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The sodium cycle. I. Na^+-dependent motility and modes of membrane energization in the marine alkalotolerant *Vibrio alginolyticus*

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Respiration, membrane potential generation and motility of the marine alkalotolerant *Vibrio alginolyticus* were studied. Subbacterial vesicles competent in NADH oxidation and $\Delta\psi$ generation were obtained. The rate of NADH oxidation by the vesicles was stimulated by Na^+ in a fashion specifically sensitive to submicromolar HQNO (2-heptyl-4-hydroxyquinoline *N*-oxide) concentrations. The same amounts of HQNO completely suppressed the $\Delta\psi$ generation. $\Delta\psi$ was also inhibited by cyanide, gramicidin D and by CCCP + monensin. CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) added without monensin exerted a much weaker effect on $\Delta\psi$. Na^+ was required to couple NADH oxidation with $\Delta\psi$ generation. These findings are in agreement with the data of Tokuda and Unemoto on Na^+-motive NADH oxidase in *V. alginolyticus*. Motility of *V. alginolyticus* cells was shown to be (i) Na^+-dependent, (ii) sensitive to CCCP + monensin combination, whereas CCCP and monensin, added separately, failed to paralyze the cells, (iii) sensitive to combined treatment by HQNO, cyanide or anaerobiosis and arsenate, whereas inhibition of respiration without arsenate resulted only in a partial suppression of motility. Artificially imposed Δp*Na*, i.e., addition of NaCl to the K^+-loaded cells paralyzed by HQNO + arsenate, was shown to initiate motility which persisted for several minutes. Monensin completely abolished the NaCl effect. Under the same conditions, respiration-supported motility was only slightly lowered by monensin. The artificially-imposed ΔpH, i.e., acidification of the medium from pH 8.6 to 6.5 failed to activate motility. It is concluded that $\Delta\bar{\mu}_{Na^+}$ produced by (i) the respiratory chain and (ii) an arsenate-sensitive anaerobic mechanism (presumably by glycolysis + Na^+ATPase) can be consumed by an Na^+-motor responsible for motility of *V. alginolyticus*.

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Abbreviations: $\Delta\bar{\mu}_{H^+}$, $\Delta\bar{\mu}_{Na^+}$, electrochemical gradients of H^+ and Na^+, respectively; $\Delta\psi$, transmembrane electric-potential difference; ΔpH and Δp*Na*, transmembrane differences in concentrations of H^+ and Na^+, respectively; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; HQNO, 2-heptyl-4-hydroxy-quinoline *N*-oxide; PCB^-, phenyldicarbaundecaborane; TPP^+, tetraphenylphosphonium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Taps, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulphonic acid; Ches, 2-(cyclohexylamino)ethanesulphonic acid.

Introduction

The progress made in bioenergetics studies in the last few years has stimulated the interest in the role of Na^+. The current point of view that H^+ is used as the coupling ion in all the energy-transducing membranes, with the only exception of the animal cell plasma membrane, is not so obvious now as it seemed before.

It was postulated that the Na^+/K^+-gradient

across the cytoplasmic membrane of bacteria serves as a $\Delta\bar{\mu}_{H^+}$-buffer which is charged when energy sources are in excess and is discharged when they are exhausted [1,2]. The data confirming this hypothesis were obtained in experiments with *Halobacterium halobium* [3,4], *Escherichia coli* and some other bacteria [4,10]. Moreover, in many bacteria it was found that $\Delta\bar{\mu}_{Na^+}$ formed at the expense of the $\Delta\bar{\mu}_{H^+}$ energy, can be utilized by Na^+, solute-symporters to accumulate some solutes inside the cell (for reviews, see Refs. 5 and 6).

Dimroth et al. [7–9,11–14] and Buckel and Semmler [15,16] reported about $\Delta\bar{\mu}_{Na^+}$ formation with no $\Delta\bar{\mu}_{H^+}$ involved in a group of anaerobic bacteria possessing Na^+-motive decarboxylases. In one of them (*Propionigenum modestum*), an Na^+-motive ATPase reaction was detected and indications of $\Delta\bar{\mu}_{Na^+}$-supported ATP synthesis by a reversal of this process were obtained [13]. Na^+-ATPase was also described in *Micoplasma mycoides* [17,18] and in *Streptococcus faecalis* [19,20]. In the latter case, it was shown that Na^+-ATPase is increased significantly under conditions unfavourable for $\Delta\bar{\mu}_{H^+}$ formation, i.e., in a mutant defective in H^+-ATPase and in the *S. faecalis* wild type growing in the presence of a protonophorous uncoupler [20].

Alkaline medium represents a natural niche where it is difficult to employ $\Delta\bar{\mu}_{H^+}$ of the usual direction (the interior of the bacterium is more negative and alkaline than the exterior). Here pumping of H^+ from the cytoplasm results in $\Delta\psi$ generation which is counterbalanced by a ΔpH of the opposite direction. As a result, $\Delta\bar{\mu}_{H^+}$ appears to be too low to support the energy-linked functions of the cytoplasmic membrane [6].

