T. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7664-7668.
Pelton, J. G., & Wemmer, D. E. (1988) *Biochemistry 27*, 8088-8096.
Saenger, W. (1984) in *Principles of Nucleic Acid Structures*, Springer-Verlag, New York.
Sarma, M. H., Gupta, G., & Sarma, R. H. (1988) *Biochemistry 27*, 3423-3432.
States, D. J., Haberkorn, R. H., Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286-292.
Weiner, S. J., Kollman, P. J., Case, D. A., Singh, U. C., Ghio, C., Alagona, G., Proteta, S., & Weiner, P. (1984) *J. Am. Chem. Soc. 106*, 765-780.

Respiratory-Driven Na^+ Electrical Potential in the Bacterium *Vitreoscilla*†

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ABSTRACT: *Vitreoscilla* is a Gram-negative bacterium with unique respiratory physiology in which Na^+ was implicated as a coupling cation for the generation of a transmembrane electrical gradient ($\Delta\psi$). Thus, cells respiring in the presence of 110 mM Na^+ generated a $\Delta\psi$ of −142 mV compared to only −42 and −56 mV for Li^+ and choline, respectively, and even the −42 and −56 mV were insensitive to the protonophore 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (DTHB). The kinetics of $\Delta\psi$ formation and collapse correlated well with the kinetics of Na^+ fluxes but not with those of H^+ fluxes. Cyanide inhibited respiration, Na^+ extrusion, and $\Delta\psi$ formation 81% or more, indicating that $\Delta\psi$ formation and Na^+ extrusion were coupled to respiration. Experiments were performed to distinguish among three possible transport systems for this coupling: (1) a Na^+-transporting ATPase; (2) an electrogenic Na^+/H^+ antiport system; (3) a primary Na^+ pump directly driven by the free energy of electron transport. DCCD and arsenate decreased cellular ATP up to 86% but had no effect on $\Delta\psi$, evidence against a Na^+-transporting ATPase. Low concentrations of DTHB had no effect on $\Delta\psi$; high concentrations transiently collapsed $\Delta\psi$, but led to a stimulation of Na^+ extrusion, the opposite of that expected for a Na^+/H^+ antiport system. Potassium ion, which collapses $\Delta\psi$, also stimulated Na^+ extrusion. The experimental evidence is against Na^+ extrusion by mechanisms 1 and 2 and supports the existence of a respiratory-driven primary Na^+ pump for generating $\Delta\psi$ in *Vitreoscilla*.

The free energy change of respiration in aerobic prokaryotes is generally conserved by coupling it to the outward translocation of protons, thereby creating a H^+ electrochemical gradient ($\Delta\mu_{H^+}$)[1] across the cell membrane (Mitchell, 1976). The $\Delta\mu_{H^+}$ then mediates various endergonic transmembrane reactions such as ATP synthesis and substrate accumulation. Na^+ has been reported to play a similar role in several anaerobes which employ Na^+-transporting decarboxylases to transduce the free energy of decarboxylation to a Na^+ electrochemical gradient ($\Delta\mu_{Na^+}$) across the cell membrane during fermentative growth (Dimroth, 1980; Hilpert & Dimroth, 1982; Hilpert et al., 1984; Buckel et al., 1982). Recently, it has been reported that even in aerobic bacteria, Na^+ can replace H^+ as the primary coupling cation in energy transduction: the alkalotolerant marine bacterium *Vibrio alginolyticus* (Tokuda & Unemoto, 1982, 1984), a few other members of the *Vibrio* family (Udagawa et al., 1986; Tsuchiya & Shinoda, 1985), and the halotolerant bacterium BA1 (Ken-Dror et al., 1986) employ a respiratory-driven primary Na^+ pump to generate a $\Delta\mu_{Na^+}$. Except for BA1, Na^+ pumping generally predominates at alkaline pH conditions where the $\Delta\mu_{Na^+}$ is believed to help the cells overcome a deficit in $\Delta\mu_{H^+}$ caused by a reversed pH gradient.

Vitreoscilla, a Gram-negative filametous bacterium, is a strict aerobe found mostly in hypoxic habitats such as the benthic regions of fresh water sources (Nichols et al., 1986), and cow dung (Pringsheim, 1951). The respiratory chain is similar to that of the *Escherichia coli*; it has both cytochrome *o* and cytochrome *d* terminal oxidases, and quinol is the direct electron donor to the cytochrome *o* oxidase (Georgiou & Webster, 1987a,b). Other aspects of the bacterium's respiratory physiology are rather unique; for example, high levels of *Vitreoscilla* hemoglobin are found in its cytoplasm under hypoxic conditions. It has been suggested that this hemoglobin acts as an O_2 "storage trap" under these conditions (Wakabayashi et al., 1986). Second, *Vitreoscilla* cells are lethally sensitive to moderate concentrations of extracellular K^+, but they can be protected by Na^+ (Efiok and Webster, unpublished results). Finally, *Vitreoscilla* is a mild alkalophile which grows optimally at a pH of about 8.0–8.5 but can grow up to pH 9.5 (Lambda & Webster, 1980).

These observations prompted us to measure the key components of $\Delta\mu_{H^+}$ ($\Delta\psi_{H^+}$ and ΔpH) across the membrane of *Vitreoscilla*. Surprisingly, only a minimal $\Delta\psi$ was detected unless Na^+ was added to the assay medium, irrespective of

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[1] Abbreviations: $\Delta\mu_{H^+}$, H^+ electrochemical potential gradient; $\Delta\mu_{Na^+}$, Na^+ electrochemical potential gradient; $\Delta\psi$, electrical potential gradient; TPP^+, tetraphenylphosphonium ion; $[Na^+]_o$, extracellular Na^+ concentration; $[Na^+]_i$, intracellular Na^+ concentration; pH_o, extracellular pH; pH_i, intracellular pH; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; DTHB, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde; BCECF, 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein.

