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# The ATP-driven primary $Na^+$ pump in subcellular vesicles of *Vibrio alginolyticus*

P.A. Dibrov, V.P. Skulachev, M.V. Sokolov and M.L. Verkhovskaya

*Department of Bioenergetics, A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR*

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Subcellular vesicles of *Vibrio alginolyticus* hydrolyze ATP and accumulate $Na^+$ in an ATP-dependent fashion. The $Na^+$ uptake is (i) strongly stimulated by $\Delta\psi$-discharging agents, i.e., the protonophorous uncoupler CCCP or valinomycin + $K^+$ and (ii) arrested by DCCD at a concentration strongly inhibiting ATP hydrolysis. Lower concentrations of DCCD stimulate the $Na^+$ accumulation supported by ATP hydrolysis as well as by NADH oxidation. It is concluded that there is an electrogenic DCCD-sensitive $Na^+$-ATPase in the cytoplasmic membrane of *V. alginolyticus*.

$Na^+$-ATPase; $Na^+$ bioenergetics; Bacterial vesicle; (*Vibrio alginolyticus*)

## 1. INTRODUCTION

In the marine alkalotolerant *Vibrio alginolyticus*, the electrochemical $Na^+$ potential difference ($\Delta\tilde{\mu}Na$) can be generated by $Na^+$-motive NADH-quinone reductase [1–3] and consumed by systems carrying out (i) accumulation of metabolites via $Na^+$, the metabolite symporters [4], (ii) rotation of the flagellum by the $Na^+$ motor [5,6] and (iii) ATP synthesis [7,8]. The latter effect, demonstrated in intact cells, was postulated to involve a $Na^+$-ATP-synthase which, if reversible, may be regarded as $Na^+$-ATPase. Below we shall describe experiments indicating that in the *V. alginolyticus* membrane, there is an ATP-dependent mechanism competent in the electrogenic transport of $Na^+$.

*Correspondence address:* P.A. Dibrov, Department of Bioenergetics, A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR

*Abbreviations:* $\Delta\tilde{\mu}H$ and $\Delta\tilde{\mu}Na$, electrochemical $H^+$ and $Na^+$ potential difference, respectively; CCCP, *m*-chlorocarbonyl-cyanide phenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide

## 2. MATERIALS AND METHODS

The *V. alginolyticus* strain 138-2, kindly supplied by Professor H. Tokuda (Chiba University, Chiba, Japan), was grown aerobically at 37°C in a salt medium [3] in the presence of 0.5% glucose and 0.5% peptone, pH 8.6. At the late-logarithmic phase, the cell suspension was diluted two-fold with the same medium supplemented with penicillin ($10^6$ units $\times$ $l^{-1}$) and incubated for 70 min at 37°C. The obtained spheroplasts were sedimented at 7500 $\times$ *g* for 10 min and washed with a solution of 25 mM Tris-$H_2SO_4$, 30 mM $MgSO_4$, 5 mM $Na_2SO_4$ and 150 mM $K_2SO_4$, pH 7.5 (medium A), or 50 mM Tricine, 30 mM $MgSO_4$, 5 mM $Na_2SOC_4$ and 250 mM $K_2SO_4$, pH 8.2 (medium B). The final spheroplast sediment was suspended in the same media supplemented with a serine proteinase inhibitor, 0.5 mM phenylmethylsulfonyl fluoride. The suspension was sonicated in an ultrasonic UZDN-2T desintegrator for 10 s, four times with 1 min intervals, 0°C, at a frequency of 22 kHz, $4 \times 10^{-5}$ A current and maximal resonance. Intact and partially destroyed spheroplasts were removed by centrifugation at 1200 $\times$ *g* for 10 min at 2°C. Subcellular vesicles were sedimented from the supernatant (48500 $\times$ *g*, 1 h, 2°C). The precipitate was suspended in medium A or B, using a glass homogenizer with a Teflon pestle and stored at 0°C. The final protein concentration in the stock solution was 10 $mg \cdot ml^{-1}$.

$Na^+$ transport was measured with the use of gel-filtration and centrifugation procedures [10]. The process was initiated by adding 10 mM substrate (ATP or NADH) to the suspension of subcellular vesicles. To stop the reaction, the suspension (0.05 ml) was centrifuged in a gel-filtration column ($D$ = 10 mm) with Sephadex G-50 coarse. As a buffer, a solution containing 5 mM Tris-$H_2SO_4$ and 120 mM $MgSO_4$, pH 8.5, was

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used. The cluate was diluted 10-fold so as to measure $Na^+$ with a PFM flame photometer.

## 3. RESULTS

In agreement with the observation of Tokuda et al. [11] on everted subcellular *V. alginolyticus* vesicles, we have found that NADH oxidation is coupled to $Na^+$ uptake by the vesicles, the process being stimulated by valinomycin and inhibited by monensin (fig.1A). Under the same conditions, however, no measurable ATP-dependent $Na^+$ uptake was observed (not shown).

