Existence of Electrogenic Hydrogen Ion/Sodium Ion Antiport in
Halobacterium halobium Cell Envelope Vesicles†

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Abstract: Illumination causes the extrusion of protons from Halobacterium halobium cell envelope vesicles, as a result of the action of light on bacteriorhodopsin. The protonmotive force developed is coupled to the active transport of Na+ out of the vesicles. The light-dependent ion fluxes in these vesicles were studied by following changes in the external pH, in the fluorescence of the dye, 3,3′-dipentyloxadicarbocyanine, in the 23Na content of the vesicles, and in 3[H]dibenzyldimethylammonium accumulation. During Na+ efflux, and dependent on the presence of Na+ inside the vesicles, the initial light-induced H+ extrusion is followed by H+ influx, which results in net alkalization of the medium at pH > 6.5. When the Na+ content of the vesicles is depleted, the original net acidification of the medium is restored and large ΔpH develops, accompanied by a decrease in the electrical potential. Data reported elsewhere suggest that the driving force for the transport of some amino acids consists mainly of the electrical potential, while for others it comprises the Na+ gradient as well. Glutamate transport appears to be energized only by the Na+ gradient. The development of the Na+ gradient during illumination thus plays an important role in energy coupling. The results obtained are consistent with the existence of an electrogenic H+/Na+ antiport mechanism (H+/Na+ < 1) in H. halobium which facilitates the uphill Na+ efflux. The light-induced protonotive force thereby becomes the driving force in forming a Na+ gradient. The presence of the proposed H+/Na+ antiporter explains many of the light-induced pH effects in intact H. halobium cells.

The central role of proton translocation across membranes in biological energy transduction has been generally recognized. According to the concept of chemiosmotic energy coupling (Mitchell, 1969, 1970, 1972), the difference in pH and electrical potential between the bulk phases across the membranes constitutes the form in which energy is conserved. The two together give the “protonotive force”, which can drive energy-requiring membrane processes through the movements of H+ or other ions. One of the energy-requiring processes in bacterial cells is the extrusion of Na+ against its electrochemical gradient (Zarlengo and Schultz, 1966; Harold et al., 1970; Harold and Papineau, 1972; Lanyi et al., 1976a). In Streptococcus faecalis (Harold and Papineau, 1972) and in Escherichia coli (West and Mitchell, 1974) the evidence suggests the electrically neutral coupled exchange of Na+ for H+ (H+/Na+ > 1), facilitated by an agent belonging to a class of hypothetical membrane components termed antiporters (Mitchell, 1970), similarly to the way the antibiotic monensin is thought to act (Harold, 1970). The extrusion of Na+ can thus be driven by a pH difference across the membranes (interior alkaline), established either by respiration or ATP hydrolysis.

† Abbreviations used are: DDA+ dibenzyldimethylammonium ion; TPB−, tetracyclineboron anion; FCCP, p-trifluoromethoxycarbonyl cyanide phenylhydrazone; DCCD, dicyclohexylcarbodiimide; ATP, adenosine triphosphate.

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Illumination causes the efflux of Na\(^{+}\) from *Halobacterium halobium* envelope vesicles and the resulting large Na\(^{+}\) gradient energizes the transport of glutamate (Lanyi et al., 1976a,b). The Na\(^{+}\) gradient also contributes to the driving force for the transport of many amino acids (MacDonald and Lanyi, 1975, and manuscript in preparation). Energy conservation in these vesicles occurs on at least three levels. (1) In the photochemical states of bacteriorhodopsin, with rapid (<10 ms) dark cycling through several intermediates, initiated by the absorption of a photon (Oesterhelt and Hess, 1973; Stoeckenius and Lozier, 1974; Lozier et al., 1975; Chu Kung et al., 1975; Dencher and Wilms, 1975). Vectorial protonation and deprotonation causes the outward translocation of H\(^{+}\) (Lozier et al., 1976). (2) In the resulting proton gradient, consisting of a pH difference (interior alkaline) and an electrical potential (interior negative), which together may reach values near 200 mV (Renthal and Lanyi, 1976). This protonmotive force decays with a half-life <20 s after illumination is terminated (Renthal and Lanyi, 1976; Lanyi et al., 1976a). (3) In the Na\(^{+}\) gradient, which appears to reach values of Na\(^{+}\)\(_{\text{out}}\)/Na\(^{+}\)\(_{\text{in}}\) > 500, based on the assumption that glutamate transport is driven entirely by Na\(^{+}\) gradient (Lanyi et al., 1976a). The high Na\(^{+}\) gradient necessary for glutamate transport decays with a half-life of 2–5 min (Lanyi et al., 1976a), although the vesicles require many hours to accumulate large amounts of Na\(^{+}\). The means of coupling the protonmotive force to Na\(^{+}\) efflux was suggested to be electrogenic antiport (Lanyi et al., 1976a,b).