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whether the external pH was neutral or alkaline. We report here the characteristics of this $\Delta\psi$ and the mechanism by which its formation is coupled to Na^+ transport in *Vitreoscilla*.

Materials and Methods

Growth and Processing of Cells. *Vitreoscilla* sp. strain C1 (culture no. 389 obtained from Dr. R. G. E. Murray) was used in all the experiments. The growth medium (1% PYA) was 1% yeast extract, 1% peptone, and sodium acetate (4 mg/L), pH 8.0. The medium was inoculated with PYA-grown log-phase starter culture and shaken at 150 rpm at 25 °C for 18–20 h, after which the cells were harvested and washed immediately as follows: 25 g of the cell pellet was homogenized and suspended in 650 mL of 50 mM Tris-HCl (pH 8.0) containing 60 mM sucrose, incubated for 5 min at 25 °C, and then centrifuged at 10700*g* for 15 min. The pellet was washed again but without any sucrose in the wash buffer. When the cells were used for $\Delta\psi$ studies, the washed pellet was homogenized in 10 mM Tris-HCl (pH 8.0) at a protein concentration of 110 ± 10 mg/mL and transferred into a plastic tube and incubated at 25 °C for about 30 min to induce anaerobiosis before being used for the experiments. Cells used for H^+ flux studies were prepared similarly to those for $\Delta\psi$ studies except that the concentration of the Tris-HCl buffer was reduced to 5 mM.

Preparation of Cation-Loaded Cells. The cell paste was diluted to 55 mg of protein/mL with buffer, and either NaCl, LiCl, or KCl solution was added to a final concentration of 100 mM salt. The suspension was incubated for 4–6 h at 0 °C or overnight (12–15 h) before being centrifuged at 10400*g* for 5 min and the paste homogenized in 10 mM Tris-HCl (pH 8.0) containing 100 mM either NaCl, LiCl, or KCl as appropriate. The paste, at a protein concentration of 110 ± 10 mg/mL, was immediately used for the experiments. The intracellular concentrations of cations in the loaded cells were measured by flame photometry, and were as follows: Na^+-loaded cells, 102 mM Na^+; Li^+-loaded cells, 2 mM Na^+ and 75 mM Li^+; K^+-loaded cells, 2 mM Na^+ and 105 mM K^+.

Measurement of $\Delta\psi$. $\Delta\psi$ was measured based on the distribution of tetraphenylphosphonium ion (TPP^+) across the cell membrane as detected by a TPP^+-sensitive electrode. The sensing element of the electrode was a poly(vinyl chloride) membrane doped with tetraphenylboron ion. The membrane was synthesized as described by Kamo et al. (1979) except that the concentrations of poly(vinyl chloride) and tetraphenylboron ion were reduced to 400 mg/10 mL of tetrahydrofuran and 8.4 mg/3.5 mL of tetrahydrofuran, respectively, and the membrane was cast in a 63 cm^2 petri dish and left to set for 24 h. These modifications gave a thinner membrane with improved sensitivity. A piece of the membrane was cut and glued onto the top of the sensing module of a blank Orion electrode (Model 92000) using a fast-acting polyacrylate glue, and dried overnight. The module was then filled with a 2 mM reference solution of TPP^+ and screwed onto the electrode body, and the membrane was conditioned in 100 μM solution of TPP^+ for 48 h before being used for $\Delta\psi$ measurement. The TPP^+ electrode and a thin combined pH/reference electrode were fitted to a rubber stopper assembly that had inlets for reagents and a gas line, and the assembly was fitted onto a 50-mL reaction vessel. The terminals of the electrodes were connected to a Fisher Accumet 753 electrode switch. The reference terminal of the pH electrode was connected to the common reference input of the switch and served as a reference for both electrodes. The electrode switch, a thermal printer, and a chart recorder were then connected to a Fisher Acumet 850 digital pH/ion meter. A four-point calibration of the electrode (range, 1–100 μM TPP^+) was routinely performed prior to $\Delta\psi$ measurements, and the electrode response was linear down to 3 μM with a slope of 58 mV/decade change in TPP^+ concentration at 25 °C.

Assay for $\Delta\psi$. In a typical assay to measure $\Delta\psi$, the total volume of the assay medium was 30 mL and contained 100 mM NaCl (or another cation), 10 mM Tris-HCl (pH 8.0), 50 μM TPP^+, and 10 mM sodium acetate (lithium acetate or Tris–acetate for other cations). The electrode assembly was fitted onto the reaction vessel and stirred at 25 °C while the pH of the assay medium was adjusted to 8.0 (or as desired), and 10 mL of O_2 was then slowly bubbled through the medium. An aliquot of the cell suspension (usually 36–45 mg of total protein) was injected from a syringe into the assay medium to start the reaction. The concentration of TPP^+ in the medium was monitored by the TPP^+ electrode and simultaneously recorded by the chart recorder and the printer, with the latter set at 10–30-s intervals. $\Delta\psi$ values corresponding to the printed TPP^+ concentrations were calculated after correcting for TPP^+ bound to intracellular components as described by Lolkema et al. (1982). The proportionality constant between [TPP^+] bound to intracellular components and free intracellular [TPP^+] (24.6) was determined with toluene-treated cells (Lolkema et al., 1982), and the intracellular H_2O space (2.6 μL/mg of cell protein) was determined as described by Rottenberg (1979).