Further experiments showed that $2 \times 10^{-5}$ M

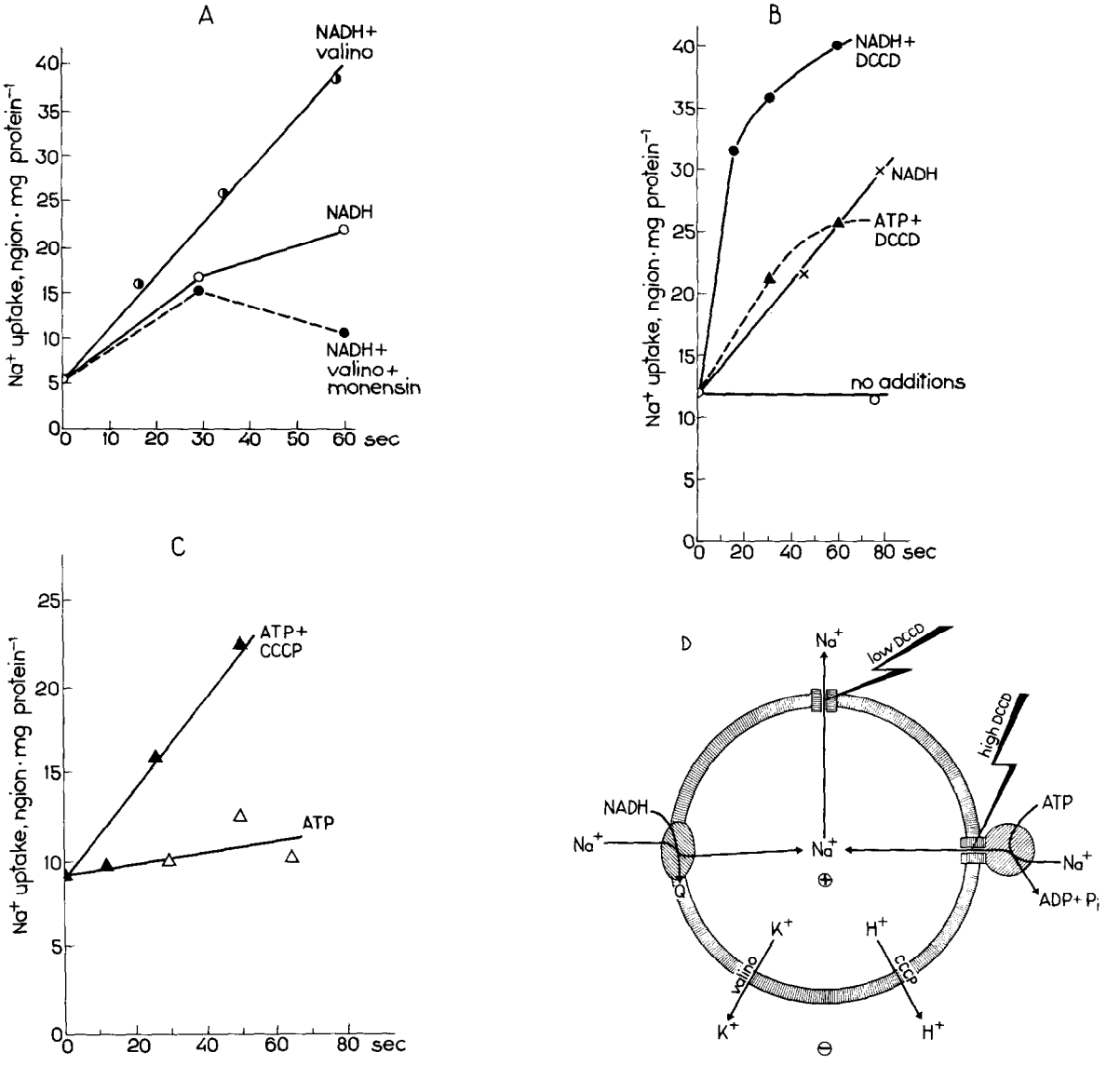


Fig.1. $Na^+$ uptake supported by NADH oxidation or ATP hydrolysis in *V. alginolyticus* subcellular vesicles. (A) Medium A; (B,C) medium B. (B) $5 \times 10^{-6}$ M valinomycin in all the samples; (C) vesicles were preincubated with $2 \times 10^{-5}$ M DCCD for 10 min (similar DCCD treatment was applied to samples with DCCD in B). Where indicated, the following additions were made at zero time: 10 mM NADH, 5 mM ATP, $2 \times 10^{-6}$ M valinomycin, $4 \times 10^{-6}$ M monensin and $2 \times 10^{-5}$ M CCCP. (D) A scheme illustrating the interplay of the energy-linked and passive ion fluxes in the vesicles.

