Journal of Bioenergetics and Biomembranes, Vol. 26, No. 6, 1994

Chemiosmotic Concept of the Membrane Bioenergetics: What Is Already Clear and What Is Still Waiting for Elucidation?

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Received July 21, 1994

The present state of the chemiosmotic concept is reviewed. Special attention is paid to (i) further progress in studies on the Na^+-coupled energetics and (ii) paradoxical bioenergetic effects when protonic or sodium potentials are utilized outside the coupling membrane (TonB-mediated uphill transports across the outer bacterial membrane). A hypothesis is put forward assuming that the same principle is employed in the bacterial flagellar motor.

KEY WORDS: Chemiosmosis; Na^+ energetics; flagellar motor; TonB protein.

INTRODUCTION

The present state of the chemiosmotic concept put forward by Mitchell (1961, 1966, 1968) in the sixties can be summarized as follows:

1. In the inner membrane of mitochondria, chloroplasts, and many bacteria, as well as in tonoplast and membranes of some intracellular vesicles, the energy-releasing and energy-consuming processes are coupled by means of the H^+ cycle.

2. The mechanism of generation of $\Delta\bar{\mu}_{H^+}$[2] by some redox enzymes consists in transport of electrons removed from a H donor across the membrane to reduce an H acceptor on the opposite membrane side. Such a transmembrane electron flow is mediated by redox groups arranged in a transmembrane fashion. Just this mechanism is shown to be responsible for the main portion of electric potential generation in photosynthetic reaction centers of bacteria and in two photosystems of chloroplast, as well as in the cytochrome $b_l \rightarrow b_h$ oxidoreduction (a partial reaction of the Q-cycle catalyzed by bc_1 and b_6f complexes).

3. Another type of mechanism of $\Delta\bar{\mu}_{H^+}$ formation includes H^+ transfer across the membrane. In this way, H^+-ATPases, H^+-pyrophosphatases, H^+-transhydrogenase, and bacteriorhodopsin operate. In certain systems, mechanisms 2 and 3 are combined so that oppositely directed $\bar{e}$ and H^+ flows appear to result in $\Delta\bar{\mu}_{H^+}$ generation (photosynthetic redox centers, bc_1 and b_6f complexes, cytochrome oxidase).

The mechanism of H^+ transfer remains obscure even for the most elaborated $\Delta\bar{\mu}_{H^+}$ generators as reaction center complexes and bacteriorhodopsin. Most probably the mechanism in question consists in a combination of (a) the H^+ transfer between some H^+ acceptor groups and (b) a conformational change causing formation of cleft or hollow in the hydrophobic region of the $\Delta\bar{\mu}_{H^+}$-generating protein. This mechanism was proposed, in particular, for bacteriorhodopsin (Skulachev, 1993).

The molecular mechanism of the main $\Delta\bar{\mu}_{H^+}$ consumer, i.e., H^+-ATP-synthase, is not yet clear. This is also true for numerous H^+, solute symporters and H^+/solute antiporters as well as for the H^+ motor rotating the bacterial flagellum.

4. In some bacteria as well as in the plasma membrane of animal and certain plant cells, Na^+ effectively substitutes for H^+ as the coupling ion. The molecular mechanism of $\Delta\bar{\mu}_{Na^+}$ generators and $\Delta\bar{\mu}_{Na^+}$ consumers awaits elucidation.

5. Discovery of the Na^+-based energetics allowed

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[2] Abbreviations: $\Delta\bar{\mu}_{H^+}$ and $\Delta\bar{\mu}_{Na^+}$, transmembrane differences in the electrochemical potentials of H^+ and Na^+, respectively; $\Delta\Psi$, transmembrane electric potential difference.

0145-479X/94/1200-0589$07.00/0

the essential missing link between some energy-supplying and energy-consuming processes in to be filled. As a result, the following general laws of biological energy interconversions can be formulated.

The first law. The living cell avoids direct utilization of external energy sources in the performance of useful work. It transforms energy of these sources to a convertible energy currency, i.e., ATP, $\Delta\bar{\mu}_{H^+}$, or $\Delta\bar{\mu}_{Na^+}$, which is then spent to support various types of energy-consuming processes.

The second law. Any living cell always possesses at least two energy currencies, one water-soluble (ATP) and the other membrane-linked ($\Delta\bar{\mu}_{H^+}$ or $\Delta\bar{\mu}_{Na^+}$).

The third law. All the energy requirements of the living cell can be satisfied if at least one of three convertible energy currencies is produced at the expense of external energy sources (Skulachev, 1992a).

The universal applicability of these three laws is confirmed by analysis of a great number of observations made by bioenergeticists during the last two decades (for reviews, see Skulachev, 1988, 1992a, b).

In this review I would like to focus attention on two important aspects that are not covered by other contributions to this mini-review series, namely the latest information from "The Sodium World" and the paradoxical bioenergetic events when $\Delta\bar{\mu}_{H^+}$ (or $\Delta\bar{\mu}_{Na^+}$) are utilized outside the coupling membrane.