Measurement of H^+ Extrusion. The procedure for assessing H^+ extrusion was similar to that described for measuring $\Delta\psi$ except that (1) a pH electrode was used and (2) the stock cell suspension was maintained in 5 mM Tris-HCl (pH 8.0). Cells (final concentration 1.8 mg of protein/mL) were suspended in the oxygenated assay medium buffered with 1.7 mM Tris–phosphate (pH 8.0) and contained either 110 mM total Li^+, or 110 mM total Na^+, or 60 mM tatal Li^+ plus 50 mM K^+. Changes in the pH of the medium were monitored. Part of the total ion concentration in each case was derived from 10 mM sodium or lithium acetate as appropriate.

Determination of Intracellular Na^+. Samples removed from the assay medium were immediately centrifuged at 15000 rpm for 30 s in an Eppendorf desktop microfuge. The supernatant was decanted, and the tube was rinsed with 1.5 mL of wash buffer (400 mM sucrose in 10 mM Tris-HCl, pH 8.0). The cells were washed by dispersing the pellet in 1.5 mL of the wash buffer and centrifuged as before. The washed pellet was extracted with 10% TCA for 30 min and the extract assayed for Na^+ by flame photometry.

Measurement of $^{22}Na^+$ Extrusion. Cells (92 mg of protein/mL) were loaded with labeled Na^+ by incubation at 0 °C for 4.5 h in the presence of 50 mM NaCl plus carrier-free $^{22}Na^+$ (13.5 μCi/mL). Other additions were as indicated in the legend to Figure 6. Loading was monitored by rapidly filtering a 10-μL aliquot every 30 min and counting the radioactivity retained in the filter. $^{22}Na^+$ retained was constant after 4 h, indicating full equilibration. The assay medium, which contained 15 mM Tris-HCl (pH 8.0) and 250 mM NaCl, was saturated with O_2, and $^{22}Na^+$-loaded cells (final concentration 4 mg of protein/mL) were added to start the reaction; 0.1-mL aliquots were removed at appropriate times, filtered, and washed within 10 s using Millipore 0.45-μm (HV) filters. The assay medium was continually bubbled with O_2 until sampling was completed. Radioactivity retained by the filtered cells was counted by liquid scintillation. The assays were performed either at 25 °C or at 12 °C using a constant-temperature circulator to maintain the temperature of the assay medium and wash buffer.

Determination of ATP. Samples were centrifuged as described above for Na^+ determination and immediately extracted with 2.5% TCA for 20 min. ATP in the extract was assayed by the firefly method as reported by DeLuca and McElroy (1978).

Determination of pH_i. pH_i was determined on the basis of fluorescent signals from the pH-sensitive dye BCECF trapped in cells following permeation and subsequent intracellular hydrolysis of the membrane-permeable acetoxymethyl-BCECF (Tsien & Poenie, 1986; Rin et al., 1982; Thomas et al., 1982). Cells were loaded by mixing 73 μL of a 1 mM DMSO solution of acetoxymethyl-BCECF with 25 mL of cell suspension (5 mg of protein/mL in 10 mM Tris-HCl, pH 8.0) and incubating the mixture for 1 h with frequent mixing. The cells were collected by centrifugation at 10000*g* for 10 min and washed twice by homogenization in 30 mL of Tris-HCl followed by centrifugation as before. Finally, the pellet was reconstituted with 10 mM Tris–phoshate (pH 8.0) at 20 mg of cell protein/mL. To calibrate the excitation intensity of the trapped dye against pH_i, the dye-loaded cells (6 mg of total protein) were mixed with 1 mL of 10 mM Tris–phosphate (pH 6.5–8.5), and CCCP (10 μM) was added to equilibrate pH_i with pH_o. The excitation fluorescent spectrum of the trapped dye was recorded from 400 to 510 nm (slit 2 nm), using a Perkin-Elmer 650-10S fluorescence spectrophotometer with the emission waveband set at 524 nm (slit 10 nm). The final pH of the assay mixture was measured with a pH electrode at the end of each scan. The ratio of the pH-dependent excitation peak at 497 nm to the isosbestic point at 434 nm (Tsien & Poenie, 1986) was plotted against the final pH and gave a standard curve that was linear from pH 6.9 to 8.1. For the experimental determination of pH_i, the dye-loaded cells were then assayed similarly but without the CCCP, and the 497/434-nm excitation intensity ratio was used to estimate the pH_i from the standard curve. The assay medium contained cells (6 mg of protein), 10 mM Tris–phosphate (pH 8.1), and 100 mM NaCl or LiCl. As a control, cells were assayed in 10 mM Tris–phosphate only. The assay medium was bubbled with a gentle stream of oxygen during the 2-min incubation.

Assay for Protein. Protein concentration was estimated as previously described (Lowry et al., 1951).

Sources of Chemicals. All chemicals were purchased from commercial sources: tetraphenylphosphonium chloride, LiCl (99.99+%), dioctyl phthalate, and DTHB, Aldrich Chemicals, Milwaukee, WI; sodium tetraphenylborate, tetrahydrofuran, lithium acetate, choline chloride (99%), CCCP, DCCD, monensin, and Trizma base, Sigma Chemical Co., St. Louis, MO; NaCl, KCl, sodium acetate, sodium phosphate, potassium phosphate, and phosphoric acid, Fisher Scientific Co., Fair Lawn, NJ; BCECF, Molecular Probes, Eugene, OR; yeast extract, FIDCO, White Plains, NY; carrier-free $^{22}NaCl$, Amersham Corp., Arlington Height, IL; peptone (HY-CASE SF), Kraft Inc., Norwich, NY.