The first indication that an ion other than H^+ energizes the bacterial membrane under alkaline conditions was obtained by Tokuda and Unemoto [21] when studying the alkalotolerant marine *Vibrio alginolyticus*. It was found that at alkaline conditions, the respiration of this bacterium is coupled with the extrusion of Na^+ from the cell to the outer medium. $\Delta\bar{\mu}_{Na^+}$ generation proved to be localized in the respiratory chain between NADH dehydrogenase and mena(ubi)quinone [21,22]. It was found in the same laboratory that the import of 19 amino acids and sucrose by *V. alginolyticus* cells proceeds in a $\Delta\bar{\mu}_{Na^+}$-dependent fashion [21,23]. Thus, respiration-generated $\Delta\bar{\mu}_{Na^+}$ was used to perform osmotic work. As it was found in our group, the mechanical work of flagellum rotation is another type of membrane-linked activity supported by $\Delta\bar{\mu}_{Na}$ in *V. alginolyticus*.

Summing up these observations we suggested that at high pH it is $\Delta\bar{\mu}_{Na^+}$, rather than $\Delta\bar{\mu}_{H^+}$, that plays the role of convertible membrane-linked energy 'currency' in *V. alginolyticus* (the sodium cycle concept) [24–27].

In this study, we continued our work on the sodium cycle problem. The final experimental proof of the Na^+-motor in *V. alginolyticus* was obtained, i.e., it was found that the monensin-sensitive motility can be supported by an artificially imposed $\Delta p\mathit{Na}$. Na^+-dependent membrane potential generation in intact bacteria and subbacterial vesicles is also to be considered. Na^+-coupled respiratory ATP synthesis and a comparative study of *V. alginolyticus* as a representative of the *Vibrionaceae* family will be the subject of the next two papers [28,29]. Some of these results were presented by one of us (V.P.S.) in a plenary lecture at the FEBS Meeting in Albufeira (Portugal) [30].

Methods and Materials

V. alginolyticus 138-2 was the generous gift of Professor Hajime Tokuda. Bacteria were grown aerobically at 37°C in a salt medium used by Tokuda et al [31], i.e., 0.5 M NaCl/10 mM KCl/2 mM KH_2PO_4/15 mM $(NH_4)_2SO_4$/5 mM $MgSO_4$/50 mM Tris-HCl (pH 7.5), supplemented with 0.5% peptone and 0.3% yeast hydrolyzate. At the late exponential growth phase, the cells were precipitated by centrifugation at 20°C. The precipitate was suspended in the growth medium. Na^+- or K^+-loaded cells were obtained as described in the next paper of the series [28].

To measure the motility rate, we estimated the time a bacterium requires to swim a known distance without changing the direction of the movement. The measurements were carried out at room temperature using a phase-contrast microscope. To calculate the motility rate, the average data on ten cells were used.

The *V. alginolyticus* subbacterial vesicles were

obtained by using a procedure similar to that of Laddaya and MacLeod [32] for isolation of membranes of the marine bacterium *Alteromonas haloplanktis*. The *V. alginolyticus* cells were washed by 0.5 M NaCl/1 mM Hepes-NaOH (pH 7.5). Here and below, the amount of washing medium was 1/7 of the growth medium. $7000 \times g$ precipitate was suspended in a small amount of the NaCl-Hepes medium and stored for 12 h at 0°C. Then the cells were washed in an NaCl-Hepes medium and $7000 \times g$ sediment was washed by 0.5 M sucrose + 1 mM Hepes-NaOH (pH 7.5). $14000 \times g$ sediment was suspended in sucrose-Hepes medium. After 30 min incubation with stirring at 25–30°C, the cells were centrifuged at $14000 \times g$ for 10 min. The precipitate was suspended and incubated at 25–30°C for 30 min in a mixture of 0.3 M NaCl/50 mM $MgSO_4$/10 mM Hepes-NaOH (pH 7.5)/0.15 mg · ml^{-1} lysozyme. Resultant spheroplasts were centrifuged at 14000 $\times g$ for 10 min, the precipitate was suspended with the aid of a glass-Teflon homogenizer in a medium containing 0.1 M sucrose/25 mM NaCl/2 mM $MgSO_4$/4 mM dithiothreitol/0.3 mM phenylmethanesulfonylfluoride/50 mM Hepes-NaOH (pH 7.5). The amount of the suspension was 1/70 of the growth medium.