*H. halobium* envelope vesicles (MacDonald and Lanyi, 1975) present a simplified system, compared to intact cells, for the study of energy coupling: the vesicles can be prepared with any desired Na\(^{+}\)/K\(^{+}\) ratio, they do not respire or contain ATP, and are inert until illuminated. They appear to be right-side-out or inverted, depending on the illumination. The vesicles are roughly proportional to the concentration of NaCl included.

Materials and Methods

Growth of *H. halobium* strain R-1 and preparation of cell envelope vesicles were as described earlier (MacDonald and Lanyi, 1975). The desired NaCl–KCl concentrations inside the vesicles were obtained by the osmotic shock method (MacDonald and Lanyi, 1975; Lanyi et al., 1976b), followed by resuspension and storage at the same NaCl–KCl concentrations.

Fluorescence and pH measurements during illumination were as described before (Renthal and Lanyi, 1976). For pH measurements, a Beckman semimicro combination electrode no. 39030 was used, which had a rapid response time. A constant upward drift of 0–0.001 ΔpH/min, depending on conditions, was present throughout the experiments and was subtracted from the traces by means of a Nicolet LAB 80 computer.

Transport determinations were carried out as previously described (MacDonald and Lanyi, 1975; Lanyi et al., 1976a,b). except that the Millipore filters were washed with chilled 3.0 M NaCl, 2 × 1 ml for [H]DDA\(^{+}\) uptake, and chilled 3.5 M NaCl, 2 × 1 ml for \(^{22}\)Na flux determinations. All determinations, including those of pH and fluorescence, were carried out at 30 °C. The vesicle suspensions were incubated in the dark for 30–40 min for pH and thermal equilibration. Except where repeated illuminations were done, the vesicles were kept at least 24 h in the dark before the experiments. All solutions, unless otherwise mentioned, contained 2 × 10\(^{-4}\) M phosphate, pH 6.0, externally added to the vesicles. Vesicle protein concentrations were set at 0.2 mg/ml.

Sources of the chemicals were as follows: DDA\(^{+}\) and TPB\(^{−}\) were from K & K Fine Chemicals, gramicidin D and valinomycin were from Calbiochem, and FCCP was from Pierce Chemicals. \(^{22}\)NaCl (carrier-free) and \(^{3}H\)DDA\(^{+}\) (12.7 mCi/mmol) were from New England Nuclear. The fluorescent dye, diO-Cy(3) (3,3′-dipentylxoyxacarbocyanine) was a gift of A. Waggoner, Amherst College.