FURTHER PROGRESS IN NA^+ CYCLE RESEARCH[3]

Na^+-Motive NADH-Q Reductase

The main result in this field is that this enzyme discovered in 1981–1982 by Tokuda and Unemoto in *Vibrio alginolyticus* (Tokuda and Unemoto, 1981, 1982) is, in fact, quite frequent among marine and moderately halophilic bacteria. In Tokuda's group it has been found in eight of the nine studied genera of marine bacteria (Tokuda, 1989, Tokuda and Kogure, 1989; Kogure and Tokuda, 1989). Independently, Unemoto's laboratory reported that of seven moderate halophiles, the activity in question is inherent in five cases (Unemoto *et al.*, 1992; Unemoto and Hayashi, 1993). In our group, this activity was found in *E. coli* and halo- and alkalotolerant *Bac. FTU* growing aerobically on succinate at low $\Delta\bar{\mu}_{H^+}$ (Avetisyan *et al.*, 1991). Dimroth reported that the Na^+-motive NADH-Q reductase is present in anaerobic *Klebsiella pneumoniae* (Dimroth and Thomer, 1989).

Unfortunately the sequence of this enzyme is still unknown. Its mechanism of action is also obscure although some ideas on the $Q^- \cdot Na^+$ complex as the Na^+-transporting intermediate have been discussed (Ken-Dror *et al.*, 1986; Rich, 1991; Skulachev, 1992b).

The enzyme is extremely sensitive to Ag^+ ($C_{1/2} = 9 \times 10^{-9}$ M), which not only inhibits the electron transfer (Asano *et al.*, 1985; Kostyrko *et al.*, 1991) but also induces the passive Na^+ conductance as if the Na^+ pump is converted to the Na^+ channel (Semeykina and Skulachev, 1990). Quite recently Hussain and coworkers (Hussain *et al.*, 1994) reported that the same concentration of Ag^+ inhibits animal Na^+/K^+-ATPase. This effect was also accompanied by a Na^+ conductance increase (Anner, personal communication).

Na^+-Motive Terminal Oxidases

This activity was disclosed in our group in 1988–1991 when *Bac. FTU* was studied (Verkhovskaya *et al.*, 1988; Semeykina *et al.*, 1989; Kostyrko *et al.*, 1991). Later we found a similar activity in *E. coli* (Avetisyan *et al.*, 1991). In both cases, the Na^+-motive oxidase has been shown to be induced in cells growing under low $\Delta\bar{\mu}_{H^+}$ conditions. In *E. coli* and *Bac. FTU*, these oxidases proved to be much less sensitive to cyanide than the H^+-motive oxidases which are present in the same bacteria growing at high $\Delta\bar{\mu}_{H^+}$. In *Bac. FTU*, a *b*-type oxidase was shown to be responsible for the Na^+ pumping, whereas an oxidase of the aa_3-type pumped H^+.

In *E. coli*, there is no aa_3 oxidase. Instead, an *o*-type oxidase can operate as an alternative to the *d*-type oxidase. It was shown that it is the *E. coli d*, rather than *o*, oxidase that functions as a Na^+ pump. As to the *o*-type oxidase, it plays in *E. coli* the role that in *Bac. FTU* is performed by cytochrome aa_3, i.e., the pumping of H^+. The similarity between the *Bac. FTU b* oxidase and the *E. coli d* oxidase and their difference from the *Bac. FTU* aa_3 oxidase and the *E. coli o* oxidase were found to consist not only in the nature of the pumped ion but also in some other properties such as the cyanide sensitivity, the rate of CO recombination, affinity for the redox mediators, etc. (Muntyan *et al.*, 1993a,b; Muntyan and Skripnikova, 1993).

[3] Reported at the 8EBEC Meeting (Skulachev, 1994a).

Strong evidence that it is cytochrome *d* that is involved in the Na^+ pumping has recently been obtained in experiments with the *E. coli* wild strain and mutants deficient in cytochrome *d* or, alternatively, in cytochrome *o* (Avetisyan *et al.*, 1992; Bogachev *et al.*, 1993).

1. Everted membrane vesicles from wild and d^+, o^- strain are competent in the Na^+ pumping when ascorbate + TMPD or succinate are oxidized. This effect is strongly stimulated by *in vitro* addition of uncoupler and arrested by a Na^+ ionophore or monensin. Under the same conditions, the d^-, o^+ strain does not pump Na^+ in spite of the high rate of succinate oxidation. In this strain, NADH oxidation is coupled to the Na^+ pumping. In all the experiments, the cells were grown in the presence of uncoupler or at high pH.

2. The d^+, o^- mutant can grow in the presence of an uncoupler as well as under alkaline conditions. On the other hand, the d^-, o^+ mutant cannot grow with an uncoupler and shows slower growth rate at high pH.

3. The wild strain is found to synthesize much more cytochrome *d* when growing with any four uncoupler studied, i.e., *m*-chlorocarbonyl-cyanide phenylhydrazone, pentachlorophenol, tetrachlorotrifluoromethyl benzimidazole, and dinitrophenol, the efficiency of the first one being maximal. The cytochrome *d* induction was arrested by chloramphenicol and potentiated by Na^+ (Avetisyan *et al.*, 1992).

4. Other conditions lowering $\Delta\bar{\mu}_{H^+}$ also induce cytochrome *d*. (i) Small (micromolar) concentrations of cyanide, specifically inhibiting cytochrome *o*, not cytochrome *d*, appear to be stimulatory for the cytochrome *d* synthesis. Similar effects are caused by (ii) ferricyanide + phenazine methosulfate or (iii) by a mutation inhibiting the flavin biosynthesis. It should be stressed that cyanide increases the reduction level of the respiratory chain whereas ferricyanide and flavin deficiency decrease this level, so that this level *per se* is hardly responsible for the above regulation.

