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Relationships between the Na^+–H^+ Antiport Activity and the Components of the Electrochemical Proton Gradient in *Escherichia coli* Membrane Vesicles†

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ABSTRACT: The kinetics of Na^+ efflux from *Escherichia coli* RA 11 membrane vesicles taking place along a favorable Na^+ concentration gradient are strongly dependent on the generation of an electrochemical proton gradient. An energy-dependent acceleration of the Na^+ efflux rate is observed at all external pHs between 5.5 and 7.5 and is prevented by uncoupling agents. The contributions of the electrical potential ($\Delta\psi$) and chemical potential (ΔpH) of H^+ to the mechanism of Na^+ efflux acceleration have been studied by determining the effects of (a) selective dissipation of $\Delta\psi$ and ΔpH in respiring membrane vesicles with valinomycin or nigericin and (b) imposition of outwardly directed K^+ diffusion gradients (imposed $\Delta\psi$, interior negative) or acetate diffusion gradients (imposed ΔpH, interior alkaline). The data indicate that, at pH 6.6 and 7.5, ΔpH and $\Delta\psi$ individually and concurrently accelerate the downhill Na^+ efflux rate. At pH 5.5, the Na^+ efflux rate is enhanced by ΔpH only when the imposed ΔpH exceeds a threshold ΔpH value; moreover, an imposed $\Delta\psi$ which per se does not enhance the Na^+ efflux rate does contribute to the acceleration of Na^+ efflux when imposed simultaneously with a ΔpH higher than the threshold ΔpH value. The results strongly suggest that the Na^+–H^+ antiport mechanism catalyzes the downhill Na^+ efflux. Furthermore, they suggest that (a) the overall exchange reaction is rate limited by the rate of coupled H^+ influx, (b) the Na^+–H^+ antiporter might function as an electrogenic process at all pHs between 5.5 and 7.5, and finally (c) the antiport function is controlled, in particular at acidic pHs, by a ΔpH-sensitive reaction or alternatively by the internal pH.

In Mitchell's chemiosmotic hypothesis, the ionic gradients across the cytoplasmic membrane are proposed to serve the purpose both of the conservation of energy and of its transmission from energy-producing to energy-consuming membrane reactions (Mitchell, 1963, 1968, 1970). It is thus well recognized that in intact bacteria or derived membrane vesicles, the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$)[1] (Mitchell, 1963)

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons; ΔpH, proton gradient across the membrane; $\Delta\psi$, electrical potential across the membrane; $\Delta\bar{\mu}_{Na^+}$, electrochemical gradient for Na^+ ions; PMS, phenazine methosulfate; FCCP, carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone; Me_2SO, dimethyl sulfoxide.

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generated as a result of H^+ extrusion during respiration or ATP hydrolysis is the immediate driving force for those transport systems (H^+–substrate symport) which catalyzes the coupled movement of protons with substrates (Harold & Altendorf, 1974; West, 1980; Kaback, 1976, 1982). On the other hand, an increasing number of observations have shown that other bacterial transport systems catalyze a Na^+–substrate symport [see review in Lanyi (1979)]; in this case, $\Delta\bar{\mu}_{Na^+}$ rather than $\Delta\bar{\mu}_{H^+}$ is acting as the driving force for these Na^+-coupled transport reactions. Since, unlike eukaryotes, most bacterial cells do not possess a primary Na^+ pump, it was proposed (Mitchell, 1973) that $\Delta\bar{\mu}_{H^+}$ functions to maintain a transmembrane Na^+ gradient ($Na^+_{in} < Na^+_{out}$) through the activity of a Na^+–H^+ antiporter.

Evidence supporting the presence of a Na^+–H^+ antiport mechanism has been presented in *Escherichia coli* cells (West & Mitchell, 1974; Zilberstein et al., 1979) or isolated membrane vesicles derived from these cells (Tokuda & Kaback, 1977; Schuldiner & Fishkes, 1978; Reenstra et al., 1980; Beck & Rosen, 1979), and there is general agreement that the driving force for antiport activity is the electrochemical gradient for protons. Furthermore, evidence has been provided indicating that both $\Delta\bar{\mu}_{H^+}$ components can individually enhance the antiport reaction (Schuldiner & Fishkes, 1978; Reenstra et al., 1980). Moreover, Schuldiner and Fishkes showed that the relative contribution of $\Delta\psi$ and ΔpH to the driving force for Na^+ efflux might vary as a function of the external pH. These authors came to the conclusion that the antiporter is solely driven by ΔpH at acidic external pHs (6.6 or lower) and behaves as an $\Delta\psi$-driven process at higher pHs (7.5). They have interpreted their results in terms of pH-dependent variation of the electrogenicity of the antiporter. On the other hand, and except for Beck and Rosen's finding that the rate of antiport reaction is influenced by $\Delta\psi$ (Beck & Rosen, 1979), no information is presently available on the effect of $\Delta\mu_{H^+}$ or of its components on the kinetic properties of the antiport mechanism. In the present study, it is shown that more insight into these kinetic properties can be obtained by analyzing the modifications of the kinetics of downhill Na^+ efflux from Na^+-loaded membrane vesicles by $\Delta\bar{\mu}_{H^+}$ or its components. The use of membrane vesicles prepared according to Kaback's procedure (Kaback, 1971) is well suited for such studies for the following reasons: (a) In vesicles oxidizing appropriate electron donors, $\Delta\psi$ and ΔpH can be manipulated by the ionophores valinomycin and nigericin, respectively, in such a manner that the energy stored across the membrane is only in a chemical or an electrical form (Ramos et al., 1976, 1977a); this approach has been shown to be very fruitful for the study of the energetics of different $\Delta\bar{\mu}_{H^+}$-driven transport systems (Ramos et al., 1977b; Lagarde, 1977; Robertson et al., 1980). (b) $\Delta\psi$ and ΔpH can be artificially generated across the deenergized membrane vesicles by imposing either outwardly directed potassium diffusion gradients ($K^+_{in} \rightarrow K^+_{out}$) in the presence of valinomycin ($\Delta\psi$, interior negative) or outwardly directed acetate diffusion gradients ($acetate_{in} \rightarrow acetate_{out}$) which lead to a transient ΔpH (interior alkaline). Valuable information concerning the relationship between $\Delta\bar{\mu}_{H^+}$ components and kinetic transport properties of various H^+ symporters in bacterial membrane vesicles has already been obtained by using this experimental tool (Hirata et al., 1974; Lancaster & Hinkle, 1977; Kaczorowski et al., 1979; Leblanc et al., 1980).

The results presented in this paper strongly suggest that the Na^+–H^+ antiporter catalyzes the downhill transport of Na^+ in membrane vesicles. They also suggest that the overall exchange reaction is rate limited by the influx of H^+ ions and functions according to an electrogenic process in the range of external pHs between 5.5 and 7.5. Finally, some observations suggest a control of the Na^+–H^+ antiport function either by a ΔpH-dependent gating phenomenon or, alternatively, by the intravesicular pH.