Table 1

DCCD effect on ATPase activity and ATP-dependent $Na^+$ uptake by *V. alginolyticus* subcellular vesicles

| [DCCD] ($\mu$M) | Preincubation (min) | ATPase (nmol ATP·mg protein$^{-1}$·min$^{-1}$) | $Na^+$ uptake (ng ion·mg protein$^{-1}$·min$^{-1}$) |
|---|---|---|---|
| – | – | 365 | 5 |
| 20 | 10 | 275 | 35 |
| 200 | 20 | 90 | 0 |

DCCD (3 nmol·mg protein$^{-1}$) strongly stimulated the NADH-oxidation supported $Na^+$ transport; addition of ATP also resulted in an $Na^+$ uptake which proceeded at a somewhat lower rate than in the NADH-containing sample (fig.1B). A higher ($2 \times 10^{-4}$ M) concentration of DCCD inhibited the ATP-dependent $Na^+$ accumulation and the ATPase activity (table 1).

Just as in the case of respiration-linked $Na^+$ uptake, the ATP effect was greatly stimulated by valinomycin and abolished by monensin. The protonophorous uncoupler CCCP was found to effectively substitute for valinomycin as an activator of the ATP-dependent $Na^+$ transport. Fig.1C shows that in the absence of valinomycin, no $Na^+$ uptake was induced by the ATP addition to the vesicles treated with a small amount of DCCD. Such an uptake occurred when $2 \times 10^{-5}$ M CCCP was present in the incubation mixture.

## 4. DISCUSSION

The above data are summarized in fig.1D. It is shown that $Na^+$ can be transported into everted vesicles by means of $Na^+$-motive NADH-quinone reductase of $Na^+$-motive ATPase. Such transport results in a positive charging of the vesicle interior. The formed $\Delta\psi$, which prevents large-scale $Na^+$ accumulation, can be discharged by an efflux of $K^+$ or $H^+$ in the presence of valinomycin or CCCP, respectively. Apparently the procedure of preparation of vesicles caused a partial loss of the catalytic parts of the $Na^+$-ATPase, whereas the $Na^+$-transporting parts remained in the membrane and increased its $Na^+$ conductance. This conductance seems to be sensitive to low concentrations of DCCD which prevents the leakage of accumulated $Na^+$. A high amount of DCCD strongly inhibits ATPase and therefore arrests the ATP-dependent $Na^+$ uptake. In this context, it should be mentioned that $Na^+$-ATPase of *Propionigenum modestum* proved to be a DCCD-sensitive enzyme of the subunit composition similar to that of the $F_0F_1$ type $H^+$-ATPase [12].

An interesting problem is the relation of the above described ATP-dependent $Na^+$ to the $H^+$-ATPase activity previously described by our group in the *V. alginolyticus* vesicles [13,14]. The ATP-linked $H^+$ transport is also stimulated by low DCCD, inhibited by high DCCD and stimulated by valinomycin. However, CCCP completely abolished the $H^+$ uptake by vesicles. This fact is in contrast to the above data on the stimulation of $Na^+$ transport by the same protonophore. The latter observation excludes any explanation of the ATP-supported $Na^+$ transport as a consequence of utilization of the $\Delta\tilde{\mu}H$ produced by $H^+$-ATPase.

Three possibilities may be considered to account for the ATP-linked $Na^+$ and $H^+$ pumps in *V. alginolyticus*: (i) there are two DCCD-sensitive ATPases in the same membrane, one for $Na^+$ and the other for $H^+$; (ii) a single ATPase can transport alternatively $Na^+$ or $H^+$; (iii) a single ATPase is competent in the symport of $Na^+$ and $H^+$.

It may be noted that $Na^+$-ATPase of *P. modestum* transports $H^+$ when $Na^+$ is absent [15] and animal $Na^+/K^+$-ATPase performs an $H^+/K^+$ antiport at acidic pH in a $Na^+$-free medium [16]. According to Boyer, substitution of $Na^+$ for $H^+$ can be explained assuming that the cation-binding sites are organized as crown ethers coordinating $Na^+$ or $H_3O^+$ [17].

The functional significance of the existence of two primary ATP-driven pump activities, one dealing with $H^+$ and the other with $Na^+$, may mean that *V. alginolyticus* can survive in both neutral and alkaline conditions. At neutral pH, the proton cycle can be operative, which involves the $H^+$-motive respiratory chain and $H^+$-ATP-synthase. At alkaline pH, when $\Delta$pH and $\Delta\psi$ are oppositely directed, the sodium cycle is actuated, with the $Na^+$-motive respiratory chain and $Na^+$-ATP-synthase being involved. In agreement with this reasoning, it was shown in our group that the *V. alginolyticus* respiratory chain, besides the $\Delta\tilde{\mu}Na$-generating NADH-quinone reductase, includes the $\Delta\tilde{\mu}H$ generator(s) between quinol and $O_2$ [18].

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