5. Induction of cytochrome *d* by uncouplers, cyanide, ferricyanide, and flavin deficiency is abolished in the mutants deficient in *Arc A* or *Arc B* proteins.

6. Increase in $[Na^+]_{out}$ from 1.5 mM to 20 mM increases the cytochrome *d* level even at high $\Delta\bar{\mu}_{H^+}$. This effect does not require the *Arc* proteins.

7. Miller and Gennis (1985) showed that the cytochrome *d* proteoliposomes can form ΔpH when oxidizing CoQ_1 in the presence of an uncoupler. Unfortunately, the authors had no idea about the Na^+ pumping and did not try the Na^+-free medium or a Na^+ ionophore.

The above findings can be compared with those reported by Verkhovskaya *et al.* in Wikstrom's group (1992) where the H^+ pumping by *E. Coli* cytochromes *o* and *d* was studied. It was found that at neutral pH, the H^+/e^- ratio is equal to 2 for cytochrome *o* and 1 for cytochrome *d* (Puustinen *et al.*, 1989, 1991; Verkhovskaya *et al.*, 1992). At higher pH *in vitro*, the ratio decreased to 1 also for cytochrome *o* (Verkhovskaya *et al.*, 1992).

Wikström's data and our data may be explained assuming that at any pH cytochrome *o* and cytochrome *d* operate as the electron-transporting Mitchellian "half-loop" forming $\Delta\bar{\mu}_{H^+}$ as a result (i) QH_2 oxidation to Q and $2H^+_{out}$ and (ii) H_2O formation from 1/2 O_2 and $2H^+_{in}$ (Mitchell, 1966). Moreover, at neutral pH, cytochrome *o* transports two more H^+_{in} per QH_2 oxidized by the H^+-pump mechanism originally discovered by Wikström in studies of mitochondrial cytochrome aa_3 (Wikström, 1977; Wikström and Casey, 1985). At high pH, cytochrome *o* fails to transport "the Wikströmian protons." This may well be a result of adaptation to the alkaline medium when the problem of unfavourable alkaline shift of pH_{in} arises and the Na^+ cycle becomes operative. Under these conditions cytochrome *d* is induced. It operates, according to the scheme, like cytochrome *o* at neutral pH, but "the Wikströmian protons" are replaced by sodium ions.

One more possibility is that in cytochrome *d*, as in cytochrome *o*, the electron transfer can, under certain conditions, be decoupled from the pump.

An indication that a terminal oxidase can operate as a Na^+ pump was also published by Efiok and Webster who studied *Vitreoscilla* (Efiok and Webster, 1990a,b).

The Na^+-Motive and Na^+-Driven Reactions in Methanogenesis

It has been found that methane formation by methanogenic bacteria includes energy-producing H^+-motive and Na^+-motive steps and an energy-consuming Na^+-driven step. For instance, methanol disproportionation to CH_4 and CO_2 is described by reactions (1) and (2):

$$CH_3OH \rightarrow CH_3 - S - CoM \xrightarrow[\Delta\bar{\mu}_{H^+}]{} CH_4 \qquad (1)$$

$$CH_3OH \rightarrow CH_3 - S - CoM \xrightarrow[\Delta\bar{\mu}_{Na^+}]{}$$

$$CH_2 = TMPT \rightarrow CHO - MFR \xrightarrow[\Delta\bar{\mu}_{Na^+}]{} CO_2 \quad (2)$$

where TMPT is for tetrahydromethanopterin and MFR is for methanofuran (Schönheit, 1993).

Reaction (1) is exergonic and pumps H^+ from the cell. As to reaction (2), its initial step is endergonic, being driven by a downhill influx of Na^+ to the cell, whereas its final step is exergonic and expels Na^+ from the cell in an uphill fashion.

The combination of the H^+ and Na^+ cycles is also inherent in some other types of methanogenesis (Schönheit, 1993; Blaut *et al.*, 1992; Becher *et al.*, 1992; Kaesler and Schönheit, 1989; Müller *et al.*, 1988) as well as acetogenesis (Heise *et al.*, 1989).

The mechanisms of the Na^+-coupled reactions in methano- and acetogenesis remain unclear. In some of them Na^+ translocation is coupled to the electron transfer (Schönheit, 1993; Kaesler and Schönheit, 1989), whereas in one case (methyl-THMP : CoM methyltransferase reaction) it is the transfer of methyl group that is coupled to the transmembrane movement of Na^+ (Becher *et al.*, 1992). Reconstitution of these very complicated systems in proteoliposomes is desirable.