Results

*Light-Induced pH Changes and Electrical Potential in Envelope Vesicles.* Vesicles, loaded with either 3.0 M KCl, 2.9 M KCl–0.1 M NaCl, or 2.0 M KCl–1.0 M NaCl, were suspended in 2.0 M KCl–0.1 M NaCl and the light-induced pH change in the medium was measured. The pH traces, shown in Figure 1, indicate that a large and steady pH change occurs only in the absence of internal NaCl. With Na\(^{+}\) inside the vesicles the initial pH changes are smaller and are followed by increasing pH changes only after periods of illumination, which are roughly proportional to the concentration of NaCl included (Figure 1B,C). The extra-vesicle salt composition makes only a small difference in the pH traces. When vesicles containing 2.0 M KCl–1.0 M NaCl (as in Figure 1C) are suspended in 3.0 M NaCl (not shown), the light-dependent pH changes are...
similar to Figure 1C, except that the magnitude of ΔpH is smaller and the second increase in the pH change occurs at a somewhat later time. When these vesicles are suspended in 3.0 M KCl (not shown), the second increase in ΔpH occurs at an earlier time. These differences from Figure 1C are as expected if the NaCl-KCl gradients partly equilibrate before illumination. The patterns of light-induced pH changes thus seem to depend largely on the presence or absence of Na⁺ inside the vesicles. The maximal increase in H⁺ concentration on the exterior of the vesicles in Figure 1 is about 50 nequiv/mg of protein, which is many times the bacteriorhodopsin content of the vesicle membrane, about 2.5 nmol/mg (MacDonald and Lanyi, 1975). The external pH changes measured are therefore assumed to reflect transmembrane pH gradients. The light-induced [H⁺] change is greatly increased (up to 130-140 nequiv/mg of protein) when the vesicle interior is strongly buffered, with 5 × 10⁻² M phosphate at pH 6.0.

Light-dependent pH changes were also determined at a constant NaCl-KCl ratio, with 2.9 M KCl-0.1 M NaCl both inside and outside the vesicles, but at varying light intensities. As shown in Figure 2A, at maximal light intensity a brief period of initial small pH change is followed by increased ΔpH, similarly to Figure 1B. At decreased light intensities, Figure 2B-D, the pH changes are progressively smaller, the initial pH decrease is slower, and the onset of the second increase in ΔpH occurs at later times.

Illumination causes the efflux of Na⁺ from H. halobium envelope vesicles, as determined by measuring the amount of ²²Na retained (Lanyi et al., 1976a). The efflux of ²²Na was followed under conditions identical to those in Figure 2 and the results are shown in Figure 3 for light intensities corresponding to Figure 2A,C,D. Comparison of the pH and ²²Na-flux measurements indicates that the onset of the second increase in ΔpH is concurrent with the depletion of Na⁺ inside the vesicles, at <2 min, ca. 6 and 10-12 min after the beginning of illumination for the light intensities shown.

The relationship between the light-induced pH changes and the Na⁺ efflux, i.e., smaller ΔpH while Na⁺ is present inside the vesicles and increasing ΔpH after Na⁺ is depleted, suggests that proton back-flow is increased by an effect of Na⁺ across the membranes. This idea is supported by experiments at higher pHs. When vesicles containing 2.0 M KCl-1.0 M NaCl are suspended in buffer containing 2.0 M KCl-1.0 M NaCl, and the pH is set at increasing values, the light-induced pH changes are progressively distorted, as seen in Figure 4A-D. Under these conditions, the initial acidification of the medium is followed by a more and more rapid rise in pH, resulting in net alkalinization for vesicles at pH > 6.5 (Figure 4C,D). Even in these cases, however, the second decline in external pH results in net acidification. There is no priori reason to expect the increasing rates of H⁺ influx with increasing initial pH and thus it must be a property of the Na⁺-dependent H⁺-translocation system. The light-induced pH response of these cell envelope vesicles and its dependence on pH greatly resemble light-induced pH changes in intact H. halobium cells (Oesterhelt and Stoeckenius, 1973; Oesterhelt, 1975; Bogomolni et al., 1976a; Bakker et al., 1976). Another pH-dependent effect in Figure 4 is the rate of relaxation for the light-induced pH gradient after illumination, which is more and more rapid with increasing pH. This is likely due to the increased cation permeability of these vesicles at higher pH (Lanyi and Hilliker, manuscript in preparation). Vesicles, loaded with 3.0 M KCl and suspended in 3.0 M KCl, cause only light-induced acidification (not shown), similar to Figure 1A, but of progressively lower magnitude with increasing pH. This latter behavior is as previously described for intact H. halobium cells and for vesicles (Bakker et al., 1976; Kanner and Racker, 1975).