Materials and Methods

Materials

^{22}NaCl (carrier free) was obtained from the Commissariat à l'Energie Atomique (Saclay, France). Stock solutions of valinomycin (Sigma), nigericin (Eli Lilly), and FCCP (Boehringer) were prepared in Me_2SO. Choline salts were prepared by titrating choline bicarbonate with reagent-grade acetic, gluconic, glucuronic, and orthophosphoric acids.

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* RA 11 was generously provided by Dr. H. R. Kaback (Roche Institute, Nutley, NJ). This strain is a mutant of *E. coli* K 12 that has a temperature-stable, inducible melibiose transport system, is α-galactosidase negative, and contains a deletion for the *lac* y gene (Lopilato et al., 1978). Cells were grown at 37 °C in medium 63 (Cohen & Rickenberg, 1956) supplemented with 1% pancreatic digest of casein (bactotryptone, Difco), 0.5 mg/mL thiamin, and 10 mM melibiose. Membrane vesicles were prepared from cells harvested during mid-exponential growth according to Kaback's procedure (Kaback, 1971). Lysozyme and sucrose were used at final concentrations of 25 μg/mL and 30%, respectively, for preparation of the spheroplasts (Ramos & Kaback, 1977). Membrane vesicles were finally resuspended in 0.1 M potassium phosphate, pH 6.6, and stored in liquid nitrogen.

Na^+ Efflux Measurements. Na^+ efflux measurements were performed by using concentrated vesicle suspensions previously equilibrated with buffered solutions of given composition and pH. To this end, membrane vesicles were diluted 30-fold with the media of desired composition (but systematically supplemented with 10 mM sodium phosphate) and pH and then equilibrated at room temperature for 30 min before centrifugation (45000*g* for 20 min). The equilibration step was repeated twice, and the final pellet was resuspended in a minimal volume of the same solution to give a suspension as concentrated as possible (usually 30–35 mg of protein per mL). Then, a small aliquot of ^{22}NaCl was added to the concentrated vesicle preparation (final activity 1500 cpm/nmol), and the suspension was incubated at 4 °C for 3–5 h in order to allow complete equilibration of Na^+ and its isotope with the intravesicular space.

Na^+ efflux from these $^{22}Na^+$-loaded vesicles was measured as follows: 2.5-μL aliquots (80 μg of protein) of concentrated vesicle suspension were rapidly diluted into a small volume (from 400 to 2000 μL) of solution buffered at the same pH, quickly agitated, and placed at 21 °C under a stream of oxygen when needed. At given times, the reaction was terminated by adding 2 mL of equilibration solution and filtering the sample. The filter (Amicon, 0.45 μm) was washed once with the same salt solution and immediately removed from the filtration apparatus. Radioactivity was determined by scintillation spectrometry following solubilization of the filters with Bray's solution. Nonspecific binding of $^{22}Na^+$ on the filter was estimated from the amount of radioactivity retained on the filter following filtration of 2.5-μL samples diluted with 4 mL of quenching solution and incubated for 2 h at 21 °C. The resulting values of radioactivity (usually about 5% of the total

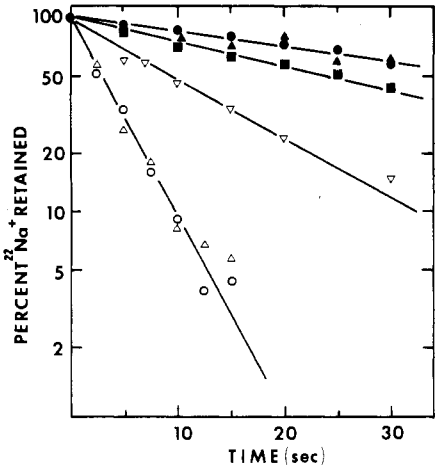

FIGURE 1: Properties of Na^+ efflux from Na^+-loaded membrane vesicles incubated at pH 6.6. Membrane vesicles prepared from *E. coli* (RA 11 strain) were resuspended in 100 mM potassium phosphate, 10 mM $MgSO_4$, and 10 mM sodium phosphate (pH 6.6) concentrated to 35 mg/mL and incubated with $^{22}Na^+$ (2000 cpm/nmol) at 4 °C until equilibration of the isotope. When the effect of FCCP had to be analyzed, the protonophore was added during this equilibration period at 1 nmol/mg of protein. 2.5-μL aliquots of the concentrated suspension of Na^+-loaded vesicles were diluted into 400 μL of buffered solution (pH 6.6) which basically contained 100 mM potassium phosphate and 10 mM $MgSO_4$: (●) no energy source added; (▲) same as (●) but in the presence of 10 mM sodium phosphate. In the other experiments, the dilution media were supplemented with (○) PMS (0.15 mM)–potassium ascorbate (ASC) (20 mM); (△) PMS–ASC plus 10 mM sodium phosphate; (▽) Li^+–D-lactate (20 mM), and (■) PMS–ASC plus FCCP (10 μM). At the times shown, the reaction was stopped by diluting the samples with 2 mL of 100 mM potassium phosphate, 10 mM sodium phosphate, and 10 mM $MgSO_4$, pH 6.6, and the samples were filtered as observed under Methods. The zero times were determined by diluting directly the 2.5-μL aliquots into 2.5 mL of quenching buffer and filtering immediately. Absolute values of $^{22}Na^+$ concentration retained by the vesicles at zero time (Na^+_i) were 20 ± 2 nmol/mg of protein. Data are expressed as the percentage of $^{22}Na^+$ retained by using zero time points for normalization. Rate constants for $^{22}Na^+$ efflux were estimated from the slope of the curve by using the relation $k = 0.693/t_{1/2}$ to calculate the Na^+ efflux rate (V_{Na} = nanomoles per milligram of protein per minute). Experiments were performed at room temperature.

radioactivity retained by $^{22}Na^+$-loaded vesicles at the onset of the efflux reaction) were used to correct the efflux data.

$^{22}Na^+$ Influx Measurements. As for $^{22}Na^+$ efflux, $^{22}Na^+$ influx was measured by using concentrated vesicle suspension preequilibrated in solutions of desired composition and pH. $^{22}Na^+$ influx measurements were initiated by vigorous mixing of 2.5-μL aliquots of the concentrated vesicle suspension into 50 μL of buffered solution containing 10 mM sodium phosphate and $^{22}NaCl$ (final activity 500 cpm/nmol). The influx reactions were terminated as explained above for $^{22}Na^+$ efflux experiments.

Protein. Protein was measured according to Lowry et al. (1951) with serum albumin as the standard.