Na^+-Motive Decarboxylations

Na^+-oxaloacetate decarboxylase of *Klebsiella pneumoniae* (Dimroth, 1980) was shown to be composed of three subunits, the largest (α) being peripheral. The other two subunits (β and γ) are integrated into the membrane. The α subunit contains the biotin prosthetic group responsible for catalysis. As to the β and γ subunits, they are somehow involved in the Na^+ translocation through the membrane hydrophobic barrier. The β and γ subunits seem to contain six and one transmembrane α-helices, respectively. Sequences of the subunits have already been published. The enzyme was reconstituted into proteoliposomes competent in Na^+ pumping (reviewed in Dimroth, 1987, 1990; and Skulachev, 1992b). Recently it has been found that at high $\Delta\bar{\mu}_{Na^+}$, decarboxylase begins to operate as a Na^+ channel (carrier). This avidin-sensitive process requires carboxylation/decarboxylation of the biotin group whereas oxaloacetate/pyruvate interconversion appears to be not necessary (Dimroth and Thomer, 1993). A similar enzyme was found in *Salmonella typhimurium* (Wifling and Dimroth, 1989). In anaerobic *Acidomonas fermentans*, *Peptococcus aerogenes*, *Clostriidium symbiosum*, and *Fusobacterium nucleatum*, the Na^+-motive glutaconyl-CoA decarboxylase was described by Buckel (1986). Dimroth reported on the Na^+-motive methylmalonyl-CoA decarboxylase from *Veilonella alcalensis* and *Propionigenium modestum* (Dimroth, 1987).

Na^+-ATPases

This is a rather numerous group of enzymes. Its members have been found in all kingdoms of living organisms. Besides the well-known Na^+/K^+-ATPase from the animal plasma membrane, the ATP-drive Na^+ pumps have been described in several bacterial genera and recently in plant species. Among them are representatives of all the three types of ATPases, i.e., F, V, and P types (reviewed in Skulachev, 1992b).

The animal Na^+/K^+-ATPase is the best example of a Na^+ pump of the P-type (reviewed in Skulachev, 1992b). Recently a similar enzyme has been discovered by Wada *et al.* (1989, 1992) in the plasma membrane of marine alga (the raphidophycean biflagellate *Heterosigma akashiwo*). This 140-kDa vanadate-sensitive Na^+ pump belongs to the P-type ATPases. In the presence of Na^+ it forms a phosphorylated intermediate which is hydrolyzed in the K^+-dependent manner. The protein possesses an immunologically identical epitope to the animal Na^+/K^+-ATPase (Wada *et al.*, 1992).

The ATP-dependent transport of Na^+, activated by uncoupler and resistant to amiloride, was quite recently described by Balnokin and Popova in everted vesicles of the plasmalemma of the marine eukaryotic microalgoa *Platymonas viridis*. The authors concluded that these cells possess an electrogenic Na^+-ATPase (Balnokin and Popova, 1994).

As to the F-type Na^+ ATPase, the *P. modestum* enzyme seems to be the most elaborated example. It is very similar to bacterial H^+-ATPases (ATP-synthases) both in sequence and subunit composition. In fact, this is a typical F_0F_1 complex. According to Dimroth (Kaim and Dimroth, 1993), active chimeric enzymes can be obtained combining *E. coli* F_0 and *P. modestum* F_1 and vice versa, the ion specificity being determined by the F_0 part. Earlier it was shown in the same group that the ion specificity of native *P. modestum* Na^+-ATPase is not absolute. When Na^+

is absent, H^+ is pumped (Laubinger and Dimroth, 1989). Apparently both Na^+ and H^+ compete for one and the same carboxylate of Glu-65 in the c-subunit of the ATPase. DCCD attacking this carboxylate in its protonated form was shown to be much less effective in the presence of Na^+ (Kluge and Dimroth, 1993).

The physiological function of the *P. modestum* enzyme consists in ATP synthesis at the expense of $\Delta\bar{\mu}_{Na^+}$ which is produced by the Na^+-motive methylmalonyl CoA-decarboxylase (Dimroth, 1987). In our laboratory it has been found that the *E. coli* F_0F_1 ATPase becomes competent in the $\Delta\bar{\mu}_{Na^+}$-driven ATP synthesis when the cell grows under low $\Delta\bar{\mu}_{H^+}$ conditions, i.e., in the presence of an uncoupler or at high pH. The everted membrane vesicles from these cells were shown to catalyze oxidative phosphorylation in the presence of protonophorous uncouplers. The ATP synthesis was completely inhibited by Na^+ ionophore ETH 157, monensin, the artificially imposed reverse Na^+ gradient, as well as by the F_0 inhibitors venturicydin and DCCD, the F_1 inhibitor aurovertin, or *unc*-mutation (deletion in the F_0F_1 operon). It was suggested that a post-translational modification of the F_0F_1 complex is responsible for the switch from H^+ to Na^+ under unfavorable conditions (Avetisyan *et al.*, 1993).

Electroneutral Na^+/K^+-ATPase of the V-type was described by Kakinuma in *Enterococcus hirae* (formerly *Streptococcus faecalis*) (Kakinuma, 1993). The enzyme is sensitive, like V-ATPases, to nitrate and *N*-ethylmaleimide and resistant to vanadate. It is composed of detachable and membrane-embedded sectors (presumably V_1 and V_0). The sequence of the largest subunit in the detachable sector is similar to that in V_1 (Takase *et al.*, 1993).

A nitrate-sensitive, vanadate-resistant Na^+-ATPase has been discovered recently by Koning's group in thermophilic anaerobic *Clostridium fervidus* (Speelmans *et al.*, 1993a). There is evidence for Na^+-ATPase (synthase) in *Vibrio alginolyticus*, *Exiguobacterium aurantiacum*, *Mycoplasma* and *Acholeplasma* (for review, see Skulachev, 1992b), methanogens (Smigan *et al.*, 1994), *Vitreoscilla* (Efiok and Webster, 1992), *Acetobacterium* (Heise *et al.*, 1992) and anaerobic alkalophile *Amphibacillus* (Koyama, 1993).