Results and Discussion

Dependence of $\Delta\psi$ on O_2. The respiratory dependence of $\Delta\psi$ was determined as described under Materials and Methods except that the assay medium was initially bubbled with a limited amount of O_2 (5 mL) to force an early depletion of O_2 to residual level. The $\Delta\psi$ formed rapidly and reached −137 mV in about 3.5 min (Figure 1). Subsequently, it partially collapsed to −88 mV as O_2 was depleting. After being pulsed with 3 mL of O_2, the negative $\Delta\psi$ recovered and reached a steady-state value of −145 mV. These results show that the formation of $\Delta\psi$ was a respiratory-driven process, and not a Donnan-type equilibrium membrane potential. The O_2 could deplete only to a residual level because the reaction chamber was not completely air-tight; consequently, $\Delta\psi$ did not collapse to zero.

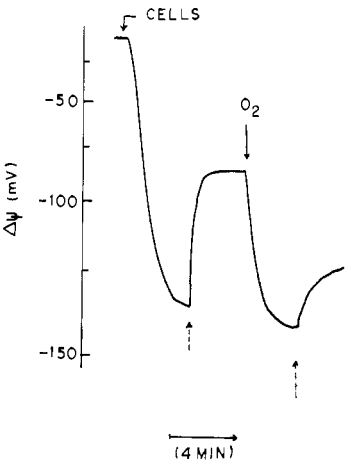

FIGURE 1: Dependence of $\Delta\psi$ on O_2: The assay medium, which contained 100 mM NaCl, 10 mM sodium acetate, 10 mM Tris-HCl (pH 8.0), and 50 μM TPP^+, was initially bubbled with a limited amount of oxygen (5 mL). Cells (final concentration 1.4 mg of protein/mL) were then injected, and $\Delta\psi$ was determined as described under Materials and Methods. Broken arrows indicate points of oxygen depletion. Solid arrow is the point at which 3 mL of oxygen was injected.

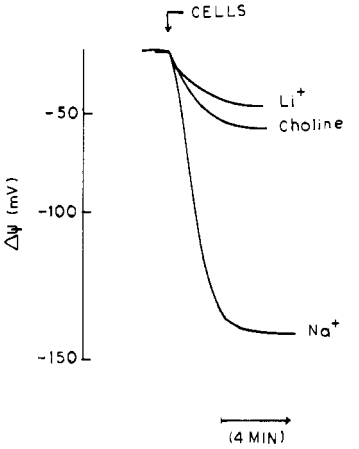

FIGURE 2: Dependence of $\Delta\psi$ on Na^+: Cells (final concentration 1.4 mg of protein/mL) were injected into the oxygenated assay medium containing a total of 110 mM NaCl, LiCl, or choline chloride in addition to 10 mM Tris–acetate (pH 8.0) and 50 μM TPP^+. $\Delta\psi$ was determined as described under Materials and Methods.

Dependence of $\Delta\psi$ on Na^+. Na^+ was specifically required for the formation of a normal $\Delta\psi$ across the cell membrane of *Vitreoscilla*. Neither Li^+ nor choline could substitute. A $\Delta\psi$ of −142 mV developed across the cell membrane in the presence of 110 mM Na^+, compared to only −42 and −56 mV, respectively, in the presence of the same concentration of Li^+ and choline (Figure 2). As shown in Figure 5B, even these small $\Delta\psi$'s observed with Li^+ and choline were insensitive to the protonophore DTHB and most likely due to trace Na^+ in the cytosol and reagents. (Trace Na^+ was 1.1 mM in the washed cells and 0.8 mM in the assay medium.) The minimal $\Delta\psi$ observed in the presence of Li^+ or choline was not due to a decreased respiratory rate, nor to the rapid influx of these cations into the cells to collapse any developing $\Delta\psi$: the rate of respiration was 78% and 18% higher in the presence of Li^+ and choline, respectively, than in the presence of Na^+, and the cells generated a high $\Delta\psi$ in the presence of high concentrations of either of these cations as long as Na^+ was present. The stimulation of respiration by Li^+ may be due to its being

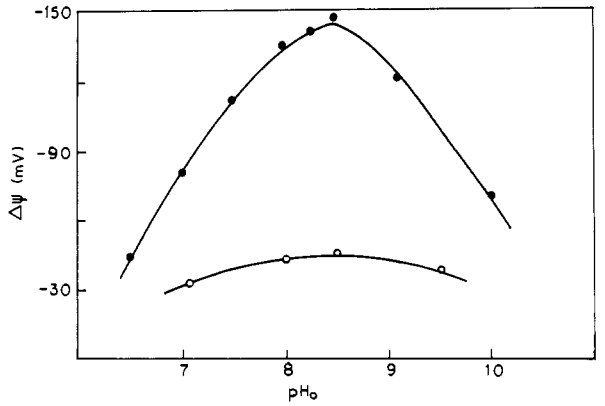

FIGURE 3: pH_o versus $\Delta\psi$ profile: $\Delta\psi$ was determined as a function of pH_o in an assay medium which contained cells (1.4 mg of protein/mL), 10 mM sodium acetate, 50 μM TPP^+, 20 mM buffer, and 100 mM total Na^+. The buffers used were the following: sodium phosphate, pH 6.5–7.5; Tris-HCl, pH 7.5–8.5; and glycine, pH 8.5–10.0. In the absence of Na^+, Tris-phosphate was used from pH 7.0 to 8.5 and glycine from pH 8.5 to 9.5. (●) $\Delta\psi$ as a function of pH_o in the presence of 100 mM Na^+. (○) $\Delta\psi$ as a function of pH_o in the absence of Na^+; NaCl and sodium acetate were replaced with LiCl and lithium acetate, respectively.