Suspension was sonicated 5 times for 20 s at a frequency of 22 kHz, $4 \cdot 10^{-5}$ A current, and maximal resonance. The mixture was centrifuged at $15000 \times g$ for 15 min and the supernatant at $160000 \times g$ for 50 min, both at 4°C. The precipitate of subbacterial vesicles was washed with 0.1 M sucrose/4 mM dithiothreitol/10 mM Hepes-NaOH (pH 7.5). Washed vesicles were suspended in the medium of the same composition to obtain the final protein concentration of about 20–30 mg per ml. The final suspension was stored at −20°C.

Oxygen consumption was measured polarographically. TPP^+ and PCB^- concentrations were monitored with TPP^+-sensitive electrode [33] and with a phospholipid-impregnated Teflon filter (diameter of the pores, 5 μm) [38], respectively. NADH oxidation was measured with a Cary-219 spectrophotometer at 340 nm.

Results

Respiration and $\Delta\psi$ generation in intact bacteria and subbacterial vesicles

In the first series of experiments, the mechanism of *V. alginolyticus* membrane energization was studied. It was shown that the storage of bacteria in 0.5 M NaCl at −5°C for 12 h results in exhaustion of endogenous respiratory substrates so that the respiration rate becomes negligible. Addition of a substrate, lactate, activated respiration, the uptake of tetraphenylphosphonium cation (TPP^+). The TPP^+ uptake was completely reversed or prevented by HQNO, a respiratory chain inhibitor affecting in *V. alginolyticus* first of all the Na^+-motive NADH-quinone oxidoreductase (see Ref. 21 and below) (Fig. 1).

In the following experiments, we studied respiration and $\Delta\psi$ generation in the inside-out subbacterial vesicles.

As seen in Fig. 2A, NaCl activates the rate of the NADH oxidation by the vesicles. The pH optimum of this activation is shifted to the alkaline region (between pH 8.5 and 9.0, Fig. 2B). Titration of the respiration rate by HQNO is given in Fig. 2C. In agreement with the observation of Tokuda and Unemoto on the whole *V. alginolyticus* cells [21] the Na^+-stimulated portion of the NADH oxidase activity proved to be specifically abolished by HQNO. Very low concentrations of HQNO were found to be inhibi-

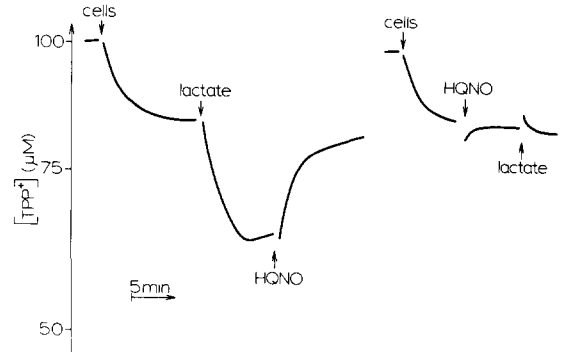

Fig. 1. $\Delta\psi$ generation by intact cells of *V. alginolyticus*. The incubation mixture contained: 0.5 M NaCl/25 mM Tricine-NaOH (pH 8.5)/$1 \cdot 10^{-4}$ M TPP^+/0.22 mg protein per ml Na^+-loaded cells. Additions: 75 mM potassium DL-lactate and $1 \cdot 10^{-4}$ M HQNO.