Results

Properties of Downhill Na^+ Efflux from Na^+-Loaded Membrane Vesicles. It is well established that the generation of $\Delta\mu_{H^+}$ across *E. coli* membrane vesicles promotes a net efflux of Na^+ ions against their electrochemical potential gradient (Tokuda & Kaback, 1976; Schuldiner & Fishkes, 1978). The data illustrated in Figure 1 show that the Na^+ efflux along the Na^+ concentration gradient is also strongly influenced by $\Delta\bar{\mu}_{H^+}$. The experiment was performed at pH_{out} 6.6, and the $^{22}Na^+$ efflux rate was monitored by following the time-dependent decrease in intravesicular $^{22}Na^+$ concentration which takes place upon dilution of vesicles preloaded with 10 mM $^{22}Na^+$. It is seen that when vesicles are diluted into Na^+-free medium containing either D-lactate (inverted open triangles) or reduced PMS (open circles) as an energy source, the rates of internal Na^+ decrease are much faster than the rate of internal Na^+ decrease measured in the absence of electron donors (closed circles). The Na^+ efflux rate increases from 20 nmol·(mg of protein)$^{-1}$·min^{-1} in the absence of energy to 80 and 280 nmol·(mg of protein)$^{-1}$·min^{-1} in the presence of D-lactate and reduced PMS, respectively. Remarkably, the extent of acceleration in Na^+ efflux rate produced by D-lactate and reduced PMS additions correlates with the magnitudes of $\Delta\bar{\mu}_{H^+}$ generated in the presence of each electron donor (data not shown). Finally, Figure 1 shows that the energy-dependent increase in Na^+ efflux rate no longer occurs in vesicles treated with the protonophore FCCP (closed squares). These data clearly indicate that the downhill Na^+ efflux mechanism is controlled by $\Delta\bar{\mu}_{H^+}$.

In addition, Figure 1 shows that neither the energy-dependent (open triangles) nor the energy-independent (closed triangles) rates of internal Na^+ decrease are modified by the presence of external Na^+ ions (10 mM) in the dilution medium. First, the lack of transstimulation of the energy-independent Na^+ efflux by external Na^+ ions suggests that in the absence of $\Delta\bar{\mu}_{H^+}$ no significant Na^+–Na^+ exchange reaction is taking place. On the other hand, in the presence of external Na^+ ions and reduced PMS (or D-lactate) in the dilution medium, the rate of $^{22}Na^+$ internal decrease corresponds to a net Na^+ efflux against both Na^+ concentration and electrical gradients. Importantly, measurements in parallel experiments of the Na^+ influx taking place during the first 20 s that follows dilution show that the Na^+ entry is too small [about 15 nmol·(mg of protein)$^{-1}$·min^{-1}] to produce important dilution effects on the internal $^{22}Na^+$ efflux. It can therefore be concluded that the $\Delta\bar{\mu}_{H^+}$-dependent acceleration of the Na^+ efflux rate is not affected by the direction of the transmembrane Na^+ concentration gradient.

The most salient feature of the experiments shown above is that the downhill Na^+ efflux is enhanced as a consequence of the generation of a $\Delta\psi$ interior negative which should logically reduce any passive outward Na^+ movement along the Na^+ concentration gradient. This excludes the possibility that the downhill Na^+ efflux is catalyzed by a passive transportlike mechanism. Rather, the observed $\Delta\bar{\mu}_{H^+}$ effect on the Na^+ efflux rate is consistent with the functioning of a Na^+–H^+ exchange system (Na^+–H^+ antiport) in which, for example, the outward Na^+ movement would be rate limited by the coupled inward movement of H^+; generation of $\Delta\bar{\mu}_{H^+}$ favorable to H^+ influx would increase Na^+ efflux.

Effects of Valinomycin and Nigericin on Downhill Na^+ Efflux at Various pHs. According to Schuldiner & Fishkes (1978), the uphill Na^+ efflux catalyzed by the Na^+–H^+ antiporter is preferentially coupled to the chemical proton gradient at acidic pHs and to the electrical gradient at more alkaline pH values. Taking as a working hypothesis that the $\Delta\bar{\mu}_{H^+}$-accelerated downhill Na^+ efflux is also mediated by the Na^+–H^+ antiporter, it is of interest to determine whether the total $\Delta\bar{\mu}_{H^+}$ or one of its two components in particular contributes to the acceleration phenomenon and if the observed contribution pattern is dependent on the external pH value.

In order to answer these questions, we investigate the effects of selective dissipation of $\Delta\bar{\mu}_{H^+}$ components with appropriate ionophores (Ramos et al., 1976) on the rate of downhill Na^+ efflux. Figure 2A illustrates the results of an experiment performed at pH_{out} 6.6. It is first observed that dissipation

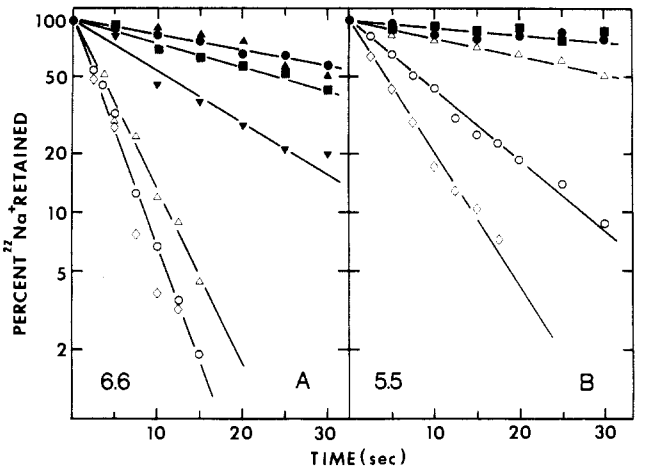

FIGURE 2: Effects of selective dissipation of ΔpH and Δψ with nigericin and valinomycin on the energy-dependent efflux from Na^+-loaded membrane vesicles at pH 6.6 and 5.5. (A) Membrane vesicles were resuspended in 100 mM potassium phosphate, 10 mM $MgSO_4$, and 10 mM sodium phosphate at pH 6.6, concentrated to 30–35 mg of protein/mL, and incubated with $^{22}Na^+$ (2000 cpm/nmol) at 4 °C until isotope equilibration. The concentrated vesicle suspension was then divided into five parts of which one was kept for control experiments; the other four were supplemented either with valinomycin (1 nmol/mg of protein) or nigericin (0.1 nmol/mg of protein), with valinomycin *plus* nigericin at the concentration mentioned above, or finally with FCCP (1 nmol/mg of protein). In control experiments, Na^+ efflux was measured by diluting 2.5-μL aliquots of untreated vesicles into 400 μL of buffered solution at pH 6.6 which basically contained 100 mM potassium phosphate, 10 mM $MgSO_4$, and PMS (0.15 mM)–ascorbate (20 mM) (○) or was devoid of an energy source (●). The Na^+ efflux from the antibiotics or FCCP-treated vesicles was measured by diluting 2.5-μL aliquots into the same saline medium (pH 6.6) containing PMS–ASC and the corresponding antibiotic or protonophore at the following final concentrations: (◇) valinomycin (5 μM); (△) nigericin (1 μM); (▼) valinomycin (5 μM) + nigericin (1 μM); (■) FCCP (10 μM); (▲) valinomycin (or nigericin) + FCCP. The decrease of the internal $^{22}Na^+$ concentration was monitored and expressed as described in Figure 1. (B) Vesicles were either treated or not treated with valinomycin, nigericin, or FCCP as described in panel A. Conditions are the same as those described for panel A, except that vesicles were equilibrated and diluted in media at pH 5.5. Symbols correspond to similar conditions as in panel A: (◇) valinomycin-treated vesicles; (△) nigericin-treated vesicles; (■) FCCP-treated vesicles; (○) control in the presence of an energy source; (●) control with no energy source.