The fluorescent cyanine dye, diO-C₅-(3), can be used to estimate light-induced electrical potentials in H. halobium...
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FIGURE 4: Dependence of light-induced pH change on initial pH. Vesicles were loaded with and suspended in 2.0 M KCl-1.0 M NaCl. Vertical bars represent pH changes after addition of 5 n equiv/ml of HCl. Vesicle protein concentration in traces B, C, and D was doubled.

envelope vesicles (Renthal and Lanyi, 1976). Figure 5 shows fluorescence traces obtained with this technique under the same conditions as used in Figure 1. In vesicles loaded with 3.0 M KCl, the light-induced fluorescence decrease is small and its rate of change is rapid, as found before (Renthal and Lanyi, 1976). The inclusion of NaCl in the vesicles greatly increases the fluorescence change, which reaches a maximal value of ~32% for vesicles containing 2.0 M KCl-1.0 M NaCl (Figure 5C). This value is near the saturation limit of the method and corresponds to about 90 mV (interior negative), as calibrated with K⁺-diffusion potentials (Renthal and Lanyi, 1976). At longer times of illumination the fluorescence change declines, concurrently with the increase of the pH change (compare Figure 5C with 1C). The pattern of fluorescence changes is not greatly affected when the vesicles are suspended at pH up to 7.5, except that the decline in the fluorescence change occurs at shorter illumination times.

The lower fluorescence change, and therefore electrical potential, observed at the later stages of illumination, is probably caused indirectly by Na⁺ efflux and the resulting establishment of a large Na⁺ gradient. The size of this gradient cannot be estimated directly, but glutamate transport rates suggest that Na⁺/Na⁺ may become higher than 500 (Lanyi et al., 1976a,b). Such a large chemical gradient of Na⁺ could increase passive Na⁺ influx sufficiently to decrease the net charge imbalance across the membranes. When vesicles containing 2.9 M KCl-0.1 M NaCl are illuminated (Figure 5B), they show only a burst of fluorescence decrease, followed immediately by a rise, again in keeping with the pH changes under these conditions (Figure 1B). The oscillation that ensues during the rise of fluorescence is reproducible, with 2 or 3 damped cycles observed. The oscillatory behavior is probably a result of the interaction of the active (uphill) Na⁺ efflux with the passive Na⁺ influx, since these two processes are self-limiting and mutually enhancing through their effect on the internal Na⁺ concentration.

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If the light-induced pH changes are related to the Na+ concentration inside the vesicles, as it appears from the experiments above, then the pH response to a second illumination should reflect the equilibration of the Na+ gradient during the intervening dark time. In Figure 6, pH traces are shown for vesicles containing 2.0 M KCl-1.0 M NaCl, inside and outside, from a first illumination of 23 min, followed by a 5-min dark time and a second 10-min illumination (trace A). The biphasic pH change during the first illumination, typical of vesicles containing high NaCl concentrations (Figure 1C and Figure 4A), is replaced on the second illumination by a pH pattern characteristic of Na+-depleted vesicles (as in Figure 1A). Increasing the dark time before the second illumination up to 5 h (Figure 6B–D) results in a gradual and partial return of the initial biphasic pH pattern. The long dark incubations required are consistent with the slow exchange of cations across the *H. halobium* vesicle membrane (half-life of internal K+, retained against a several-hundred-fold K+ gradient, is about 5 h at 30°C; Lanyi and Hilliker, manuscript in preparation).

Effects of FCCP and Valinomycin on Light-Induced pH Changes and Electrical Potential. Vesicles, loaded with 2.0 M KCl-1.0 M NaCl and suspended at the same KCl-NaCl concentration, were illuminated in the presence of the uncoupler, FCCP. The pH traces obtained (not shown) are similar to Figure 1C, but with increasing FCCP concentrations, from $6 \times 10^{-7}$ to $3 \times 10^{-6}$ M, the second increase in $\Delta \text{pH}$ occurs at increasingly longer times and the overall magnitude of $\Delta \text{pH}$ decreases. Qualitatively, the second increase in $\Delta \text{pH}$ seems to be more sensitive to FCCP than the pH gradient itself. Valinomycin, at concentrations between $8 \times 10^{-7}$ and $5 \times 10^{-6}$ M, also causes increasing delays in the Na+-dependent second increase of $\Delta \text{pH}$ (not shown), but the total magnitude of the light-induced pH change increases with increasing valinomycin concentration, as observed before (Renthal and Lanyi, 1976). Table I shows net [*H]* accumulation by fluorescence change (Figure 5C). Also, in the absence of NaCl in the vesicles, no [*H]* accumulation is seen (not shown), even though there is a small electrical potential (Figure 5A). Adding valinomycin will abolish [*H]* uptake (Figure 7), consistent with the fluorescence data (Figure 5D).