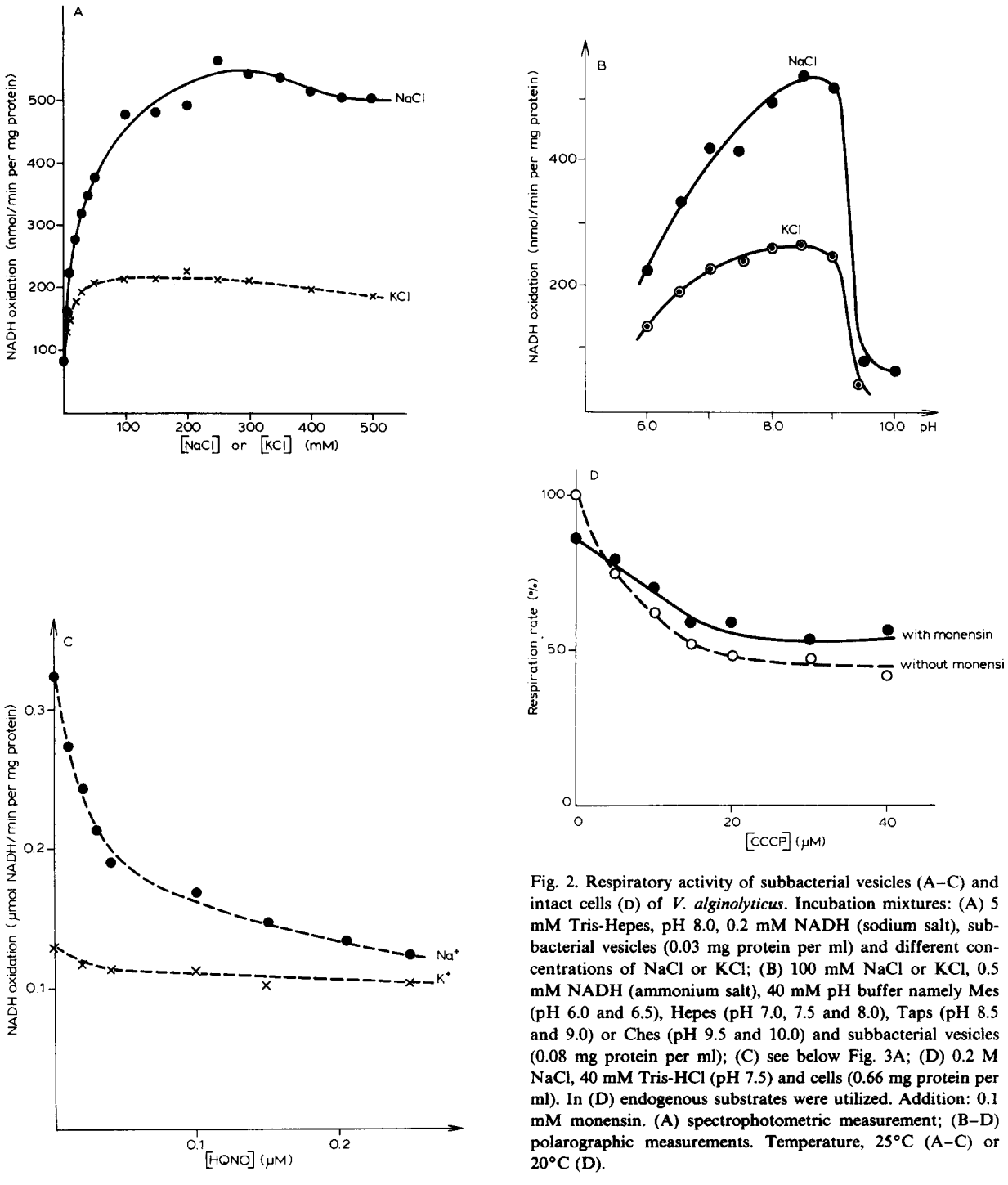

Fig. 2. Respiratory activity of subbacterial vesicles (A–C) and intact cells (D) of *V. alginolyticus*. Incubation mixtures: (A) 5 mM Tris-Hepes, pH 8.0, 0.2 mM NADH (sodium salt), subbacterial vesicles (0.03 mg protein per ml) and different concentrations of NaCl or KCl; (B) 100 mM NaCl or KCl, 0.5 mM NADH (ammonium salt), 40 mM pH buffer namely Mes (pH 6.0 and 6.5), Hepes (pH 7.0, 7.5 and 8.0), Taps (pH 8.5 and 9.0) or Ches (pH 9.5 and 10.0) and subbacterial vesicles (0.08 mg protein per ml); (C) see below Fig. 3A; (D) 0.2 M NaCl, 40 mM Tris-HCl (pH 7.5) and cells (0.66 mg protein per ml). In (D) endogenous substrates were utilized. Addition: 0.1 mM monensin. (A) spectrophotometric measurement; (B–D) polarographic measurements. Temperature, 25°C (A–C) or 20°C (D).

tory: half-maximal inhibition was observed to be induced by $2.5 \cdot 10^{-8}$ M HQNO or $9 \cdot 10^{-11}$ mol HQNO per mg protein. Such concentrations of HQNO had no effect upon Na^+-independent respiration.