of Δψ with valinomycin does not lead to substantial modification of the energy-dependent increase in the Na^+ efflux rate [V_{Na} = 300 nmol·(mg of protein)$^{-1}$·min^{-1}, open diamonds] as compared to control conditions (open circles). On the other hand, when ΔpH generation has been prevented by nigericin, V_{Na} is only reduced by 20% (open triangles). Finally, and as expected from its uncoupling effect, a mixture of valinomycin and nigericin strongly reduces the Na^+ efflux rate (closed inverted triangles). It is important to stress that, in these conditions, neither valinomycin nor nigericin catalyzes significant Na^+ efflux. Thus, first, the energy-independent Na^+ efflux is not accelerated as a consequence of the addition of either ionophore (closed triangles); also, the energy-dependent Na^+ efflux rates from either valinomycin- or nigericin-treated vesicles are reduced by FCCP to levels not significantly different from those taking place in the absence of energy. In conclusion, since $\Delta\bar{\mu}_{H^+}$ is reduced to a sole ΔpH in valinomycin-treated vesicles and comprises only an electrical term in nigericin-treated vesicles (Ramos et al., 1976), it appears that both the chemical and the electrical components of $\Delta\bar{\mu}_{H^+}$ independently accelerate the downhill Na^+ efflux at pH_{out} 6.6.

A similar experiment performed at pH_{out} 5.5 is illustrated in Figure 2B. In contrast to the observations at pH_{out} 6.6, it can be observed that selective dissipation of Δψ and ΔpH leads to very different effects. Thus, the energy-dependent Na^+ efflux is shown to occur at a rate 2 times faster in valinomycin-treated vesicles (open diamonds) than in control conditions [V_{Na} = 90 nmol·(mg of protein)$^{-1}$·min^{-1}, open circles]. Under these circumstances, it is important to recall that a significant increase in ΔpH magnitude (30%) takes place besides the collapse of Δψ (Ramos & Kaback, 1977). This suggests some correlation between the magnitude of ΔpH and the rate of Na^+ efflux. In line with this conclusion, Figure 2B shows that the collapse of ΔpH with nigericin leads to a 4-fold reduction in the Na^+ efflux rate (open triangles); this inhibition compares in magnitude with that observed with FCCP (closed squares). These data, therefore, suggest a preferential role of ΔpH in the accelerating effect of $\Delta\mu_{H^+}$ on the downhill Na^+ efflux rate at pH_{out} 5.5. Although not illustrated, it should finally be mentioned that the energy-dependent downhill Na^+ efflux from vesicles equilibrated at pH 7.5 [V_{Na} = 520 nmol·(mg of protein)$^{-1}$·min^{-1}] is not modified by addition of nigericin but is drastically reduced if Δψ is dissipated using valinomycin and/or the lipophilic cation tetraphenylphosphonium [V_{Na} = 90 nmol·(mg of protein)$^{-1}$·min^{-1}] (Ghazi et al., 1981). The observation that the energy-dependent acceleration of the downhill Na^+ efflux rate is primarily associated with generation of Δψ at alkaline pH is consistent with the previous demonstration that $\Delta\bar{\mu}_{H^+}$ comprises only an electrical term at this pH_{out} (Ramos & Kaback, 1977).

Effects of Artificially Imposed Δψ and ΔpH on Na^+ Efflux at Various pHs. Independent and complementary information on the contribution of the $\Delta\bar{\mu}_{H^+}$ components to the acceleration of the downhill Na^+ efflux at different external pHs can be derived from an examination of the effects of artificially imposed ΔpH (interior alkaline) or Δψ (interior negative) on the Na^+ efflux rate (Figure 3A–C). In these experiments, vesicles were equilibrated with 100 mM potassium acetate–10 mM labeled sodium phosphate at either pH 5.5, 6.6, or 7.5 and incubated in the presence of valinomycin. Artificial Δψ's (interior negative; open triangles) were generated as a consequence of the imposition of outwardly directed potassium diffusion gradients across the membrane vesicles ($K^+_{in} \rightarrow K^+_{out}$) in the presence of an excess of valinomycin; the presence of high concentrations of acetate in both the intravesicular and diluting media is expected to prevent the generation of any ΔpH. On the other hand, artificial ΔpHs (alkaline interior; open circles) were created by imposing outwardly directed acetate diffusion gradients ($acetate_{in} \rightarrow acetate_{out}$). The presence of high potassium concentrations on both sides of the membrane together with valinomycin is expected to short-circuit any Δψ generated.

The middle panel (B) in Figure 3 shows that at pH 6.6 the rate of Na^+ efflux is increased from 55 nmol·(mg of protein)$^{-1}$·min^{-1} in the absence of any imposed gradient (closed circles) to 220 and 460 nmol·(mg of protein)$^{-1}$·min^{-1} in response to an imposed Δψ (open triangles) and imposed ΔpH (open circles), respectively. Importantly, both the Δψ- (Figure 3B) and ΔpH-induced accelerations in the Na^+ efflux rate were prevented in the presence of the uncoupling agent FCCP (10 μM). When the same operations were performed at pH 5.5 (Figure 3A), it can be observed that the Na^+ efflux responds differently to artificially imposed Δψ and ΔpH (both of 167 mV). The imposed ΔpH again promotes an increase in the V_{Na} value [340 nmol·(mg of protein)$^{-1}$·min^{-1} as compared to 40 nmol·(mg of protein)$^{-1}$·min^{-1} in the absence of any imposed gradient]. In contrast, the imposition of artificial Δψ