Discussion

*H. halobium* envelope vesicles, suspended in NaCl or in KCl solutions, exhibit simple kinetics for light-dependent pH changes in pH and electrical potential (Renthal and Lanyi, 1976), which appear consistent with the role of bacteriorhodopsin as a light-energized proton pump (Oesterhelt and Stoeckenius, 1973; Oesterhelt and Hess, 1973). When both NaCl and KCl are present, the observed changes in pH and potential are quite complex and must reflect secondary events, which follow the proton translocation. Since the anomalous kinetics during illumination are closely related to the active transport of Na+ out of the vesicles, they provide information about the mechanism and energetics of the Na+ efflux.

Earlier evidence suggested that the Na+ gradients arise by electrogenic antiport of Na+ with another cation or by electrogenic symport with an anion (Lanyi et al., 1976a). Thus, Na+ can move uphill, against its electrical and chemical gradient, while the other ion moves downhill. The possibilities were: antiport with K+ or H+, or symport with Cl- or OH~.

![Figure 6: pH changes during a second illumination of *H. halobium* envelope vesicles.](image)

**Figure 6:** pH changes during a second illumination of *H. halobium* envelope vesicles. pH calibration as in Figure 1. Vesicles were loaded with and suspended in 2.0 M KCl-1.0 M NaCl. An initial 23-min illumination (shown only for trace A) was followed by incubation in the dark and a second illumination. Dark times between illuminations: trace A, 5 min; trace B, 80 min; trace C, 160 min; trace D, 300 min.

### TABLE I: Effect of Valinomycin on the Light-Induced $\Delta [\text{H}^+]$ in *H. halobium* Envelope Vesicles.

<table>
<thead>
<tr>
<th>Salt Concentration</th>
<th>No Addition</th>
<th>Valinomycin (5 $\times 10^{-6}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 M KCl-1.0 M NaCl</td>
<td>$69 \pm 5^a$</td>
<td>$133 \pm 5$</td>
</tr>
<tr>
<td>2.9 M KCl-0.1 M NaCl</td>
<td>$104 \pm 7$</td>
<td>$147 \pm 5$</td>
</tr>
<tr>
<td>3.0 M KCl</td>
<td>$132 \pm 5$</td>
<td>$139 \pm 5$</td>
</tr>
</tbody>
</table>

*a* Salt composition same inside and outside the vesicles. Internal phosphate concentration $5 \times 10^{-2}$ M, external $2 \times 10^{-3}$ M, pH set at 6.0. Vesicles incubated 10 min with valinomycin. $^a$ Means and standard deviations from triplicates.

**Renthal and Lanyi, manuscript in preparation.**
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mechanism of Na+ efflux involved K+ or Cl−, the Na+ flux would result in lowered net membrane potentials and increased ΔpH. The results obtained are the opposite: in the presence of Na+ inside the vesicles the initial light-induced H+ influx is followed by H+ influx, decreasing the ΔpH, or even reversing its sign until the intravesicle Na+ content is depleted, consistent with Mitchell's analysis of the action of an electrogenic H+-cation antiporter (Mitchell, 1969). In qualitative terms, the reversal of the pH gradient is the result of the fact that when the H+ pump is not very active (such as at high pH), it can keep up with the positive charge influx (H+ minus Na+) but not with the H+ ions which return via the antiporter. Thus, under these special conditions, the interior potential can stay negative despite a net influx of H+.