In Fig. 2D the effect of CCCP on respiration of

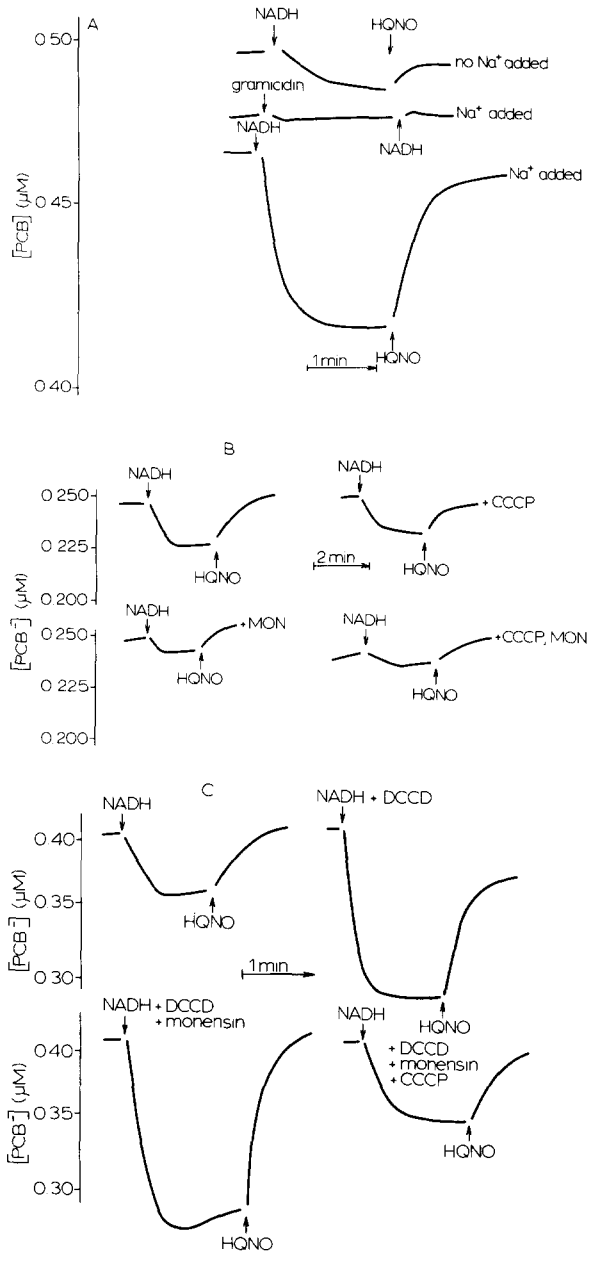

Fig. 3. $\Delta\psi$ formation by subcellular vesicles of *V. alginolyticus* Incubation mixtures contained: (A) and (B), 0.1 M sucrose/20 mM Tris-HCl (pH 7.5)/20 mM NaCl/$1 \cdot 10^{-6}$ M PCB^-/0.3–0.4 mg protein per ml subbacterial vesicles; (C), 0.1 M sucrose/25 mM NaCl/50 mM Hepes-NaOH (pH 7.5)/$1 \cdot 10^{-6}$ M PCB^-/0.3 mg protein per ml^{-1} subbacterial vesicles. Additions: 1 mM NADH/$2.5 \cdot 10^{-6}$ M HQNO/$5 \cdot 10^{-6}$ M CCCP/$1 \cdot 10^{-6}$ M monensin/$2 \cdot 10^{-6}$ M gramicidin D/$4 \cdot 10^{-5}$ M DCCD. In the samples without gramicidin, Fig. 1A, the mixture was supplemented with 10 mM $(NH_4)_2SO_4$, and the NH_4^+ salt of NADH was added. In the sample with gramicidin (Fig. 3A), a 20 min time interval followed between the additions of gramicidin and NADH (not shown in the figure). In Fig. B, the mixture was supplemented with $1.25 \cdot 10^{-7}$ M HQNO inducing partial inhibition of the Na^+-motive NADH-quinone reductase to facilitate discharge of $\Delta\psi$ by CCCP and monensin.

intact cells is shown. It is seen that this compound causes a 2-fold inhibition of the respiratory rate. Monensin, the Na^+/H^+-antiporter [34] exerts no significant influence upon this effect.

Fig. 3 shows membrane potential generation coupled with NADH oxidation by subbacterial vesicles. To monitor the membrane potential, synthetic penetrating anion PCB^- was used [33]. Addition of NADH was found to initiate the PCB^- uptake by the vesicles. This uptake was energy-dependent, being sensitive to gramicidin D, HQNO (Fig. 3A), and to cyanide (not shown). Na^+ addition strongly increased $\Delta\psi$ (Fig. 3A). CCCP + monensin inhibited the $\Delta\psi$ formation. CCCP and monensin added separately were less efficient (Fig. 3B). Combination of CCCP and monensin was without effect upon respiration rate in subbacterial vesicles (data not shown).

As shown in the following experiments, the addition of DCCD increased the respiratory $\Delta\psi$. Addition of monensin after DCCD induced a further $\Delta\psi$ increase. DCCD and monensin effects were completely abolished by CCCP (Fig. 3C).

Motility of the V. alginolyticus cells

In the below experiments, the motility of *V. alginolyticus* cells was studied. As is seen from Fig. 4A, Na^+ is necessary for motility at either pH 8.6 or 7.5, the higher Na^+ level being required at less alkaline pH. Half-maximal motility rates were observed at about 17 mM and 50 mM NaCl at pH 8.6 and 7.5, respectively. Addition of CCCP was found to lower the motility rate and strongly increase the Na^+ level necessary for the maximal rate to be attained.