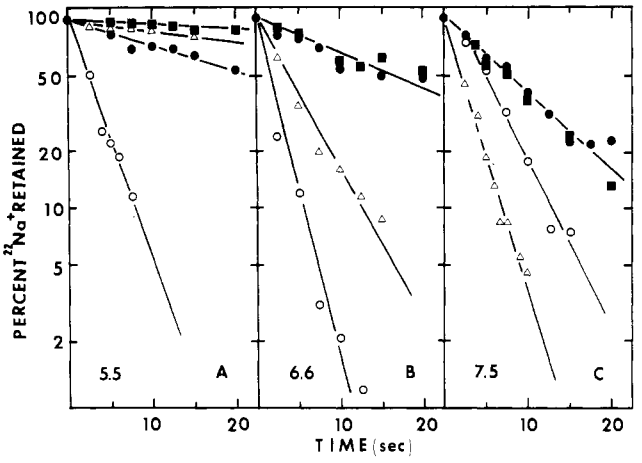

FIGURE 3: Effects of imposed $\Delta\psi$ (interior negative) or ΔpH (interior alkaline) on Na^+ efflux from Na^+-loaded vesicles incubated at pH 5.5 (A), 6.6 (B), or 7.5 (C). Membrane vesicles were equilibrated with 100 mM potassium acetate, 10 mM $MgSO_4$, and 10 mM sodium phosphate at given pH values and concentrated to about 30 mg of protein/mL. The vesicles were then incubated for 4 h with $^{22}Na^+$ (2000 cpm/nmol) at 4 °C and finally treated with valinomycin (1 nmol/mg of protein) and in some cases (■) with FCCP (1 nmol/mg of protein). Na^+ efflux was measured by diluting 2.5-μL aliquots of concentrated vesicles into 2 mL of saline solution which in addition to the salts indicated below contained 10 mM $MgSO_4$ and 5 μM valinomycin and had the same pH as the media used during equilibration. The experiments were conducted at pH 5.5 (panel A), 6.6 (panel B), and 7.5 (panel C). The salt composition of the different media was the following: (A and B) (●) 100 mM potassium acetate–10 mM potassium phosphate (no gradient generated); (○) 100 mM potassium gluconate–10 mM potassium phosphate (generation of ΔpH, interior alkaline); (△) 100 mM choline acetate–10 mM choline phosphate (generation of $\Delta\psi$, interior negative); (■) same composition as (○) except that FCCP was added at a final concentration of 10 μM; (C) same as panels A and B except (○) 10 mM potassium acetate–90 mM potassium gluconate–10 mM potassium phosphate (generation of ΔpH 60 mV, interior alkaline). The time-dependent decrease of intravesicular $^{22}Na^+$ concentration was monitored by filtration and expressed as described in Figure 1. Except at pH 7.5 where the ΔpH is only 60 mV, notice that the imposed $\Delta\psi$ and ΔpH have theoretical values (167 mV) computed by using the relationships $\Delta\psi = 58 \log ([K^+_{in}]/[K^+_{out}])$ and $\Delta pH = 58 \log ([acetate_{in}]/[acetate_{out}])$.

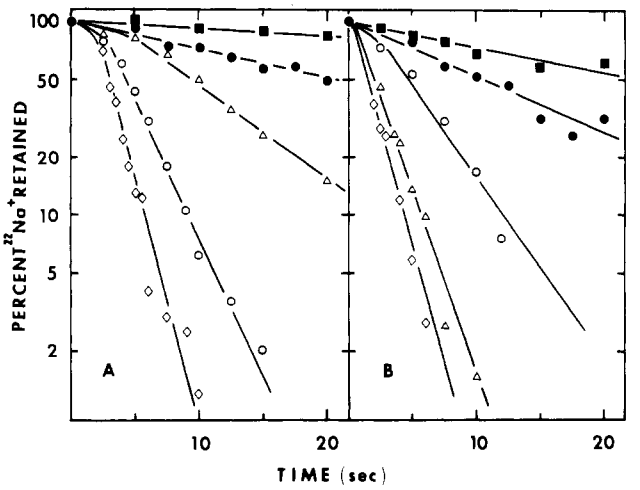

FIGURE 4: Effects of simultaneously imposed $\Delta\psi$ (interior negative) and ΔpH (interior alkaline) on Na^+ efflux from Na^+-loaded vesicles incubated at pH 6.6 (A) and 7.5 (B). Membrane vesicles were equilibrated in 100 mM potassium acetate, 10 mM $MgSO_4$, and 10 mM sodium phosphate at the given pH, equilibrated with $^{22}Na^+$, and treated with valinomycin (1 nmol/mg of protein) as described in Figure 3. 2.5-μL aliquots of concentrated vesicle suspension were diluted in media (400 μL) of appropriate composition for the generation of $\Delta\psi$ (interior negative, open triangles), ΔpH (interior alkaline, open circles), and $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline, open diamonds). The salt composition of the different media used for dilution was as follows. (A) (●) 100 mM potassium acetate–10 mM potassium phosphate (no gradient generated); (○) 100 mM potassium glucuronate–10 mM potassium phosphate (ΔpH 128 mV, interior alkaline); (△) 100 mM choline acetate–10 mM choline phosphate ($\Delta\psi$ 128 mV, interior negative); (◇) 100 mM choline glucuronate–10 mM choline phosphate [$\Delta\bar{\mu}_{H^+}$, interior negative (128 mV) and alkaline (128 mV)]; (■) same as (◇) except that FCCP was added to the diluting medium to a final concentration of 10 μM. (B) (●) 100 mM potassium acetate–10 mM potassium phosphate (no gradient generated); (○) 90 mM potassium glucuronate–10 mM potassium acetate–10 mM potassium phosphate (ΔpH 60 mV, interior alkaline); (△) 100 mM choline acetate–10 mM choline phosphate ($\Delta\psi$ 128 mV, interior negative); (◇) 90 mM choline glucuronate–10 mM choline acetate–10 mM choline phosphate [$\Delta\bar{\mu}_{H^+}$, interior negative (128 mV) and alkaline (60 mV)]; (■) same as (◇) except that FCCP was added to the diluting medium to a final concentration of 10 μM. All solutions contained in addition 10 mM $MgSO_4$ and 5 μM valinomycin. The decrease in intravesicular $^{22}Na^+$ concentration was monitored by filtration as described in Figure 1.

does not significantly modify the Na^+ efflux rate [V_{Na} = 20 nmol·(mg of protein)$^{-1}$·min^{-1}]. Finally, Figure 3C represents the effects of imposed $\Delta\psi$ (167 mV) and ΔpH (60 mV) on the Na^+ efflux rate from Na^+-loaded vesicles equilibrated and diluted in media at pH 7.5. As expected, imposition of an artificial $\Delta\psi$ produces a significant acceleration of the Na^+ efflux rate [from 80 to 400 nmol·(mg of protein)$^{-1}$·min^{-1}]. Surprisingly, however, it is observed in this same figure that imposition of an artificial ΔpH of 60 mV also substantially increases the Na^+ efflux rate [V_{Na} = 260 nmol·(mg of protein)$^{-1}$·min^{-1}]; this enhancement in the Na^+ efflux rate by the chemical gradient is again completely prevented in FCCP-treated membranes. These experiments therefore not only confirm that the downhill Na^+ efflux is accelerated by $\Delta\psi$ as well as by ΔpH at pH 6.6 but also extend this conclusion to the mechanism of acceleration of the downhill Na^+ efflux at higher pHs.