Developing a quantitative model for the light-dependent events in _H. halobium_ envelope vesicles is not yet possible, since it would require knowledge of (1) the passive permeabilities of H+, Na+, and K+, (2) the stoichiometry and efficiency of the proposed H+–Na+ antiporter, and (3) the probable control of H+ translocation by bacteriorhodopsin through changes in internal pH. What follows is a qualitative model, based on our present understanding of the system. As depicted in Figure 8, light absorption by bacteriorhodopsin causes H+ extrusion from the vesicles, establishing a pH difference (interior alkaline) and an electrical potential (interior negative), which are limited by the passive permeabilities of H+, Na+, and K+. The light-dependent protonotive force drives the influx of Na+ through the electrogenic H+/Na+ antiporter, causing an influx of H+ which depends on the presence of Na+ inside the vesicles. At the beginning of the illumination, methionine, leucine, and other amino acids, which are transported in an electrogenic fashion (MacDonald and Lanyi, 1975), can be actively transported, but not glutamate, which depends entirely on Na+ gradients (Lanyi et al., 1976a,b). The rapid Na+ efflux during illumination results in the depletion of Na+ inside the vesicles, giving rise to Na+ gradients >500, as suggested by the rate of glutamate transport (Lanyi et al., 1976b). This large Na+ gradient can provide additional driving force to Na+-dependent amino acid transport systems, such as that of leucine, as well as energize glutamate uptake. On the other hand, passive Na+ influx now begins to contribute to the net charge flux across the membranes and results in decreased membrane potential. Large-scale movement of ions across membranes must, of course, be approximately electrically neutral and the rapid depletion of the vesicles of Na+ (Figure 3) could take place only if other ions are also displaced. Since in the absence of K+ the Na+-dependent anomalies in ΔpH and electrical are not observed (Renthal and Lanyi, 1976), the Na+ influx probably occurs at the expense of K+ influx. K+ is known to be accumulated by _H. halobium_ vesicles during illumination (Kanner and Racker, 1975).

Net influx of H+ during the early part of illumination, followed by net efflux, such as is now found in envelope vesicles (Figure 4), has been observed in intact _H. halobium_ cells (Oesterhelt and Stoeckenius, 1973; Oesterhelt, 1975; Wagner, 1976; Bogomolni et al., 1976; Bakker et al., 1976). Because of kinetic similarities between the H+ influx and light-induced ATP synthesis, and because of the DCCD sensitivity of both, the pH rise was interpreted to reflect the consumption of protons during ATP synthesis (Oesterhelt, 1975) or proton influx associated with ATP synthesis (Bogomolni et al., 1976). We suggest that the results with intact cells can be at least partly explained by Na+-dependent H+ influx.

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Hummel–Dreyer Gel Chromatographic Procedure as Applied to Ligand-Mediated Association†

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ABSTRACT: A mass transport theory of the gel chromatographic procedure of Hummel and Dreyer for measuring the binding of small ligand molecules to macromolecules has been formulated for ligand-mediated macromolecular association. It is concluded that the Hummel–Dreyer procedure is the method of choice for quantitating ligand binding in such systems. Guidelines are drawn for unambiguous interpretation of the nonclassical elution profiles in terms of the number of binding sites on the macromonomer and their intrinsic binding constant. Most of these guidelines also apply to static equilibrium methods such as equilibrium dialysis.

Hummel and Dreyer (1962) introduced the following gel chromatographic procedure for quantitating the binding of a small ligand molecule, X, by a protein or other macromolecule, M. After equilibration of a column of Sephadex (G-25 or other grade which excludes M) with X, M dissolved in the solution used to equilibrate the column is applied. The column is then eluted with the same solution as used for equilibration, and the effluent is analyzed for X. As the band of M moves down the column in the exterior mobile phase, it removes X from the solution within the gel until the binding equilibrium is satisfied by the concentration of unbound X ahead of M. Subsequently, M and its complexes with X emerge from the column at its void volume to give a peak in the elution profile of X, followed at some point by a trough. The amount of X removed from solution by the protein as manifested by the trough is equal to the excess X in the peak. The mean number of mol of X bound/mol of M, ν, can be calculated using the area of the trough or the void volume peak depending, in practice, upon how the effluent is monitored for X; if by ultraviolet spectrophotometry (Fairclough and Fruton, 1966) the area of the trough must be used, since the protein contributes to the absorbance of the void volume fractions; if by determination of radioactivity (Levi et al., 1974), the area of the peak can be