Na^+, Solute Symporters

Na^+, solute symporters are quite typical for marine bacteria as well as for the animal plasma membrane (reviewed in Skulachev, 1988, 1992b; Imae, 1991; Poolman and Konings, 1993). In some bacteria, this seems to be the main [*V. alginolyticus* (Unemoto and Hayashi, 1993)] or even the sole [*Clostrisium fervidus* (Speelmans *et al.*, 1993a,b)] mechanism of the uphill import of metabolites into the cell.

An example exists where the efflux of an end-product of metabolism in symport with Na^+ is employed to generate $\Delta\bar{\mu}_{Na^+}$. In *Selenomonas ruminantium*, fermentation results in succinate^{2-} which is exported with $3Na^+$ so that $\Delta\bar{\mu}_{Na^+}$ is generated, being composed of $\Delta\Psi$ (the interior negative) and ΔpNa (low $[Na^+]$ inside) (Michel and Macy, 1990).

Why Living Cells Employ the Sodium Cycle

For a bacterium growing at low $\Delta\bar{\mu}_{H^+}$ the reason why the Na^+ cycle substitutes for the H^+ cycle seems quite obvious: this is a way to survive under unfavorable conditions (Skulachev, 1984). In those bacteria where the Na^+ cycle is inducible, the $\Delta\bar{\mu}_{H^+}$ level is apparently monitored by a special receptor of the protonmotive force (we called it "protometer") which sends a signal to systems responsible for switching the energetics from H^+ to Na^+ when $\Delta\bar{\mu}_{H^+}$ is lowered (Skulachev, 1988). There are at least three examples where mechanistically different effects, resulting in a $\Delta\bar{\mu}_{H^+}$ decrease, were found to induce the Na^+ cycle. In our laboratory it was found that the Na^+-motive respiratory chain of *Bac. FTU* and *E. coli* is induced under alkaline conditions or, at neutral pH, by adding to the growth medium either a protonophorous uncoupler or a low concentration of cyanide which specifically inhibits the H^+-motive oxidase (Semeykina and Skulachev, 1991; Avetisyan *et al.*, 1991). The induction requires *Arc A* and *Arc B* proteins (Bogachev *et al.*, 1993b), a regulatory system which is known to be involved in control of expression of several catabolic enzymes (Iuchi *et al.*, 1990).

In Kakinuma's group the Na^+/K^+-ATPase of anaerobic *E. hirae* was shown to be induced by uncouplers, high pH, or mutation in the H^+-ATPase. Mutant deficient in the Na^+/K^+-ATPase could not grow at high pH (for reviews, see Kakinuma, 1993; and Skulachev, 1992b).

The reason to employ the Na^+ cycle in *Cl. fervidus* is thermophilism. It was found that at high temperature the H^+ conductance of the bacterial membrane is much higher than the Na^+ conductance (Speelmans *et al.*, 1993a,b).

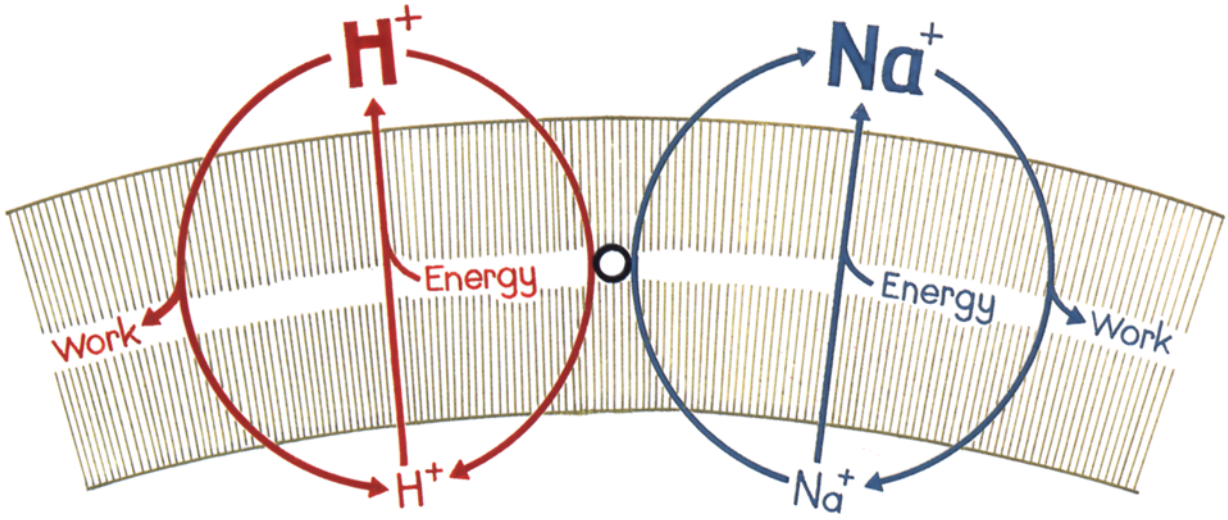

Fig. 1. Interrelations of the H^+ and Na^+ energetics.