In Fig. 4B, CCCP titration of the motility rate is shown, At pH 7.5, a quite obvious plateau was revealed when the CCCP concentrations increased above $1 \cdot 10^{-6}$ M. Such a CCCP-resistant motility was completely suppressed by monensin. In the absence of CCCP, monensin was without any measurable effect upon motility. At pH 8.6 the CCCP titration curves were shifted to the higher

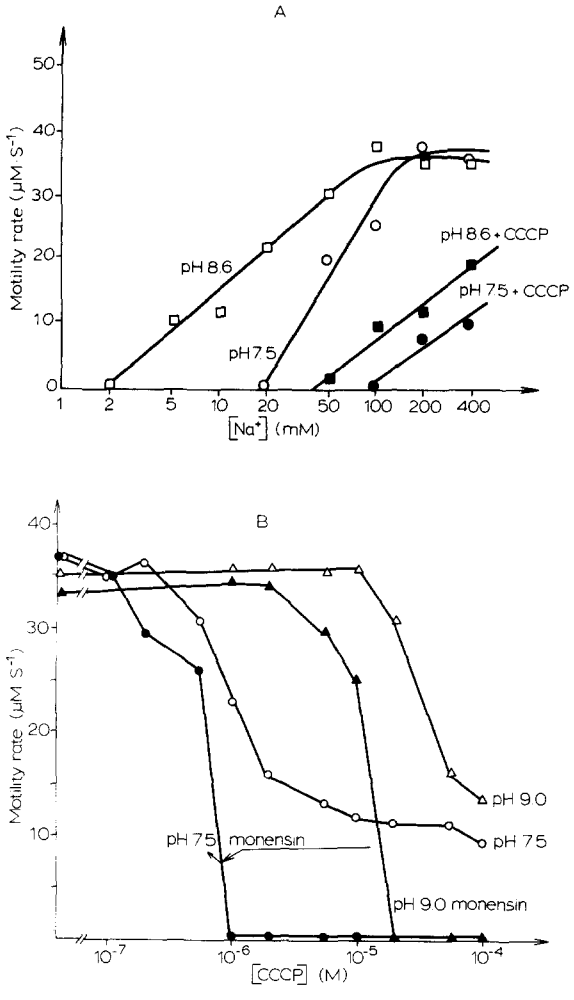

Fig. 4. Motility of *V. alginolyticus* driven by the respiration-produced $\Delta\bar{\mu}_{Na^+}$. (A) Incubation mixture contained: 50 mM Tris-HCl (pH 7.5 or 8.6), K^+-loaded cells (10^5 cells per ml) and different concentrations of NaCl and KCl so that the total NaCl + KCl concentration was 0.4 M; [NaCl] is indicated in the abscissa; CCCP concentration was $1 \cdot 10^{-5}$ M. (B) Bacteria (10^5 cells per ml) were incubated for 3 min at room temperature in a medium containing 0.5 M NaCl/50 mM Tris-HCl (pH 7.5 or 8.6), in the presence of different CCCP concentration with or without $3 \cdot 10^{-5}$ M monensin.