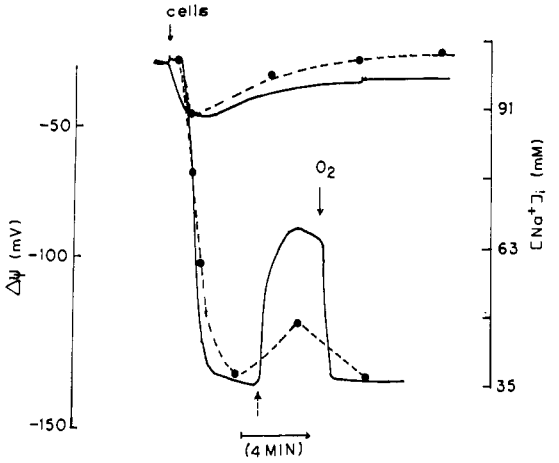

FIGURE 4: Kinetics of Na^+ extrusion versus $\Delta\psi$ formation: Cells loaded with 100 mM NaCl were injected into the oxygenated assay medium containing 100 mM NaCl and 10 mM sodium acetate. The medium also contained 10 mM Tris-HCl (pH 8.0) and 50 μM TPP^+, and the cell concentration was 1.7 mg of protein/mL. A 1-mL aliquot of the suspension was removed at appropriate times and processed for $[Na^+]_i$ as described under Materials and Methods. (--) $[Na^+]_i$ versus time. (—) $\Delta\psi$ versus time. The upper two curves were obtained in the presence of 5 mM NaCN; in which case, the pH of the assay medium was readjusted to 8.0 before addition of the cells. The broken arrow indicates the point of oxygen depletion, and 4 mL of oxygen was injected after $\Delta\psi$ had collapsed to a new steady-state level (solid arrow).

slightly permeable to the cells which respond by increasing the respiratory rate to maintain the high $\Delta\psi$.

$\Delta\psi$ versus pH_o and pH_i. pH_i was determined as described under Materials and Methods. Cells respiring at a pH_o of 8.0 in the absence of added Na^+ or Li^+ maintained their pH_i at 7.4, and in the presence of 100 mM Na^+ or Li^+, the pH_i was 7.3 and 8.0, respectively. Thus, formation of a large $\Delta\psi$ in the presence of Na^+ (Figure 2) was not accompanied by cytoplasmic alkalinization which would occur if redox H^+ pumps in the respiratory chain required Na^+ for optimal H^+ pumping activity. Conversely, the cytoplasmic alkalinization in the presence of Li^+ was not accompanied by the formation of a H^+-coupled $\Delta\psi$ since the −42 mV that formed was insensitive to DTHB (Figure 5B). The Na^+-dependent $\Delta\psi$ was observed over a wide range of pH_o values (6.5–8.5) with an optimum at pH 8.5 (Figure 3) and was not limited to alkaline conditions which would be expected if the alkalotolerant nature of this bacterium necessitated the use of a Na^+/H^+ antiport system to sustain a H^+-coupled $\Delta\psi$ when the pH_o exceeded the pH_i (Krulwich, 1983).

Kinetics of Na^+ Extrusion vs $\Delta\psi$ Formation. If $\Delta\psi$ in *Vitreoscilla* is Na^+-coupled, then as a first requirement the cells must be able to extrude Na^+ against its electrochemical gradient during respiration, and the kinetics of Na^+ extrusion should correlate with those of $\Delta\psi$ formation. Cells were preloaded with 100 mM NaCl and assayed concurrently for $\Delta\psi$ and $[Na^+]_i$ in an aerobic assay medium containing 100 mM total Na^+. Thus, any Na^+ extrusion would be performed against its electrochemical gradient (see Figure 6 for extrusion against a 5-fold gradient). The results are shown in Figure 4 (lower two curves) where a graph of $[Na^+]_i$ versus time has been imposed on that of $\Delta\psi$ versus time. The $[Na^+]_i$ fell rapidly in the first 2 min, paralleling the rapid formation of $\Delta\psi$ during this time. Both $[Na^+]_i$ and $\Delta\psi$ reached steady-state levels (38 mM and −143 mV, respectively) at about the same time, and remained constant until O_2 depleted to the residual level, at which time $[Na^+]_i$ rose to 46 mM as extracellular Na^+ returned into the cells, and the negative $\Delta\psi$ decreased rapidly to −88 mV. Upon pulsing the medium with O_2, $[Na^+]_i$ decreased to the previous steady-state value of 38 mM in tandem with the return of $\Delta\psi$ to the previous steady-state value. These data support the coupling of $\Delta\psi$ to Na^+ because the cells extruded Na^+ with kinetics that correlated with those of $\Delta\psi$ formation, and, further, the collapse of $\Delta\psi$ in response to decreasing O_2 also correlated with the influx of Na^+ into the cells. The upper two curves in Figure 4 show that when the same experiment was performed in the presence of 5 mM CN^-, both Na^+ extrusion and $\Delta\psi$ formation were strongly inhibited, as was respiration (Table I). A strong coupling between these parameters is suggested by the fact that all three were inhibited 81% or more.