Induction of the Na^+ cycle enzymes in *E. coli* and *E. hirae* was shown to require Na^+ (Skulachev, 1992b; Bogachev *et al.*, 1993b). An example exists where a decrease in $[Na^+]_{out}$ results in the opposite switch, i.e., from the Na^+ to the H^+ cycle. Imae and coworkers (Atsumi *et al.*, 1992) showed that marine *Vibrio parahaemolyticus* produces two different types of flagella, namely a single polar flagellum to swim in liquid medium or numerous lateral flagella for swarming over viscous surfaces. The first type of flagellum is driven by the Na^+-motor (Chernyak *et al.*, 1983), whereas the second—by the H^+-motor (Atsumi *et al.*, 1992). The lateral flagella are induced by growing under viscous conditions (Atsumi *et al.*, 1992). One might speculate that the switch from Na^+ to H^+ is due to a decrease in $[Na^+]_{out}$ when the bacterium changes habitat from seawater to the surface of a substrate outside the sea.

Such a regulation is absent from *E. coli* and *Bac. FTU*. According to our data, switching the energetics from H^+ to Na^+ when $\Delta\bar{\mu}_{H^+}$ is lowered does not cause any change in the flagellar motor which in *E. coli* is always $\Delta\bar{\mu}_{H^+}$-driven whereas in *Bac. FTU* it is always $\Delta\bar{\mu}_{Na^+}$-driven. As a result, *E. coli* and *Bac. FTU* are motionless when $\Delta\bar{\mu}_{H^+}$ and $\Delta\bar{\mu}_{Na^+}$ are low (Bogachev *et al.*, 1993a).

There are some cases when reasons to use the Na^+-cycle instead of the H^+-cycle are not obvious. For instance, it is rather difficult to explain in functional terms why all the membranous energy-coupled decarboxylases pump Na^+, not H^+, or why some of the energy-transducing steps of methanogenesis are H^+-linked whereas others are Na^+-linked. Perhaps, these questions cannot be settled now just as the question of why glycolytic phosphorylation is of the substrate type whereas respiratory chain phosphorylation is of the chemiosmotic type. The H^+ cycle and the Na^+ cycle look like two kinds of chemiosmotic mechanisms of energy transductions of ubiquitous distribution among living cells. The existence of such parallel pathways should greatly stabilize the bioenergetic systems. It seems quite natural that, for example, the Na^+ energetics substitutes for the H^+ energetics when the latter appears to be inefficient. However, in certain chemiosmotic reactions only one of these two possibilities is realized. Thus, the question of why it is Na^+, not H^+, that is pumped by decarboxylases may be answered in a very simple way: because the Na^+-motive decarboxylases were invented by the evolution while the H^+-motive decarboxylases were not.

Interrelations of the H^+ cycle and the Na^+ cycle are illustrated in Fig. 1. It is shown that an H^+ pump or a Na^+ pump carries out uphill transport of the corresponding coupling ion at the expense of utilization of some external energy sources. Then the coupling ion moves in the opposite direction, i.e., downhill, to support useful work. Two cycles can be coupled via the Na^+/H^+ antiporter. To support life at low $\Delta\bar{\mu}_{H^+}$, two cycles should be decoupled by suppression or inactivation of the antiporter, whereas the Na^+ cycle should be induced or activated.

UTILIZATION OF $\Delta\bar{\mu}_{H^+}$ ($\Delta\bar{\mu}_{Na^+}$) OUTSIDE THE COUPLING MEMBRANE[4]

Until recently, it seemed quite obvious that both the energy-releasing process and the energy-consuming process coupled by $H^+(Na^+)$ circulation must be localized in one and the same membrane. However, progress in bioenergetic research has resulted in novel observations indicating that in some systems this is not the case. Namely, $\Delta\bar{\mu}_{H^+}$ or $\Delta\bar{\mu}_{Na^+}$ can be utilized at some distance from the coupling membrane or even in another (non-coupling) membrane separated from the coupling one by an aqueous compartment. This paradoxical effect might be a clue for the understanding of some molecular mechanisms still awaiting elucidation, such as the bacterial flagellar motor.

The first set of facts concerns ATP-synthase of the F_0F_1-type. It is already known that the $\Delta\bar{\mu}_{H^+}$ ($\Delta\bar{\mu}_{Na^+}$)-driven ATP production carried out by this enzyme occurs not in the membrane but outside it, i.e., at a distance of at least 5 nm from the membrane surface. The energy-supplying downhill $H^+(Na^+)$ transfer occurs in the F_0 part plugged through the membrane, whereas ATP is produced in the F_1 part, i.e., in the head-piece of the F_0F_1 "mushroom" protruding into the aqueous phase and connected to F_0 with a rather long stalk. The mechanism of energy transmission from F_0 to F_1 is not yet clear. However, in any case, this system may be considered as a precedent of $\Delta\bar{\mu}_{H^+}$ ($\Delta\bar{\mu}_{Na^+}$) utilization outside the coupling membrane. It is important that those H^+ ions which are transported via F_0 can hardly participate in formation of H_2O when ADP is phosphorylated by inorganic phosphate in F_1 since in some cases Na^+ effectively substitutes for H^+ in this system (see the preceding section).