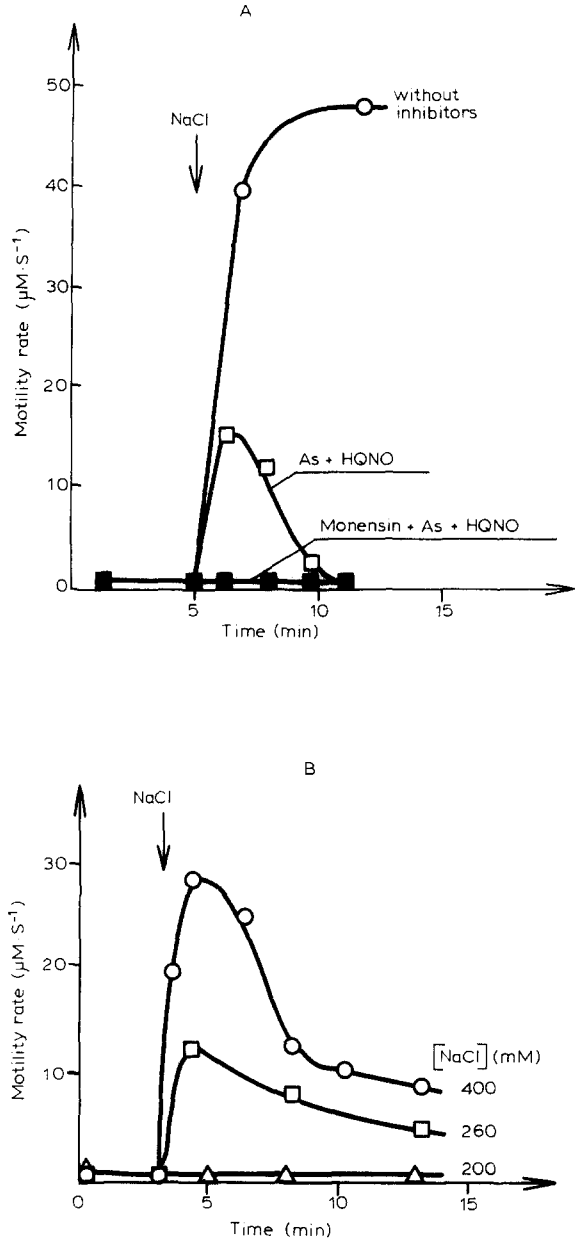

Fig. 5. *V. alginolyticus* motility supported by the artificially imposed $\Delta p Na$. (A) K^+-loaded cells (10^6 cell per ml) were incubated at room temperature for 10 min in 0.4 M KCl and 50 mM Tris-HCl (pH 7.5) with or without the inhibitors. $3 \cdot 10^{-5}$ M monensin and 10 mM potassium arsenate were added at zero time; $1 \cdot 10^{-5}$ M HQNO was added 5 min later. Then the cells were diluted to 10^5 bacteria per ml with the same solution (the starting point in the figure) or with a solution containing NaCl instead of KCl (the dilution time is indicated by abscissa). (B) As (A), but HQNO and arsenate were present in all the samples, differing in the outer NaCl concentration.

protonophore concentrations apparently due to a lower level of the protonated form of CCCP.

Some results of the inhibitor analysis of *V. alginolyticus* motility are given in Table I. It was found that a combination of (i) any agent inhibiting the respiratory chain (KCN, HQNO or anaerobiosis), and (ii) arsenate is required to

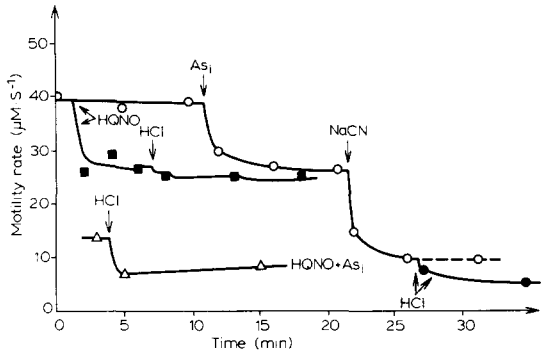

Fig. 6. Artificially imposed ΔpH fails to support *V. alginolyticus* motility. The cells were diluted with a solution of 0.4 M NaCl/0.05 M Tris-HCl (pH 8.6). HQNO + As_i curve, cells were pretreated with HQNO and arsenate as in Fig. 5A. Additions, $1 \cdot 10^{-4}$ M HQNO, $1 \cdot 10^{-2}$ M potassium arsenate, $3 \cdot 10^{-4}$ M NaCN and HCl to shift pH from 8.6 to 6.5.

paralyze the cells completely while arsenate or, say, KCN, added separately, exerts only a partial inhibitory effect on motility.

In cells paralyzed by HQNO and arsenate, the effect of the artificially imposed gradient of the Na^+ concentration was studied. It was shown (Fig. 5 A and B) that the addition of NaCl to an Na^+-free medium initiated motility of the HQNO and arsenate-treated cells which were motionless before this addition. The NaCl-induced motility proved to be transient and disappeared within 5 min. Monensin completely prevented the motility burst (Fig. 5A). Without HQNO and arsenate, NaCl induced motility which did not decrease in the investigated time interval (Fig. 5A) and was monensin-resistant (see above Fig. 4B). At the same time, an artificially imposed pH gradient failed to support motility (Fig. 6).

Discussion

The data in the first series of experiments seem to confirm the observations by Tokuda an Unemoto on intact *V. alginolyticus* cells [21] and extend them to subbacterial inside-out vesicles. They are in agreement with the suggestion [21] that in the cytoplasmic membrane of these bacteria there is an electrogenic Na^+-motive system of NADH oxidation.

(i) NADH oxidation by vesicles is stimulated by Na^+ (Fig. 2A–C).

(ii) This stimulation is specifically abolished by submicromolar HQNO (Fig. 2C).

(iii) Na^+ is required to couple NADH oxidation with $\Delta\psi$ generation (Fig. 3A).

(iv) $\Delta\psi$ supported by NADH oxidation in the presence of Na^+ is suppressed by low concentrations of HQNO or gramicidin D (Fig. 3A).

The effects of CCCP and monensin may be explained in the same terms. An addition of CCCP which is known to completely suppress $\Delta\psi$ formation by the protonic potential generators proved to be insufficient to obtain the maximal inhibition of $\Delta\psi$ in *V. alginolyticus* vesicles. Apparently this is due to the production of ΔpH resulting from electrophoretic CCCP-mediated H^+ efflux down $\Delta\psi$ formed by the Na^+-motive respiration.

If such is the case, monensin, discharging ΔpH, should potentiate CCCP inhibition. This was in fact found in the experiment (Fig. 3B). Control measurements on intact cells showed that monensin had no effect upon the respiration rate in the presence of CCCP (Fig. 2D).