Effects of Simultaneous Imposition of Artificial $\Delta\psi$ and ΔpH. While the results presented in the preceding section show that $\Delta\psi$ and ΔpH independently accelerate the downhill Na^+ efflux rate at pH 6.6 and 7.5, the observations illustrated in Figure 4A,B indicate that the two $\Delta\bar{\mu}_{H^+}$ components can act concurrently. In these experiments, vesicles were equilibrated with 100 mM potassium acetate and 10 mM sodium phosphate at pH 6.6 (or 7.5), treated with valinomycin, and then diluted into media appropriate for the generation of either ΔpH (interior alkaline, open circles), $\Delta\psi$ (interior negative, open triangles), or $\Delta\bar{\mu}_{H^+}$ (interior alkaline and negative, open diamonds). It is seen in Figure 4A (pH 6.6) that whereas independent imposition of $\Delta\psi$ and ΔpH (128 mV) increases the Na^+ efflux rate from 40 to 144 and 400 nmol·(mg of protein)$^{-1}$·min^{-1}, respectively, simultaneous imposition of $\Delta\psi$ (128 mV) and ΔpH (128 mV) brings the Na^+ efflux rate to values as high as 740 nmol·(mg of protein)$^{-1}$·min^{-1}. Importantly, these large accelerations in the Na^+ efflux rate are not observed in membranes uncoupled with FCCP. As shown in Figure 4B, similar potentiation of the accelerating effect of $\Delta\psi$ and ΔpH was recorded at pH_{out} 7.5.

The experiments referred to above do not specify if generation of either $\Delta\psi$ or ΔpH modifies a single transport reaction or whether two different pathways coexisting in the membrane are affected, one being activated by $\Delta\psi$ and the other by ΔpH. Figure 5 illustrates an experiment performed at pH 6.6 which argues in favor of the first hypothesis. In this case, internal as well as external cation compositions were manipulated in such a way that $\Delta\psi$ of inverse polarity (i.e., interior positive instead of negative) could be imposed concurrently with a ΔpH (interior alkaline). Figure 5 shows that

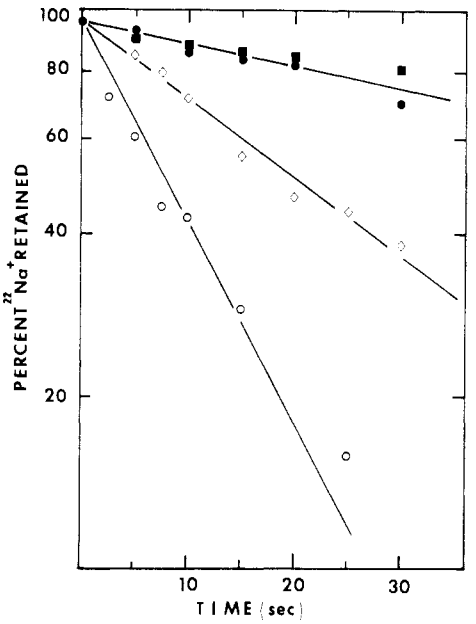

FIGURE 5: Effect of simultaneous imposition of $\Delta\psi$ (interior positive) and ΔpH (interior alkaline) on Na^+ efflux in vesicles incubated at pH 6.6. Membrane vesicles were equilibrated with 100 mM choline acetate, 10 mM $MgSO_4$, 10 mM sodium phosphate, and 1 mM potassium phosphate (pH 6.6). Following concentration and equilibration with $^{22}Na^+$, the vesicles were then treated with valinomycin (1 nmol/mg of protein). 2.5-μL aliquots were diluted into 2 mL of buffered media (pH 6.6) which had the following composition: (●) 100 mM choline acetate, 10 mM choline phosphate, and 1 mM potassium phosphate (no gradient); (○) 100 mM choline glucuronate, 10 mM choline phosphate, and 1 mM potassium phosphate (ΔpH 167 mV, interior alkaline); (◇) 100 mM potassium glucuronate–11 mM potassium phosphate [$\Delta\bar{\mu}_{H^+}$, interior alkaline (167 mV) and interior positive (120 mV)]; (■) same as (○) but in the presence of FCCP (10 μM). All dilution media contained 10 mM $MgSO_4$ and 5 μM valinomycin. The internal [$^{22}Na^+$] decrease was monitored by filtration as described in Figure 1. Notice that the ΔpH-induced rate of internal [$^{22}Na^+$] decrease is lower than those in Figures 3 and 4.

the Na^+ efflux rate is higher [V_{Na} = 100 nmol·(mg of protein)$^{-1}$·min^{-1}] when ΔpH is imposed alone as compared to conditions where $\Delta\psi$ (positive inside) is imposed simultaneously with the artificial ΔpH [V_{Na} = 40 nmol·(mg of protein)$^{-1}$·min^{-1}]. Although the Na^+ efflux rates measured in those experiments are small—perhaps due to the absence of internal K^+ ions or to an inhibitory effect of cholinium ions—these observations which were repeatedly observed suggest that the transport reaction activated by ΔpH is also affected by a transmembrane electrical field.

Finally, further information regarding the relative role of $\Delta\psi$ and ΔpH in the $\Delta\bar{\mu}_{H^+}$-dependent acceleration of the downhill Na^+ efflux at acidic pH (pH_{out} 5.5) comes from examination in Figure 6 of the effect of simultaneously imposed $\Delta\psi$ (interior negative) and ΔpH (interior alkaline). It is thus observed in Figure 6A that simultaneous imposition of $\Delta\psi$ (128 mV, interior negative) and ΔpH (128 mV, interior alkaline) promotes a faster Na^+ efflux (open diamonds) than that resulting from imposition of only ΔpH (open circles). Calculation of the corresponding absolute Na^+ efflux rates [385 and 265 nmol·(mg of protein)$^{-1}$·min^{-1} indicates that the potentiation effect of $\Delta\psi$ is highly significant. Since imposition of a $\Delta\psi$ is in itself unable to accelerate the Na^+ efflux mechanism at this pH (open triangles), it can be suggested that an imposed ΔpH leads to a change in the antiporter properties so that the downhill Na^+ efflux mechanism can be activated by the electrical potential. Moreover, Figure 6B,C shows that the accelerating effect of an imposed $\Delta\psi$ on the Na^+ efflux rate is dependent on the magnitude of the ΔpH