Important observations were recently made when the uphill transport of solutes across the outer membrane of Gram-negative bacteria was studied. It is well known that vitamin B_{12} and the iron-transporting complexes (siderophores) can be accumulated in the bacterial periplasm before being transported to the cytoplasm across the inner (cytoplasmic) membrane (for reviews, see Kadner, 1990; Postle, 1993; Klebba *et al.*, 1993). For example, for vitamin B_{12} a 1,000-fold gradient between the outer medium and periplasm was shown (Reynolds *et al.*, 1980). The problem was how to supply this uphill process with energy since no energy-producing devices have been described for the outer membrane. The situation became even more intriguing when it was revealed (Kadner, 1990; Reynolds *et al.*, 1980; Bradbeer, 1993) that the transport of vitamin B_{12} and siderophores is supported by $\Delta\bar{\mu}_{H^+}$ which certainly could not exist on the outer membrane because of the presence of porin channels highly conductive for solutes of up to 600 Da molecular mass.

The solution to the problem seems to have been found when the role of a protein called TonB was elucidated. This protein, being required for accumulation of vitamin B_{12}, siderophores, and some other outer medium compounds in the periplasm of Gram-negative bacteria (Kadner, 1990; Postle, 1993; Klebba *et al.*, 1993), is somehow involved in a rather complex transport system including a high-affinity outer membrane protein ("receptor"), a periplasmic binding protein, and an inner membrane protein homologous to other known traffic ATPases. TonB contains about 250 amino acid residues. It forms two terminal domains which can be arranged in a transmembrane fashion and a central, elongated, structurally rigid, core region. It was shown that the N-terminal and central domains are localized in the cytoplasmic membrane and periplasm, respectively. As to the C-terminal domain, it is putatively localized in the outer membrane or, at least, contacts its inner surface (for reviews, see Kadner, 1990; Postle, 1993; Klebba *et al.*, 1993). It was found that TonB can be cross-linked with the outer membrane "receptors" combining with, for example, siderophores (Skare *et al.*, 1993). It was suggested that TonB transmits the energy of $\Delta\bar{\mu}_{H^+}$ from the cytoplasmic membrane to the outer membrane proteins, causing translocation of, say, vitamin B_{12} across the outer membrane and its desorption to the periplasm (Fig. 2). Then vitamin B_{12} is transported to the cytoplasm by a quite different energy-dependent mechanism, most probably a vitamin B_{12}-motive ATPase. As a result, a very efficient vitamin-accumulating system is organized allowing two uphill transport mechanisms to operate in series. It is noteworthy that the vitamin B_{12} molecule is too large to diffuse via the porin channel (Postle, 1993). A mechanism similar to that mediated by TonB seems to be involved in export of some enzymes from the bacterial cytoplasm to the outer medium (reviewed in Palmen *et al.*, 1994).

Recently I suggested that the same principle as in the TonB mechanism may have been employed in the

[4] Reported at the Symposium on bacterial energetics and transport, the Society of General Microbiology (Skulachev, 1994b).

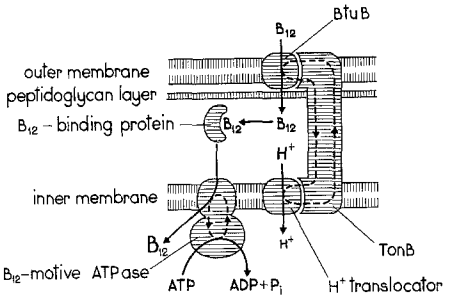

Fig. 2. The TonB-mediated transport across bacterial membranes. It is assumed that downhill translocation of H^+ through the inner (cytoplasmic) membrane from periplasm to cytosol is coupled (dashed line) with the uphill transport of a solute (e.g., vitamin B_{12}) through the outer membrane from medium to periplasm by the BtuB protein. The coupling is carried out by the TonB protein crossing the membranes and periplasm. Vitamin B_{12} is presumably bound in periplasm by a B_{12}-binding protein to be transported uphill to cytosol by a hypothetical B_{12}-motive ATPase (Skulachev, 1994c).

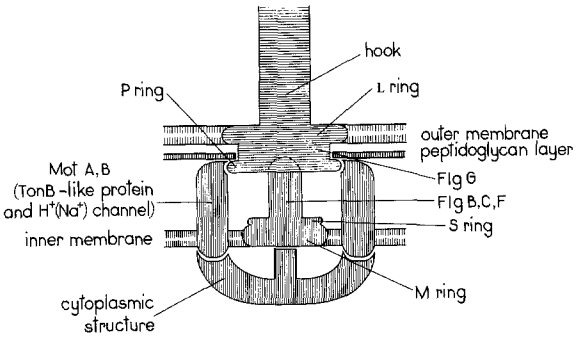

Fig. 3. Hypothetical scheme of the bacterial flagellar motor. It is assumed that the rotor part of the motor (horizontal shading) is localized outside the coupling (inner) membrane of the bacterial cell. The downhill H^+ (Na^+) influx through the inner membrane causes rotation via a TonB-like protein (putatively, a Mot protein) which transmits energy from the inner membrane to rotor. All the components localized in the inner membrane are assumed to be parts of stator (vertical shading). Probable constituents of stator belonging to the outer membrane are not shown. The essential feature of the hypothesis is that the rotating part of the motor is localized outside the inner membrane so that rotation may be organized without the risk of nonspecific leakage of ions through this membrane (Skulachev, 1994b).