Table I: Pattern of Inhibition of Respiratory Energy Parameters by Cyanide[a]

parameter	value in absence of NaCN	value in presence of NaCN	inhibition (%)
$\Delta\psi$	−143 mV	−27 mV	81
$\Delta[Na^+]_i$[b]	62 mM	1 mM	98
O_2 consumed	0.690 μmol	0.037 μmol	95

[a] Data from Figure 4 were used to calculate the percent inhibition of $\Delta\psi$ and $\Delta[Na^+]_i$. Oxygen uptake was measured polarographically at pH 8.0 in the presence and absence of 5 mM CN^-, using a cell concentration of 1.0 mg of protein/mL. Rates were calculated based on a 5-min interval. [b] $\Delta[Na^+]_i$ = initial $[Na^+]_i$ − final $[Na^+]_i$.

Three coupling mechanisms between respiration and Na^+ extrusion are known to occur in other organisms. In the first two, the free energy of electron transport is used to generate a $\Delta\mu_{H^+}$ intermediate. In one case, this is transduced to ATP, and a Na^+-transporting ATPase couples the free energy of hydrolysis of the ATP to the translocation of intracellular Na^+ to the exterior (Heefner & Harold 1982; Shirvan et al., 1989). In the second case, a Na^+/H^+ antiport system couples the $\Delta\mu_{H^+}$ to the translocation of intracellular Na^+ to the exterior in exchange for external H^+ (Krulwich, 1983). Third, a primary Na^+ pump directly couples the free energy change of electron transport to the translocation of intracellular Na^+ (Tokuda & Unemoto, 1982). The F_0F_1-ATPase inhibitors DCCD and arsenate, the Na^+-specific ionophore monensin, and the protonophores DTHB and CCCP were used to determine which of these systems is operating in *Vitreoscilla*.

Effects of DCCD and Arsenate on $\Delta\psi$. $\Delta\psi$ and ATP were determined concurrently in the presence of increasing con-

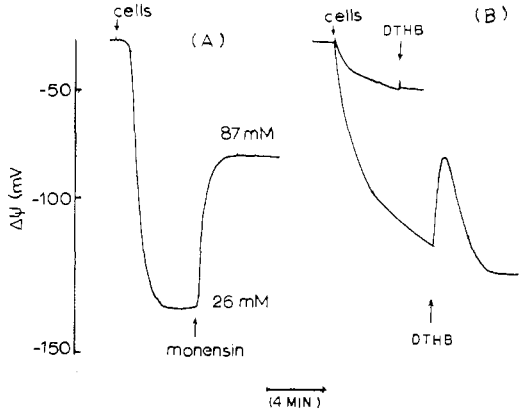

FIGURE 5: Effects of monensin and DTHB on $\Delta\psi$: $\Delta\psi$ was determined as described under Materials and Methods using cells at a concentration of 0.9 mg of protein/mL and a total Na^+ concentration of 100 mM. When $\Delta\psi$ reached steady state, either 10 μM monensin (A) or 75 μM DTHB (B) was added. Duplicate 1-mL aliquots were removed and processed for $[Na^+]_i$ before and after addition of the monensin (A). The numbers with mM unit refer to the $[Na^+]_i$. In the upper curve (B), 110 mM LiCl replaced the NaCl.

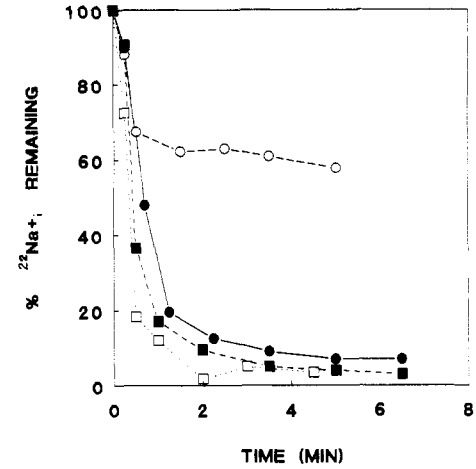

FIGURE 6: Effect of DTHB on $^{22}Na^+$ extrusion. $^{22}Na^+$ extrusion was assayed as described under Materials and Methods, in the presence of 75 μM DTHB (■---■), 75 μM DTHB + 100 mM KCL (□···□), 5 mM NaCN (○---○), or no additions (control) (●—●). The indicated reagents were added to the assay medium prior to addition of cells. Data used here obtained at 12 °C and were similar to those obtained at 25 °C.

centrations of DCCD using cells pretreated with DCCD 1 min prior to starting the assay. DCCD at 1.5 mM decreased cellular ATP by 65% but had no effect on $\Delta\psi$ even at 10 mM. Arsenate at 10 mM decreased ATP 86% but still had no effect on $\Delta\psi$. These results make it unlikely that Na^+ extrusion and $\Delta\psi$ formation in *Vitreoscilla* are coupled to respiration by a Na^+-transporting ATPase.

Effect of Monensin on $\Delta\psi$. Monensin is a Na^+-specific ionophore that normally catalyzes Na^+/H^+ exchange across a membrane (Pressman, 1968; Sandeaux et al., 1982). It has been predicted that monensin should not collapse a Na^+-coupled $\Delta\psi$ generated by a Na^+/H^+ antiporter but should collapse a Na^+-coupled $\Delta\psi$ generated by a primary Na^+ pump (Skulachev, 1987). Monensin did collapse $\Delta\psi$ in *Vitreoscilla* (Figure 5A), from −142 to −85 mV with an accompanying rise in $[Na^+]_i$ from 26 to 87 mM. The magnitude of collapse increased with increasing concentration of monensin (from 2 to 10 μM). In a related experiment, 10 μM monesin inhibited Na^+-coupled ATP synthesis 60% in respiring *Vitreoscilla* cells (Volny and Webster, unpublished results).

Effects of CCCP and DTHB on $\Delta\psi$ and Na^+ Extrusion. CCCP was not suitable for our studies; it gave anomalous results, and we subsequently found that in stoichiometric amounts it markedly perturbed the spectrum of purified cytochrome *o* (Efiok and Webster, unpublished results). Another protonophore, DTHB, was, therefore, chosen. DTHB is in the CCCP family with a pK_a of 7.9 (Miyoshi et al., 1987) and did not detectably alter the spectrum of purified cytochrome *o*. If Na^+ is extruded by a Na^+/H^+ antiport system in *Vitreoscilla*, DTHB should collapse $\Delta\psi$ by collapsing the driving force for Na^+ extrusion, $\Delta\mu_{H^+}$. However, up to 20 μM DTHB had virtually no effect on $\Delta\psi$. At higher concentrations, it collapsed $\Delta\psi$ transiently, but $\Delta\psi$ recovered completely within 3–4 min (Figure 5B). The upper curve in this figure shows that 75 μM DTHB was ineffective in collapsing the residual $\Delta\psi$ generated in the absence of added Na^+, an indication that this $\Delta\psi$ is due to the extrusion of trace cytoplamic Na^+ rather than H^+. These results are all inconsistent with a Na^+/H^+ antiport mechanism for the generation of the Na^+-dependent $\Delta\psi$ but are consistent with a respiratory-driven primary Na^+ pump; the transient collapse of $\Delta\psi$ by DTHB stimulated respiration and Na^+ extrusion, leading to the subsequent recovery of $\Delta\psi$ as observed. Stimulation of Na^+ extrusion by DTHB was demonstrated by using cells loaded with 50 mM labeled Na^+ (Figure 6). Although not shown, 100 mM K^+ also stimulated $^{22}Na^+$ extrusion but at a slightly lower rate (K^+ even without valinomycin collapses $\Delta\psi$ rapidly). When 75 μM DTHB and 100 mM K^+ were both present, the rate of $^{22}Na^+$ efflux was further increased. The assay was performed at 12 °C to slow down Na^+ extrusion and enable more sampling during the first minute. Results obtained at 25 °C were similar to those at 12 °C except that the rates of extrusion were faster and 100 mM K^+ was as effective as DTHB in stimulating Na^+ efflux.

Kinetics of H^+ Fluxes. In bacteria which transduce energy via $\Delta\mu_{H^+}$, the onset of respiration elicits a primary H^+ extrusion which is normally rapid and correlates with the formation of $\Delta\psi$ (Mitchell, 1976; Kell & Hitchens, 1982). Our experiments indicated a lack of correlation between H^+ fluxes and $\Delta\psi$. In the absence of Na^+ (100 mM Li^+ added), only a gradual H^+ extrusion was observed, and this was preceded by a lag phase of about 45 s. In the presence of 50 mM K^+ to induce maximum H^+ extrusion, the lag phase persisted, and the rate of the slow H^+ extrusion increased only about 8%. In the presence of 100 mM Na^+, the H^+ extrusion was even slower and preceded by H^+ uptake during the first 45 s even though this is the period when the rate of $\Delta\psi$ formation is most rapid (Figures 1 and 2). Normally, this would be interpreted as evidence for a Na^+/H^+ antiport activity, but a Na^+/H^+ antiport system has already been discounted (Figures 5 and 6). This H^+ uptake is most likely a counter-cationic influx of H^+ driven by $\Delta\psi_{Na^+}$ that is normally observed for bacteria employing primary Na^+ pumps (Tokuda & Unemoto, 1982; Shirvan et al., 1989). The observed slow H^+ efflux may be due to the scalar protolytic reaction between cytochrome *o* and Q_8H_2 and/or the downhill efflux of H^+ since pH_i was more acidic than pH_o.

We conclude that the formation of $\Delta\psi$ is directly coupled to respiratory-driven Na^+ extrusion on the basis of the following: the excellent correlation between the kinetics of respiratory-driven Na^+ extrusion and $\Delta\psi$ formation (Figure 4); the collapse of $\Delta\psi$ by monensin (Figure 5A); the transient collapse of $\Delta\psi$ by the protonophore DTHB and the subsequent stimulation of Na^+ extrusion leading to a complete recovery of $\Delta\psi$ (Figures 5B and 6); and the lack of correlation between $\Delta\psi$ and H^+ fluxes. To explain these observations by the "classical" chemiosmotic hypothesis (Mitchell, 1967, 1976)

would require an electrogenic Na^+/H^+ antiport mechanism in *Vitreoscilla*. This is not supported by the following evidence: (1) the most common characteristic of Na^+/H^+ antiporters is the ability of Li^+ to substitute for Na^+ (Krulwich, 1983). In none of the experiments was Li^+ able to substitute for Na^+. (2) Monensin would not have collapsed $\Delta\psi$ if Na^+ were extruded by a Na^+/H^+ antiport system (Skulachev, 1987). (3) If $\Delta\psi$ were coupled by a Na^+/H^+ antiport system, then the elimination of the driving force, $\Delta\mu_{H^+}$, by DTHB would have inhibited Na^+ extrusion and collapsed $\Delta\psi$ permanently; in fact, $\Delta\psi$ collapsed only transiently, and Na^+ extrusion was stimulated. (4) In other experiments not reported above, up to 10 mM amiloride, a known inhibitor of both eukaryotic and *Escherichia coli* Na^+/H^+ antiport systems (Benos, 1982; Mochizuku & Oosawa, 1985; McMorrow et al., 1989), did not affect $\Delta\psi$. The insensitivity of $\Delta\psi$ to DTHB (including the −42 mV generated in the absence of added Na^+) may indicate that *Vitreoscilla*'s respiratory chain is fully Na^+-motive, in contrast to that of *Vibrio alginolyticus* which has a Na^+-motive as well as a H^+-motive segment (Tokuda & Unemoto, 1982). Further, we have found that the activities of cytochrome *o* and NADH dehydrogenase, the major redox centers in *Vitreoscilla*'s respiratory chain, are significantly enhanced by Na^+ (Efiok and Webster, unpublished results). However, our present data do not exclude the possibility that some H^+ pumping occurs.

The forces behind the evolution of a Na^+-coupled energy transducing system in *Vitreoscilla* are not well understood at this time, especially since the bacterium is nonmarine. Its weakly alkalotolerant growth habit may be a contributing factor since in an alkaline environment, the extrusion of protons to generate a $\Delta\mu_{H^+}$ might result in a lethal alkalinization of the cytoplasm. A switch to another cation would therefore be a major advantage. Since Na^+ is ubiquitous in the environment, its use as a primary transmembrane energy coupling cation in bacteria may be more prevalent than is currently realized.

Registry No. Na, 7440-23-5.

References

Benos, D. J. (1982) *Am. J. Physiol. 242*, C131–C145.

Buckel, W., & Semler, R. (1982) *FEBS Lett. 148*, 35–38.

DeLuca, M., & McElory, W. D. (1978) *Methods Enzymol. 57*, 3–14.

Dimroth, P. (1980) *FEBS Lett. 122*, 234–236.

Georgiou, C. D., & Webster, D. A. (1987a) *Biochemistry 26*, 6521–6526.

Georgiou, C. D., & Webster, D. A. (1987b) *Arch. Microbiol. 148*, 328–333.

Heefner, D. L., & Harold, F. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2798–2802.

Hilpert, W., & Dimroth, P. (1982) *Nature (London) 296*, 584–585.

Hilpert, W., Schink, B., & Dimroth, P. (1984) *EMBO J. 3*, 1665–1670.

Kamo, N., Muratsugu, R. H., & Kobatake, Y. (1979) *J. Membr. Biol. 49*, 105–121.

Kell, D. B., & Hitchens, G. D. (1982) *Faraday Discuss. Chem. Soc. No. 74*, 377–388.

Ken-Dror, S., Preger, R., & Avi-Dor, Y. (1986) *Arch. Biochem. Biophys. 244*, 122–127.

Krulwich, T. A. (1983) *Biochim. Biophys. Acta 726*, 245–264.

Lamba, P., & Webster, D. A. (1980) *J. Bacteriol. 142*, 169–173.

Lolkema, J. S., Hellingwerf, K. J., & Konnings, W. N. (1982) *Biochim. Biophys. Acta 681*, 85–94.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

McMorrow, I., Shuman, H. A., Sze, D., Wilson, D. M., & Wilson, T. H. (1989) *Biochim. Biophys. Acta 981*, 21–26.

Mitchell, P. (1967) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 26*, 1370–1379.

Mitchell, P. (1976) *Biochem. Soc. Trans. 4*, 399–428.

Miyoshi, H., Nishioka, T., & Fujita, T. (1987) *Biochim. Biophys. Acta 891*, 293–299.

Mochizuku-Oda, N., & Oosawa, F. (1985) *J. Bacteriol. 163*, 395–397.

Nichols, P., Stulp, B. K., Jones, J. G., & White, D. C. (1986) *Arch. Microbiol. 146*, 1–6.

Pressman, B. C. (1968) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 27*, 1283–1288.

Pringsheim, E. G. (1951) *J. Gen. Microbiol. 5*, 124–149.

Rink, T. J., Tsien, R. Y., & Pozzan, T. (1982) *J. Cell Biol. 95*, 189–196.

Rottenberg, H. (1979) *Methods Enzymol. 55*, 547–569.

Sandeaux, R., Sandeau, J., Gavach, C., & Brun, B. (1982) *Biochim. Biophys. Acta 684*, 127–132.

Shirvan, M. H., Schuldiner, S., & Rottem, S. (1989) *J. Bacteriol. 171*, 4417–4424.

Skulachev, V. P. (1987) in *Ion Transport in Prokaryotes* (Rosen, B. P., & Silver, S., Eds.) pp 131–164, Academic Press, San Diego.

Thomas, J. A., Kolbeck, P. C., & Langworthy, T. A. (1982) in *Intracellular pH: Its measurement, Regulation, and Utilization in Cellular Functions* (Nuccitelli, R., & Deamer, D. W., Eds.) pp 105–123, A. R. Liss, New York.

Tokuda, H., & Unemoto, T. (1982) *J. Biol. Chem. 257*, 10007–10014.

Tokuda, H., & Unemoto, T. (1984) *J. Biol. Chem. 259*, 7785–7790.

Tsien, R. Y., & Poenie, M. (1986) *Trends Biochem. Sci. (Pers. Ed.) 11*, 450–455.

Tsuchiya, T., & Shinoda, S. (1985) *J. Bacteriol. 162*, 794–798.

Udagawa, T., Unemoto, T., & Tokuda, H. (1986) *J. Biol. Chem. 261*, 2616–2622.

Wakabayashi, S., Matsubara, H., & Webster, D. A. (1986) *Nature 322*, 481–483.