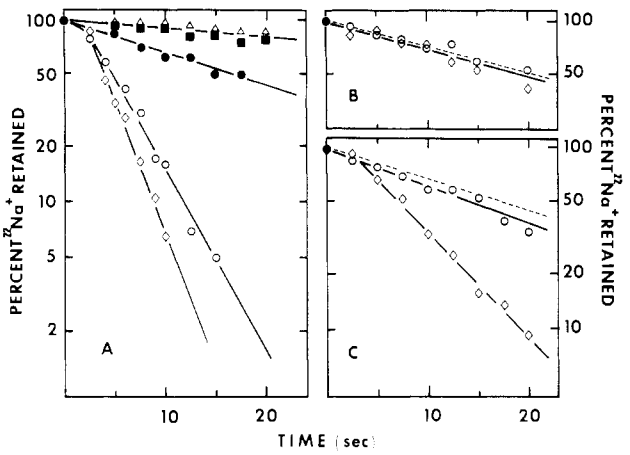

FIGURE 6: Effect of imposed $\Delta\psi$ as a function of the magnitude of a simultaneously imposed ΔpH on the Na^+ efflux from vesicles incubated at pH 5.5. Membrane vesicles were equilibrated in 100 mM potassium acetate, 10 mM $MgSO_4$, and 10 mM sodium phosphate (pH 5.5), concentrated to about 30 mg/mL, and incubated in the presence of $^{22}Na^+$ at 4 °C. When the isotopic equilibration was reached, valinomycin was added to a final concentration of 1 nmol/mg of protein. Aliquots (2.5 μL) were then rapidly diluted (160-fold) into the media indicated below and of appropriate composition for the generation of either $\Delta\psi$ and/or ΔpH of given amplitudes [calculated by using $\Delta\psi$ = 58 log ([K^+_{in}]/[K^+_{out}]) and ΔpH = 58 log ([$acetate_{in}$]/[$acetate_{out}$])]. (A) (●) 100 mM potassium acetate–10 mM potassium phosphate (no gradient generated); (○) 100 mM potassium glucuronate–10 mM potassium phosphate (ΔpH 128 mV, interior alkaline, no $\Delta\psi$); (◇) 100 mM choline glucuronate–10 mM choline phosphate (ΔpH 128 mV, interior alkaline; $\Delta\psi$ 128 mV, interior negative); (■) same as (◇) but performed in the presence of 10 μM FCCP; (△) 100 mM choline acetate–10 mM choline phosphate ($\Delta\psi$ 128 mV, interior negative, no ΔpH). (B) (○) 16 mM potassium acetate, 84 mM potassium glucuronate, and 10 mM potassium phosphate (ΔpH 45 mV, interior alkaline); (◇) 16 mM choline acetate, 84 mM choline glucuronate, and 10 mM choline phosphate (ΔpH 45 mV, interior alkaline; $\Delta\psi$ 128 mV, interior negative). (C) (○) 5 mM potassium acetate, 95 mM potassium glucuronate, and 10 mM potassium phosphate (ΔpH 73 mV, interior alkaline); (◇) 5 mM choline acetate, 95 mM choline glucuronate, and 10 mM choline phosphate (ΔpH 73 mV, interior alkaline; $\Delta\psi$ 128 mV; interior negative). The dashed lines in panels B and C represent the Na^+ efflux rate measured in the absence of an imposed gradient (see panel A).

simultaneously imposed. Thus, if the external acetate to glucuronate concentration ratio is combined so that the transmembrane acetate gradient, and therefore the resulting ΔpH, is 45 mV, it is seen that concurrent imposition of $\Delta\psi$ (128 mV) has no effect on the Na^+ efflux rate (Figure 6B). In contrast, if the imposed ΔpH is 73 mV, simultaneous imposition of $\Delta\psi$ increases the Na^+ efflux rate to 160 nmol·(mg of protein)$^{-1}$·min^{-1}, as compared to 60 nmol·(mg of protein)$^{-1}$·min^{-1} in response to imposition of only ΔpH (Figure 6C). These results indicate that a ΔpH of sufficient magnitude ($\geq$70 mV) should be imposed before any accelerating effect of the electrical gradient be observed. As a corollary, it should be noted that this threshold ΔpH value is also the minimal ΔpH value required for acceleration of the downhill Na^+ efflux in response to an imposed chemical gradient (Figure 6C).

Discussion

The effects of $\Delta\bar{\mu}_{H^+}$ or of each of its components on the kinetics of downhill Na^+ efflux from Na^+-loaded membrane vesicles incubated at the different external pHs reported in this paper strongly suggest that the downhill transport of Na^+ ions is mediated by the Na^+–H^+ antiport mechanism. Furthermore, the results fit a model in which the outward movement of Na^+ catalyzed by the antiporter would be rate limited by the coupled inward movement of H^+. Finally, some

observations suggest that the intravesicular pH plays a regulatory role in the antiport function.

Many observations illustrated indicate that the downhill Na^+ efflux taking place along a favorable Na^+ concentration gradient (about 90 mV) is strongly enhanced in the presence of $\Delta\bar{\mu}_{H^+}$. This effect is observed in conditions where $\Delta\bar{\mu}_{H^+}$ is generated either during respiratory activity (Figures 1 and 2) or immediately following imposition of artificial $\Delta\psi$ and ΔpH (Figures 3 and 4). As already stressed under Results, the acceleration of the Na^+ efflux rate by $\Delta\bar{\mu}_{H^+}$ cannot be accounted for by an increase in passive outward Na^+ movements or leaks. Indeed, $\Delta\psi$ generated in these experiments makes the vesicles' interior electrically negative with respect to the outer medium and, hence, should oppose the outward movement of positively charged Na^+ ions and not accelerate it. This conclusion is strenghtened by the observation that imposition of $\Delta\psi$ of opposite polarity (Figure 5), i.e., interior positive, reduces rather than accelerates Na^+ efflux.

In the consideration of possible mechanisms implicated in the efflux of Na^+ ions, the acceleration of the downhill Na^+ efflux by an imposed ΔpH (Figures 2–6) is particularly suggestive of the participation of a Na^+–H^+ exchange process. Thus, ΔpH enhances Na^+ efflux in membrane vesicles electrically uncoupled with valinomycin and a high concentration of K^+ ions. In the absence of any possible phenomenon of nonspecific electrical coupling between Na^+ efflux and H^+ influx down their respective concentration gradients, it follows that the mechanism of flux interaction can only be direct. This implies that the downhill Na^+ efflux is mediated by a Na^+–H^+ exchange process, probably the Na^+–H^+ antiporter which catalyzes the uphill Na^+ efflux in these bacterial membranes (Tokuda & Kaback, 1977; Schuldiner & Fishkes, 1978). This interpretation gives a straightforward explanation for all the kinetic modifications of Na^+ efflux associated with the generation of transmembrane $\Delta\bar{\mu}_{H^+}$, ΔpH, and $\Delta\psi$. Thus, generation of $\Delta\bar{\mu}_{H^+}$, interior negative and alkaline, which favors H^+ influx, would increase the rate of Na^+–H^+ exchange and hence the Na^+ efflux (Figures 1–4). Furthermore, it can be expected that separate imposition of $\Delta\psi$ (interior negative) or ΔpH (interior alkaline) might independently enhance Na^+ efflux (Figures 2–4) since they individually tend to accelerate inward H^+ movement. Incidentally, the absence of a stimulating effect of external Na^+ ions on the energy-independent Na^+ efflux (Figure 1) suggests that no substantial Na^+–Na^+ exchange is catalyzed in the absence of $\Delta\bar{\mu}_{H^+}$. It is therefore improbable that $\Delta\bar{\mu}_{H^+}$ shifts the mode of operation of the antiporter from a Na^+–Na^+ exchange system in the absence of $\Delta\bar{\mu}_{H^+}$ to a Na^+–H^+ exchange mechanism in the presence of $\Delta\bar{\mu}_{H^+}$.