flagellar motor of bacteria (Skulachev, 1994b). It is known that locomotion of the great majority of motile bacteria is supported by rotation of flagella. This rotation proved to be powered by $\Delta\bar{\mu}_{H^+}$ (Larsen *et al.*, 1974; Skulachev, 1975; Belyakova *et al.*, 1976; Matsuura *et al.*, 1977; Manson *et al.*, 1977; Glagolev and Skulachev, 1978) or $\Delta\bar{\mu}_{Na^+}$ (Hirota *et al.*, 1981; Hirota and Imae, 1983; Chernyak *et al.*, 1983; Bakeeva *et al.*, 1986). In fact, downhill flux of charged species (H^+ or Na^+) into the cell is somehow coupled to operation of the so-called $H^+(Na^+)$ motor which rotates the flagellum. The motor is a constituent of the basal body composed of several proteins and localized in the cell wall, being attached to the proximal end of the flagellum.

The mechanism of operation of the flagellar motor remains obscure. It is even unclear which of its parts form the rotor and which the stator. The simplest possibility appeared to be that it is the M-ring contacting the cytoplasmic membrane that functions as rotor. This assumption was based on the fact that the driving force for the motor, i.e., $\Delta\bar{\mu}_{H^+}$ or $\Delta\bar{\mu}_{Na^+}$, is localized on the cytoplasmic membrane.

However, it was unclear how as large a body as the M-ring (diameter about 30 nm) is rotating at high speed (up to 300 Hz) in the coupling membrane without large-scale leakage of H^+. Such leakage would seem especially dangerous in peritrichial bacteria like *E. coli* having many flagella, all being operative when the cell swims.

A scheme allowing one to overcome this difficulty is illustrated in Fig. 3. It proposes that the cytoplasmic membrane bears only stator components of the motor, including $H^+(Na^+)$ channels and TonB-like proteins which transmit energy from the channels to the rotor. Moreover, in the stator there are M and S rings which, according to recent observations, are formed by the same protein (see, e.g., Hazelbauer *et al.*, 1993), and the proximal part of the rod formed by the FlgB, C, and D proteins (Jones and Aizawa, 1991). As to the rotor, it is formed by the L and P rings and the distal part of the rod, i.e., FlgG protein. In this context it is noteworthy that, according to Okino *et al.* (1989), sonication of isolated basal bodies of *S. typhimurium* flagellar motors gave rise to their partial destruction. The resulting basal bodies were found to lack M and S rings and proximal rod proteins, whereas the L and P rings and FlgG protein were still present. In the same study, it was found that a mutation in the M-ring protein failed to stop motility but decreased the mechanical stability of the motor in viscous media.

In the scheme (Fig. 3) the TonB-like energy-transmitting protein contacts the P ring. However, in some bacteria this may be a "superdisc" of diameter much larger than the L and P rings. The "superdisc" was shown to be localized outside the cytoplasmic membrane (Kupper *et al.*, 1989; Schuster and Baeuerlein, 1992). As to the ion channel and the TonB-like energy transmitter, they might be

constituents of 10–12 intramembrane particles surrounding the basal body and protruding from the cytoplasmic membrane (Khan and Dapice, 1988; Khan *et al.*, 1992; Khan, 1993). The particles in question, most probably, include MotA and MotB proteins, which are absolutely required for motility (Hazelbauer *et al.*, 1993). Mutations in these proteins were shown to result in disappearance of the particles. Simultaneous introduction of the *mot*A and *mot*B genes led to recovery of both motility and the particles, but neither gene alone was sufficient (Khan and Dapice, 1988).

Thus, according to the scheme in Fig. 3, there is nothing rotating in the cytoplasmic membrane and, hence, risk of the H^+ (Na^+) leakage through the operating motor is minimized.

It remains to be elucidated what the mechanism of energy transmission from the cytoplasmic membrane to transport proteins of the outer membrane or to the rotor of the flagellar motor is. In the concrete versions of this mechanism one should take into account that the torque of the flagellar motor in tethered cells is practically temperature-independent within the 4–38°C range (Khan and Berg, 1983; Iwazawa *et al.*, 1993).

Some predictions of the above hypothesis can be experimentally verified.

1. Some inner membrane protein(s) essential for rotation of flagellum (tentatively, MotA or MotB) can be cross-linked with the basal body constituents localized outside the inner membrane (ring P, ring L, FlgG, or "superdisc").

2. Such a cross-linking must arrest any rotation.

3. Deletion in the gene encoding for the protein competent in cross-linking to, say, Mot, must also arrest the rotation.

4. Reconstituting MotA and MotB with phospholipids, one might form proteoliposomes which are H^+-permeable.

5. Inhibitors of the H^+ permeability in Mot proteoliposomes must also inhibit the motor.

6. Proteoliposomes with Mot proteins isolated from bacteria which have Na^+ motors must be leaky for Na^+. Amiloride derivatives inhibiting the Na^+ motor (Sugiyama *et al.*, 1988) must arrest this leakage.

The precedent that the membrane-linked energy is utilized at some distance from the coupling membrane may force us to reconsider the current point of view on many bioenergetic mechanisms. The same principle may well be operative in import of proteins and nucleic acids into the bacterial cells, export of proteins from these cells, protein import into mitochondria and chloroplasts, etc.

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