Some inhibitory effect of monensin added without CCCP on the $\Delta\psi$ level (Fig. 3B) may be due to the existence of an endogenous H^+ conducting system in subbacterial vesicles. This system seems to be DCCD-sensitive. Such an assumption explains a DCCD-induced increase in the $\Delta\psi$ level in vesicles (Fig. 3C). In full consistences with this explanation, it was found that DCCD treatment reverses the direction of the monensin effect upon the $\Delta\psi$ level in the vesicles. Monensin caused some $\Delta\psi$ decrease in the absence of DCCD and some $\Delta\psi$ increase in its presence (Fig. 3).

In the second part of the paper, the motility of *V. alginolyticus* cells was studied. In conformity with the preliminary observations made in this group [24,25], it was found that $\Delta\bar{\mu}_{Na^+}$, rather than $\Delta\bar{\mu}H$, is consumed by the flagellar motor of this bacterium.

(i) Na^+ is necessary for motility (Fig. 4A).

(ii) There is a CCCP-resistant motility which is abolished by combined treatment with CCCP + monensin (Fig. 4B).

(iii) HQNO inhibits the motility rate increase which accompanies transition from anaerobic to aerobic conditions (Table I),

(iv) Artificially imposed ΔpNa, but not ΔpH,

TABLE I

INHIBITOR ANALYSIS OF *V. ALGINOLYTICUS* MOTILITY AT PH 8.6

Conditions	Additions	Motility rate ($\mu m \cdot s^{-1}$)
Aerobic	–	35
	0.2 mM KCN	15
	$1 \cdot 10^{-5}$ M HQNO	20
	10 mM arsenate	25
	KCN + arsenate	0
	HQNO + arsenate	0
Anaerobic	–	10
	3 mM arsenate	0

initiates motility of the HQNO- and arsenate-poisoned cells for several min, the effect being monensin-sensitive (Fig. 5 A and B).

The latter observation can be regarded as the final proof of the $\Delta\bar{\mu}_{Na^+}$-supported motility of *V. alginolyticus* cells.

Recently in this group Drs. I.I. Brown, I.I. Kirik and I.I. Severina obtained indications that a similar mechanism is responsible for motility of the alkalotolerant and halotolerant cyanobacterium *Oscillatoria brevis*.

The literature gives two other indications of the Na^+-motor existence. In both cases, alkalophilic bacilli were studied, namely *Bacillus firmus* [35] and *Bacillus* YN-1 [36,37]. Artificially imposed ΔpNa was not tested. As to the respiration-supported motility, it proved to be Na^+-dependent. Both $\Delta\psi$ and ΔpNa produced by respiration were equally effective in supporting motility [37].

A rather high $\Delta\bar{\mu}_{Na^+}$ threshold (about 100 mV) was found for the Na^+-motor of *Bacillus* YN-1 [37]. This fact may explain our observation that even a 2-fold decrease in $[Na^+]_{out}$ (from 400 to 200 mM, see Fig. 5B) prevents motility from being activated by artificial ΔpNa.

The high threshold may also account for the fact that CCCP added without monensin still causes some decrease in the motility rate (see Fig. 4B). Maybe a ΔpNa rise which must accompany the CCCP-induced $\Delta\psi$ decrease failed to compensate this decrease in a time interval when the motility rate was being measured. If we are not far from the threshold, even a small lowering of $\Delta\bar{\mu}_{Na^+}$ may induce an appreciable inhibition of motility. Proceedings from the above reasoning it was found that a higher outer $[Na^+]$ and hence a higher ΔpNa was required to support motility when CCCP was present (Fig. 4A). Another reason for the CCCP-induced inhibition may be connected with the CCCP-mediated change in the intracellular pH which may have an allosteric effect on the Na^+-motor or respiratory chain. The direct inhibitory effect of CCCP on the respiratory chain (see Fig. 2D) may also be involved.

Summarizing the data on the motility measurements one may conclude that *V. alginolyticus*, like alkalophilic bacilli, employed the Na^+-motor to swim. In the case of bacilli, it remained unclear whether the motor-utilized $\Delta\bar{\mu}_{Na^+}$ was directly produced by an Na^+-motive respiratory chain or its formation was supported by co-operation of an H^+-motive respiration and an Na^+/H^+-antiporter. On the other hand, it seems clear in the case of *V. alginolyticus* that $\Delta\bar{\mu}_{Na^+}$-generation may be due to the Na^+-motive respiratory chain first described by Tokuda and Unemoto [21] and confirmed by experiments reported in the first part of this paper. Apart from respiration, there is yet another mechanism of membrane energization in *V. alginolyticus* which can support motility. It is insensitive to anaerobiosis, HQNO and cyanide but sensitive to arsenate (Table I). One possibility is that under anaerobiosis, hydrolysis of glycolytic ATP by an Na^+-ATPase is responsible for membrane energization, supplying the Na^+-motor with energy. If such were the case, Na^+-ATPase, operating in the opposite directions might be able to catalyze Na^+-coupled oxidative phosphorylation under aerobic conditions. This possibility was analyzed in following paper [28].

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