The assumption that the kinetic properties of the downhill Na^+ efflux reflect those of the Na^+–H^+ antiporter calls for two important suggestions regarding the relationships between the antiport activity and $\Delta\bar{\mu}_{H^+}$ or its components. Thus, first, in the absence of $\Delta\bar{\mu}_{H^+}$, the Na^+ efflux rate along its chemical gradient is low; importantly, this energy-independent Na^+ efflux rate is not accelerated when the membrane vesicles are incubated with FCCP. It is thus apparent that limitation of the energy-independent Na^+ efflux rate cannot be consecutive to the buildup of an adverse electrochemical proton gradient created by the forced inward movement of H^+ ions via the antiporter. Rather, the data would be consistent with the existence of a kinetic barrier for Na^+ efflux in deenergized membranes. Since, on the other hand, Na^+ efflux is accelerated when H^+ influx is favored, it seems reasonable to conclude that the overall reaction of Na^+–H^+ exchange—and hence Na^+ efflux—is rate limited by the reaction involved in the inward translocation of H^+ ions. The second suggestion concerns the respective contribution of ΔpH and $\Delta\psi$ to the mechanism of acceleration of the Na^+ efflux rate. Indeed, Figures 3 and 4 indicate that at pH 6.6 or higher both $\Delta\bar{\mu}_{H^+}$ components independently stimulate the Na^+ efflux rate. These data indicate that neither a specific $\Delta\psi$-sensitive step nor a ΔpH-sensitive step preferentially and absolutely controls the rate-limiting reaction, i.e., the H^+ influx, of the antiport function at these pHs. This suggestion highlights the importance of determining whether $\Delta\psi$ and ΔpH affect the same or different steps in the H^+ translocation process.

At pH 5.5 (Figures 3A and 6), the situation is apparently more complex. First, an imposed ΔpH stimulates the Na^+ efflux rate provided that the ΔpH value is of sufficient magnitude (Figure 3A). Second, even very high imposed $\Delta\psi$'s do not accelerate by themselves the Na^+ efflux rate (Figure 3A). However, when a ΔpH of at least 70 mV is simultaneously imposed, $\Delta\psi$ does accelerate the Na^+ efflux (Figure 6A,C). These observations suggest the existence of a threshold ΔpH value beyond which both ΔpH and $\Delta\psi$ can influence the Na^+ efflux mechanism. Two different regulatory mechanisms can be hypothesized. First, and in line with Lanyi's suggestion (1979), one can invoke gating effects of the gradients on the carrier. These gating effects would nevertheless be solely restricted to a chemical gradient since $\Delta\psi$ of a corresponding magnitude does not accelerate per se the Na^+ transport reaction; hence, one can conclude that the overall transport reaction is rate limited by a ΔpH-sensitive step. Alternately, it can be postulated that the threshold ΔpH value does not refer to a minimal energy that should be provided for the activation of the carrier but rather corresponds to a shift in the internal pH value (from 5.5 to about 6.8) above which both ΔpH and $\Delta\psi$ can enhance the Na^+–H^+ antiporter activity. Distinction between these two possibilities will be considered in a more quantitative analysis of the antiport activity as a function of the magnitude of artificially imposed $\Delta\psi$ and ΔpH which will be presented in a forthcoming paper.

In spite of the limitations observed at acidic pH, it is apparent that the kinetics of the downhill Na^+ efflux catalyzed by the antiporter are influenced by both $\Delta\psi$ and ΔpH in the pH range between 5.5 and 7.5. In this context, the dependence of the rate of Na^+–H^+ exchange on $\Delta\psi$ is frequently used as an argument to suggest that the system is electrogenic, i.e., that the stoichiometry of the exchange is higher than unity (Lanyi, 1979; Beck & Rosen, 1979; Mandel et al., 1980). In line with this interpretation, our observations would suggest that the Na^+–H^+ antiport of *E. coli* functions as an electrogenic process at all pHs between 5.5 and 7.5. This conclusion does not support the contention of Schuldiner & Fishkes (1978) that the antiporter catalyzes an electroneutral Na^+–H^+ exchange at acidic pHs. The contradiction might be, however, only apparent. Indeed, these authors clearly establish that ΔpH does drive the uphill Na^+ efflux at pH 6.6; however, they did not provide evidence indicating that only ΔpH is able to drive the Na^+ efflux reaction at this pH. Furthermore, it should be emphasized that interpretation of the ionophore effects on the Na^+ efflux rate should take into account the interconversion of $\Delta\psi$ into ΔpH which takes place at this pH (Ramos & Kaback, 1977). Indeed, in the presence of valinomycin, the increase in the contribution of ΔpH—because of an increase in its magnitude due to the interconversion process—might well compensate, partially or totally, the loss of the contribution of $\Delta\psi$ collapsed by the antibiotic. These remarks emphasize the need for more direct information

concerning the specific contribution of $\Delta\psi$ to the driving force for uphill Na^+ efflux at acidic pHs. Their data would also be of interest to analyze whether kinetic and thermodynamic aspects of the antiport function can be related to each other.

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Characterization of Two Nucleotide Binding Sites on the Isolated, Reconstitutively Active β Subunit of the F_0·F_1 ATP Synthase†

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ABSTRACT: The reconstitutively active β subunit that has been removed from the *Rhodospirillum rubrum* membrane-bound ATP synthase (RrF_0·F_1) by LiCl extraction [Philosoph, S., Binder, A., & Gromet-Elhanan, Z. (1977) *J. Biol. Chem. 252*, 8747–8752] and purified to homogeneity [Khananshvili, D., & Gromet-Elhanan, Z. (1982) *J. Biol. Chem. 257*, 11377–11383] binds both ATP and ADP. In the absence of $MgCl_2$ 1 mol of ATP or ADP is bound per mol of β subunit with K_d values of 4.4 μM and 6.7 μM, respectively. The binding of both nucleotides is optimal at a pH range between 7.6 and 8.3 and has a characteristic half-time of 3 min. Addition of $MgCl_2$ has no influence on the stoichiometry, kinetics, or affinity of the binding of ADP to β. It has also no effect on the binding of ATP at limiting concentrations. But at high ATP concentrations, an additional binding site, which is dependent on the concentration of both ATP and $MgCl_2$, is revealed. Thus, a maximal binding stoichiometry of 2 mol of ATP per mol of β subunits is obtained with ≥2 mM ATP at a $MgCl_2$ to ATP ratio of at least 0.5. Under these conditions, one ATP molecule binds to the β with a K_d and $t_{1/2}$ identical with those recorded in the absence of $MgCl_2$, whereas the second ATP molecule binds with a K_d of 200 μM and a $t_{1/2}$ of 20 min. These results indicate that the isolated β subunit of the RrF_0·F_1 ATP synthase contains two nucleotide binding sites: one that binds either ATP or ADP and is independent on the presence of $MgCl_2$ and one that binds rather specifically ATP in the presence of $MgCl_2$.

The terminal step of ATP synthesis in energy-transducing membranes is generally accepted to be catalyzed by a membrane-bound reversible proton-translocating ATPase (Mitchell, 1966), which has been isolated from membranes of mitochondria, bacteria, and chloroplasts and found to consist of two portions: F_1 and F_0 (Penefsky, 1979; Fillingame, 1981; Nelson, 1981). The catalytic F_1 portion is an extrinsic membrane protein composed of five different subunits: α, β, γ, δ, and ϵ. The F_0 portion is an intrinsic membrane complex that

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