. & Guest, J. R. 1987 Domains in pyruvate dehydrogenase complex. *Biochem. J.* **247**, 641–649.
Schneider, E. & Altendorf, K. H. 1987 Bacterial adenosine 5′-triphosphate synthase (F_1F_0). *Microbiol. Rev.* **51**, 477–497.
Schwarz, E., Oesterhelt, D., Reinke, H., Beyreuther, K. & Dimroth, P. 1988 Sequence of the α-subunit of oxaloacetate decarboxylase. *J. biol. Chem.* **263**, 9640–9645.
Stephens, P. E., Darlison, M. G., Lewis, H. M. & Guest, J. R. 1983 The pyruvate dehydrogenase complex of *Escherichia coli*. *Eur. J. Biochem.* **133**, 481–489.
Thauer, R. K., Jungermann, K. & Decker, K. 1977 Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* **41**, 100–180.
Tokuda, H. & Unemoto, T. 1982 Respiration-dependent Na^+ pump in *Vibrio alginolyticus*. *J. biol. Chem.* **257**, 10007–10014.
Tokuda, H. & Unemoto, T. 1984 Coupling site of the respiratory Na^+ pump of *Vibrio alginolyticus*. *J. biol. Chem.* **259**, 7785–7790.
Tsuchiya, T. & Wilson, T. H. 1978 Cation-melibiose cotransport. *Membr. Biochem.* **2**, 63–79.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES