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Light-Driven Primary Sodium Ion Transport in Halobacterium halobium Membranes

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Light-induced sodium extrusion from H halobium cell envelope vesicles proceeds largely through an uncoupler-sensitive pathway involving bacteriorhodopsin and a proton/sodium antiporter. Vesicles from bacteriorhodopsin-negative strains also extrude sodium ions during illumination, but this transport is not sensitive to uncouplers and has been proposed to involve a light-energized primary sodium pump. Proton uptake in such vesicles is passive, and under steady-state illumination the large electrical potential (negative inside) is just balanced by a pH difference (acid inside), so that the proton-motive force is near zero. Action spectra indicated that this effect of illumination is attributable to a pigment absorbing near 585 nm (cf 568 for bacteriorhodopsin). Bleaching of the vesicles by prolonged illumination with hydroxylamine results in inactivation of the transport; retinal addition causes partial return of the activity. Retinal addition also causes the appearance of an absorption peak at 588 nm, while the absorption of free retinal decreases. The 588 nm pigment is present in very small quantities (0.13 nmole/mg protein), and behaves differently from bacteriorhodopsin in a number of respects. Vesicles can be prepared from bacteriorhodopsin-containing H halobium strains in which primary transport for both protons and sodium can be observed. Both pumps appear to cause the outward transport of the cations. The observations indicate the existence of a second retinal protein, in addition to bacteriorhodopsin, in H halobium, which is associated with primary sodium translocation. The initial proton uptake normally observed during illumination of whole H halobium cells may therefore be a passive flux in response to the primary sodium extrusion.

Key words: H halobium, sodium transport, retinal protein, light-energy transduction

Much of the generation and transmission of metabolically useful energy takes place in and across membranes. According to the chemiosmotic hypothesis [1–3], the barrier properties of membranes allow the spatial separation of reactants and products in enzyme-catalyzed processes, and thereby the conservation and utilization of free energy normally lost in a homogeneous phase becomes possible. Those membrane components that will

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conserve energy in vectorial form – eg, phosphate bond energy in sodium/potassium gradients across membranes (Na^+/K^+ ATPase) or light-energy in proton gradients (bacteriorhodopsin) – were termed "pumps." It is now generally accepted that energy-requiring membrane reactions will function at the expense of the chemical and electrical gradients created by these pumps between the phases separated by cellular or subcellular closed membrane systems. Examples for such reactions include not only the active accumulation and excretion of metabolites but also ATP synthesis, signal propagation in excitable cells, and the functioning of synaptic junctions.

Most of the pumps studied are multicomponent systems consisting of either distinct interacting membrane proteins and redox pigments or protein aggregates with complex subunit architecture. The retinal protein, bacteriorhodopsin [4, 5], in H halobium membranes represents one of the exceptions to this rule. This proton pump, which utilizes light-energy, consists of a single polypeptide chain of 248 amino acids [6] arranged in seven nearly completely helical segments looped across the width of the membrane [7]. The retinal is bound via a Schiff-base linkage to lysine 41 in the protein, and it appears that this relatively simple structure contains all that is required for the light-driven proton translocation [5]. It is of great importance for investigations of this system, however, that bacteriorhodopsin is normally part of a more complex structure and is found in two-dimensional crystalline arrays containing $\sim 10^5$ molecules [8]. Such a unique organization for a membrane protein has not only permitted the easy isolation of membranes containing exclusively bacteriorhodopsin but also made possible revealing X-ray, electron, and neutron diffraction studies [7, 8–11]. Recently the amino acid sequence of bacteriorhodopsin has become available [6], and since the three-dimensional crystallization of the protein has also been achieved [12], it is likely that within a few years the structure of bacteriorhodopsin will be known to the same degree of resolution as for many soluble proteins.

In contrast, the mechanism of function in bacteriorhodopsin is not well understood at present. Flash spectroscopic studies [5] have shown that the chromophore (570 nm) is converted after a few picoseconds of absorbing a photon into another (K) form, which decays within 10 msec at room temperature through a series of other spectral intermediates (L, M, N, and O). During this time a proton is released on the side of the membrane facing the cell exterior, followed by proton uptake on the cytoplasmic side. It has been proposed that the reversible protonation of the retinal Schiff-base is the central event in proton translocation [5, 13]. Protons to and from this group will be supplied by proton conduction either via an ice-like matrix in the protein [14] by an Onsager mechanism or, less likely, via an aqueous channel. Initiation of the proton migration after absorption of a photon by the retinal may be induced by charge delocalization along the retinyl chain or by configurational change of the retinal relative to other groups in the protein. The reversible protonation of a tyrosine observed during the photocycle [15, 16], the pH dependence of the photointermediates [17], as well as a distinct pK for light-induced proton release [18] suggest that additional donor and acceptor groups exist for protons other than the Schiff-base.

Bacteriorhodopsin will transport protons against an electrochemical potential difference up to at least 300 mV [19], and continuous illumination of intact H halobium cells, cell envelope vesicles, and liposomes reconstituted with purified bacteriorhodopsin will result in the development of a pH difference and/or electrical potential across the membranes. The energy conserved in this gradient of protons has been thought to drive ATP synthesis via a proton-translocating ATPase [20–23], to generate a sodium gradient via a sodium/proton antiport system [24, 25], and indirectly through the gradient of sodium to drive

various sodium/amino acid symport systems [26–28]. Thus, bacteriorhodopsin represents a source of metabolically useful energy, alternative to the respiratory chain in H halobium. Understanding the structure and mechanism of this proton pump has and will continue to contribute significantly to the study of membraneous pumps in general.

Recently the observation of light-driven sodium transport independent of proton gradients in vesicles prepared from various H halobium strains prompted Lindley and MacDonald to propose the existence of a light-driven sodium pump in these membranes [29]. The significance of this possible second light-energy converter in H halobium is far-reaching, both for the photophysiology of the organism and for the mechanisms by which light-driven ionic pumps will function. This review is concerned with evidence now available in favor of the existence of such a sodium pump in H halobium, with the spectroscopic properties of a retinal pigment associated with the sodium translocation, and with the consequences of primary sodium transport for the physiology of H halobium cells. Structural and functional comparisons between the two retinal pigments of H halobium would be highly interesting, but such studies will require the isolation of the sodium pump pigment.

EVIDENCE FOR PRIMARY SODIUM TRANSPORT IN ILLUMINATED H HALOBIUM VESICLES

Lindley and MacDonald found [29] that membrane vesicles from a bacteriorhodopsin-deficient, red-pigmented strain of H halobium described by Mukohata and co-workers [30, 31] lost ^{22}Na during illumination 6–7 times more rapidly than in the dark. The sodium transport was insensitive to the proton conductor, 1799. Membrane potential (negative inside) during illumination was calculated from radioactively labeled triphenylmethylphosphonium ion* uptake, measured by the flow dialysis method. A membrane potential of about −90 mV developed, which could be abolished with valinomycin and K^+ but not with proton conductors. From these results Lindley and MacDonald argued that, in this system, the membrane potential was caused by the extrusion of sodium ions rather than protons. Protons, in fact, were not extruded but were taken up by the vesicles during the illumination, as detected by pH increase in the medium and labeled N-methylmorpholine accumulation. The proton uptake was dependent on the presence of sodium and was enhanced by uncouplers and diminished by valinomycin and K^+. Proton movements thus appeared to be secondary, and driven by the electrical potential created by sodium extrusion.

MacDonald and co-workers also obtained results consistent with a light-driven sodium pump in vesicles prepared from the bacteriorhodopsin-containing H halobium R-1 strain [32]. Unlike previously studied vesicles, which sediment at 35,000g [26, 27] and release protons during illumination [26, 33], vesicles collected at much higher centrifugal forces showed light-induced proton uptake. The pH rise in the medium during illumination was enhanced by uncouplers and inhibited by gramicidin or valimycin and K^+. A number of control experiments were performed to ascertain that the vesicle preparations were homogeneous and that the various ionophores used functioned in the expected manner. Thus, the uptake of radioactively labeled acetate and N-methylmorpholine by the vesicles was determined simultaneously. The results showed that contamination of the preparations with acetate-accumulating (and therefore proton-extruding) vesicles was less than 10%, a level easily detectable when such vesicles were added. The fluorescent dyes ANS^- and

*Abbreviations: $TPMP^+$ = triphenylmethylphosphonium ion; ANS^- = 8-anilino-1-naphthalene sulfonic anion; $diO\text{-}C_5$ = 3,3′-dipentyloxadicarbocyanine.

diO-C_5 were used to measure light-induced membrane potentials, positive and negative inside, respectively. Fluorescence quenching for ANS^- was calibrated with potassium diffusion potentials, but no positive inside membrane potential was detectable in the vesicles during illumination. Negative inside electrical potential was detectable with diO-C_5, as well as with labeled $TPMP^+$ uptake. The authors concluded [32] that vesicles that would extrude or actively accumulate protons did not contribute significantly to the light-dependent effects observed. Proton uptake, similar to that induced by illumination, could be demonstrated in the dark, however, when an uncoupler was added after the imposition of a potassium-diffusion potential (negative inside).

As expected from the hypothesis of a sodium pump in these membranes, the development of a light-driven membrane potential was shown to be dependent on the presence of sodium ions. Vesicles extensively washed with KCl exhibited pH drop instead of rise during illumination [32], and the membrane potential was much diminished. Adding NaCl and incubation restored the original light-dependent pH rise and high membrane potential.

We have also confirmed the existence of a primary sodium pump in membrane vesicles prepared from various H halobium strains, particularly from those lacking bacteriorhodopsin. Electrical and pH gradients were quantitatively measured during illumination in parallel experiments with the fluorescent dyes, diO-C_5 and 9-aminoacridine, respectively [34]. Vesicles from a bacteriorhodopsin-deficient red strain [30, 31] developed −140 to −150 mV inside negative electrical potentials upon illumination, balanced by acid inside pH difference, so as to make the net protonmotive force in the steady state near zero. This result is consistent with the absence of active transport for protons. Uncouplers had no effect on the magnitude of these gradients, but they accelerated proton uptake. Slowly sedimenting vesicles from the bacteriorhodopsin-containing R-1 strain developed inside negative membrane potentials upon illumination, which was about −100 mV in excess of the opposing pH gradient. With uncoupler present, the light-induced electrical potential decreased and the pH gradient increased, and the net protonmotive force approached zero. We suggested, therefore, that in these vesicles pumps are present for both protons (bacteriorhodopsin) and sodium ions (the proposed sodium pump), and that these both function in the outward direction [34]. Presumably, this orientation is the same in the cytoplasmic membrane of intact H halobium cells as well. The two pumps must have different pH optima, as at pH below 4.5 proton extrusion was observed, whereas above this value uptake predominated [34]. Indeed, illumination of bacteriorhodopsin-containing envelope vesicles has been known to create much larger pH difference at pH below 6 [35], and sodium transport and membrane potential in the bacteriorhodopsin-deficient vesicles showed a distinct optimum near pH 7.5–8.0 [34]. MacDonald and co-workers reported, however, that in slowly sedimenting vesicles from the R-1 strain the sodium efflux rate was constant between pH 5.0 and 8.0 [32]. In these vesicles sodium extrusion in the lower pH range may take place via the bacteriorhodopsin-sodium/proton antiporter pathway described before [24, 25].

The passive uptake of protons during illumination was virtually abolished by treatment of the vesicles with 10 μM dicyclohexylcarbodiimide (DCCD), an agent that inhibits the Mg-dependent ATPase in this organism [20–22], and should block the proton-conducting channel. Uncouplers restored the light-induced proton uptake in DCCD-treated vesicles, while the membrane potential was unaffected by these treatments. It would appear, therefore, that proton uptake in these vesicles normally takes place through the F_O channel of the ATPase, but sodium extrusion does not involve this component. However, treatment with large excess of DCCD (100 μM) will abolish sodium transport as well.

SPECTROSCOPIC AND MOLECULAR PROPERTIES OF A PIGMENT ASSOCIATED WITH THE LIGHT-DRIVEN SODIUM PUMP

While MacDonald's observations, together with our results, discussed above, strongly suggest the existence of a light-driven sodium pump in H halobium membranes, the presence of a pigment associated with this pump and its relationship to bacteriorhodopsin had still to be established by spectroscopic studies. We obtained action spectra for the creation of electrical and pH gradients during illumination [34], which exhibited maxima at 585–590 nm, different from the 568 nm absorption band of bacteriorhodopsin.

For more accurate spectroscopic work, interference from the red carotenoids normally present in H halobium was eliminated by the use of a "colorless" strain isolated by H.J. Weber, labeled ET-15. This strain lacks bacteriorhodopsin as well, to a detection limit of a few molecules per cell (Weber and Bogomolni, personal communication), but shows all the light-dependent proton movements that had suggested the existence of a sodium pump. We found [36] that difference spectra between vesicles prepared from the ET-15 strain and vesicles from a retinal-deficient, light-unresponsive H halobium strain contained an absorption band near 590 nm. The amplitude of this band was very low, amounting to about 0.006 OD units/mg membrane protein.

A more useful method for obtaining spectroscopic information about the pigment proved to be bleaching and reconstitution with added retinal. These experiments were patterned after methods worked out for bacteriorhodopsin. The latter is known to be bleached after prolonged (3–6 h) illumination in the presence of hydroxylamine [37, 38], a treatment that cleaves the lysine Schiff-base to form the retinal oxime. After removal of excess hydroxylamine, the chromophore absorption band of bacteriorhodopsin is quantitatively restored with added retinal. Difference spectra of bleached H halobium ET-15 vesicles, with and without added trans-retinal, exhibited a new 588 nm absorption band, which grew during a 2–3-h incubation time at the expense of the 385 nm absorption band of free retinal. With unbleached, but hydroxylamine-treated vesicles, the retinal band remained undiminished and the 588 nm band did not arise [36]. This result confirmed the existence of a retinal protein absorbing near 590 nm, predicted from the earlier action spectra [34], and is shown in Figure 1. From such experiments an approximate extinction coefficient of 48,000 M^{-1} cm^{-1} (retinal equivalents) was calculated for the pigment. For bacteriorhodopsin the corresponding number is 63,000 M^{-1} cm^{-1} [39]. Using this value, the amount of 588 nm pigment in ET-15 membranes was estimated to be 0.13 nmole/mg protein, or about 5% of the bacteriorhodopsin content of typical vesicle preparations from the R-1 strain.

Partial bleaching of ET-15 vesicles followed by retinal reconstitution, as well as measurement of absorbance changes at 588 nm and light-induced proton uptake, established a linear relationship between spectroscopic and transport properties of the 588 nm pigment. This finding, and the agreement between the action spectra obtained earlier and the absorption spectra, strongly suggests that the 588 nm absorption band detected is associated with the sodium pump. Absolute absorption spectra of bleached and reconstituted ET-15 membranes, free of light-scattering effects, exhibited no particularly strong absorption bands near 590 nm, besides the 588 nm pigment, as shown in Figure 2.

The absorption spectrum of bacteriorhodopsin in purple membrane patches of envelope vesicles does not change in 4 M NaCl upon addition of alkali, up to pH 11. In contrast, we found that the 588 nm pigment absorption band reversibly shifted to 548 nm and decreased in magnitude at a midpoint pH of about 9.6 [36]. It is interesting to note

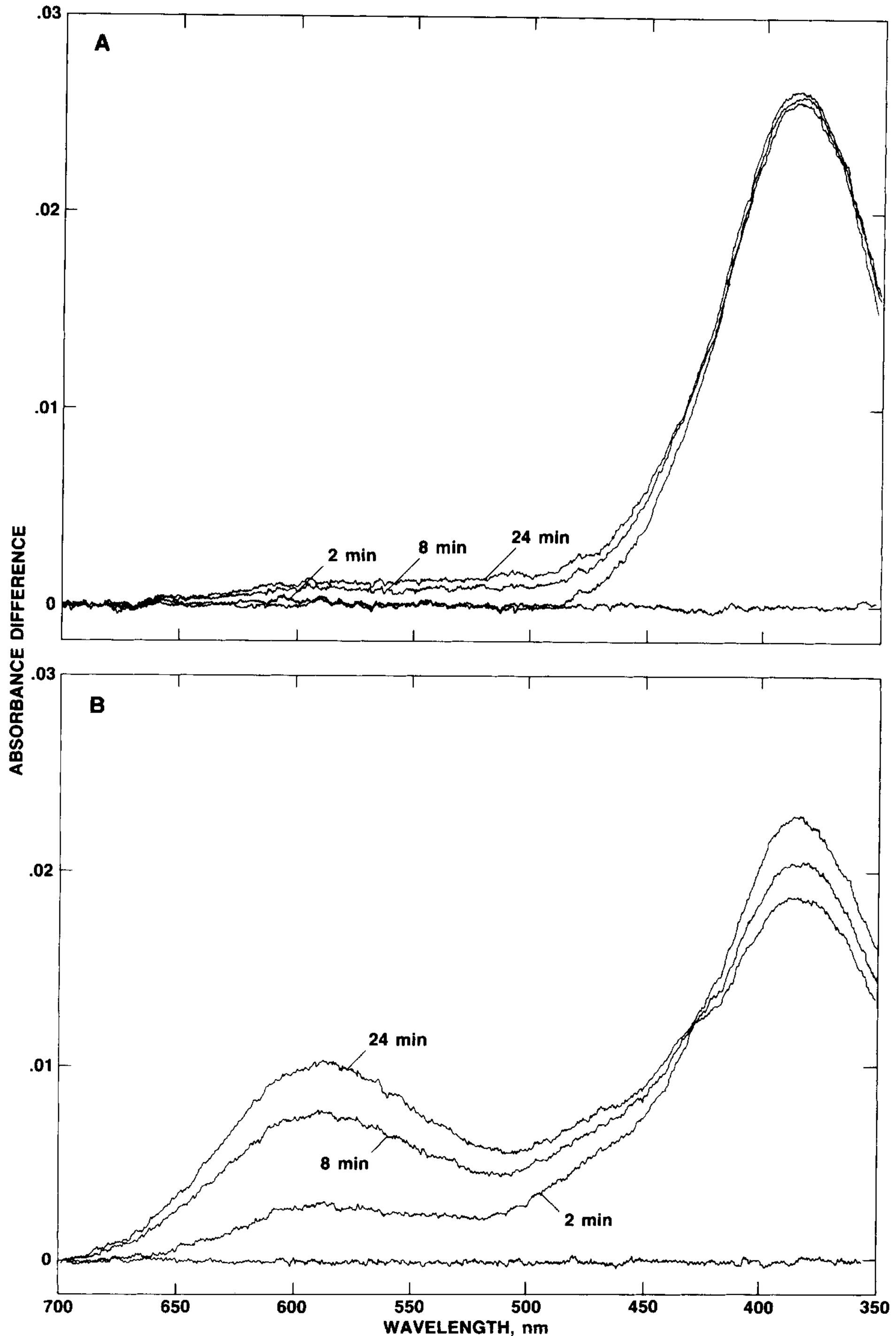

Fig. 1. Retinal reconstitution of the 588 nm pigment in bleached H halobium ET-15 membranes. A. Vesicles treated with 0.2 M hydroxylamine, but not bleached by illumination. B. Vesicles bleached by illuminating 15½ h in the presence of hydroxylamine. The washed membrane suspensions were divided into two portions, and trans-retinal in methanol (0.15 μM final concentration) was added to one of these. Difference spectra between this and the other (reference) portion were determined at the times indicated after retinal addition. Reprinted with permission from Lanyi and Weber, J Biol Chem 255:243, 1980.

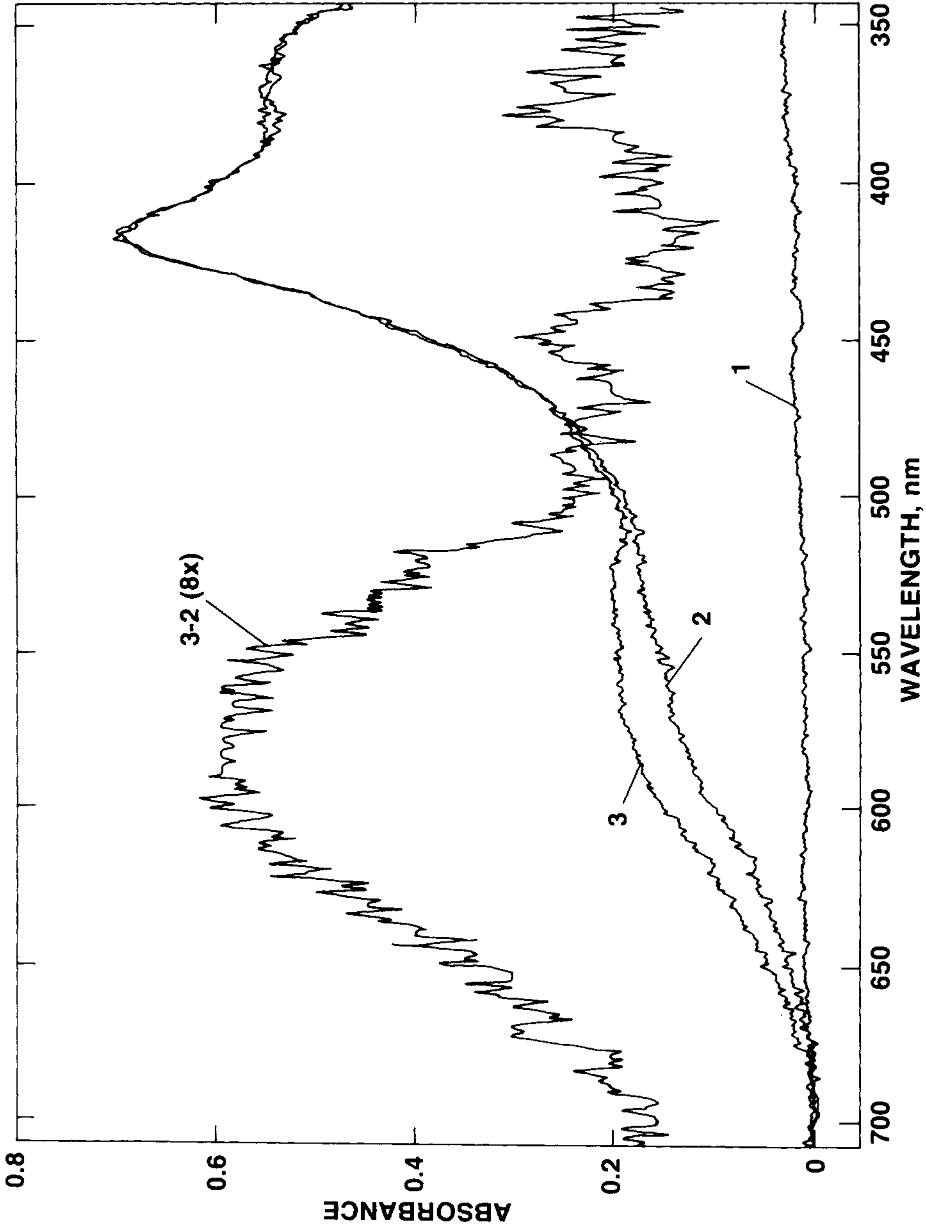

Fig. 2. Absolute absorption spectra of bleached (2) and retinal-reconstituted (3) H halobium ET-15 membranes. Spectra were obtained with a light-integrating sphere, as described elsewhere [36]. Conditions: 10 mg/ml protein in 4 M NaCl, retinal added to 1.0 nmole/ml. The difference between spectra 3 and 2 shows the absorption band of the reconstituted pigment near 590 nm.

that monomeric bacteriorhodopsin (produced by incorporating purified purple membrane into sonicated liposomes in 4 M NaCl) also shows a blue shift on alkalinization, but the shift is smaller: from 562 to 548 nm (Lanyi, unpublished observations).

Bacteriorhodopsin is heat-stable at 75°C. After bleached bacteriorhodopsin-containing envelope vesicles are heated for 5 min, retinal reconstitution of the chromophore (and of proton transport activity) will proceed normally. In contrast, the reconstitution of bleached ET-15 vesicles is virtually completely abolished by heating, as is sodium transport in the unbleached vesicles [34, 36].

The above results indicate that a retinal protein, with an absorption band at 588 nm, is implicated in the light-driven sodium pump. While bacteriorhodopsin and the 588 nm pigment appear to be quite similar, they show obvious differences in transport function, spectroscopic properties, and stability. It is tempting to speculate that the two pumps, one for protons and one for sodium ions, might function analogously, but with different cation specificities. However, given the proposed central role of the protonated Schiff-base in the transport by bacteriorhodopsin, it would be difficult to envisage analogous mechanisms for proton and sodium translocation. Without yet excluding this possibility, it seems wise to consider more complex models for the sodium pump, which contain a bacteriorhodopsin-like proton translocator intimately coupled to a sodium/proton exchanger. Little direct information is available to decide in favor of such a model, but we have recently found that the transport activity of the sodium pump is abolished by phenylmercuric ion (at a half-maximal concentration of 50 μM) but is reactivated by mercaptoethanol This observation, as well as one recently reported by Matsumo-Yagi and Mukohata [31] on the inhibition of secondary proton movements by triphenyl tin in bacteriorhodopsin-negative H halobium, implicate sulfhydryl group(s) in the functioning of the sodium pump. If the retinal protein complex in this pigment is similar to bacteriorhodopsin in that it lacks cysteine, this result would suggest the presence of at least one other functional component in the sodium pump.

The rigidity of the structure of bacteriorhodopsin [7–10], and the poor accessibility of many reagents to its active groups buried inside the membrane, have posed difficulties in the study of this pump. We expect the sodium pump to be more amenable to disruption and chemical modification, since, unlike bacteriorhodopsin, it will become inactivated when heated or exposed to low salt conditions, and it shows reversible spectral changes at moderately alkaline pH. These observations indicate that the sodium pump has a more labile structure, and they suggest, but do not prove, that the pump is not located in a crystalline array, as is bacteriorhodopsin. As expected if this is so, the former pigment is found in a band together with the majority of membrane fragments on a sucrose gradient formed without salts, well above the band of the heavier purple membrane patches (Oesterhelt and Lanyi, unpublished observations).

SIGNIFICANCE OF LIGHT-DRIVEN PRIMARY SODIUM EXTRUSION FOR THE H HALOBIUM CELLS

Since another mechanism for active sodium extrusion in H halobium – ie, proton extrusion by bacteriorhodopsin plus sodium/proton antiport – has been described [24, 25], the physiological significance of the light-driven sodium pump is not clear. Various lines of evidence suggest that the larger part of sodium transport in these cells is energized by the electrochemical gradient of protons. We have found that after vesicles containing both sodium transport systems were heated at 75°C the sodium extrusion during illumi-

nation was not significantly lowered, even though the sodium pump under these conditions is inactivated. In vesicles prepared from a retinal-negative H halobium strain no light-driven reactions were observed, and retinal addition to the purified membrane preparation reconstituted the proton pump but not the sodium pump. This is presumably because the apoprotein for the sodium pump had not been synthesized in the absence of retinal, whereas bacterio-opsin is present, although in decreased amounts. Vesicles of this kind, when reconstituted with retinal, extruded sodium ions during illumination at rates similar to those in vesicles from the normal R-1 strain (Luisi, Lanyi, and Weber, manuscript in preparation). The sodium transport was virtually completely inhibited by proton conductors. Oxidation of the nonphysiological electron donor, dimethylphenylene diamine, in the dark also caused the rapid transport of sodium in all vesicles tested, including those not reconstituted with retinal. Thus, proton-gradient-dependent sodium transport accounted for most of the sodium extrusion in these membranes. Consistent with these results, we found that the relative rates of sodium transport via the two systems described are quite different: transport attributed to the sodium pump is at least ten times slower than that associated with the sodium/proton antiporter. MacDonald and co-workers reported higher activity for the sodium pump, however, [32].

In spite of the demonstrably small quantity of sodium pump pigment in H halobium membranes compared to the amount of bacteriorhodopsin normally present, large transmembrane electrical potentials will be produced by the light-driven sodium extrusion, as expected from the low permeability [40] of the membranes to this cation. An early report by Matsuno-Yagi and Mukohata [30] showed that ATP synthesis could be induced by illumination even in a bacteriorhodopsin-deficient H halobium strain, and the ATP synthesis was associated with monophasic proton influx. It now appears that this observation can be explained by light-induced electrical potential attributable to the sodium pump. Rough calculations indicate that the rate of sodium extrusion under these conditions should support the proton uptake rate observed. Matsuno-Yagi and Mukohata could distinguish between two light-dependent processes in different H halobium strains: 1) proton extrusion, sensitive to prior bleaching of the cells in the presence of hydroxylamine but not to heating at 75°C, and 2) proton uptake, much less sensitive to bleaching but abolished by heat treatment. The presence of both of these processes (reflecting active and passive proton movements) in the normal R-1 strain, as well as the findings with slowly sedimenting vesicles from this strain [32, 34], suggest to us that H halobium normally contains both proton and sodium pumps. In support of this are our observations that in cells of the bacteriorhodopsin-deficient strain light-induced proton uptake is enhanced up to 5–6-fold when proton conductors are added, but it is considerably inhibited when the membrane-permeant cation $TPMP^+$ is present, and thereby the electrical potential is lowered. Proton influx induced by illumination therefore must be passive, whereas efflux is active. This explanation differs from previous interpretations of the complex pH changes observed during the illumination of R-1 cells [41], which separate proton influx from efflux but attribute both to the operation of the proton pump. Since the proton influx has been related to ATP synthesis [20–23], it follows from the hypothesis of the sodium pump that at least part of ATP synthesis is energized by the increased electrical potential due to sodium exit.

Matsuno-Yagi and Mukohata have recently reported further results with their bacteriorhodopsin-deficient strain [31], which confirm these ideas. They found that growing these cells with 3 mM nicotine resulted in loss of both light-induced effects: proton uptake and ATP synthesis. Addition of trans-retinal to the cells caused the return of both of these functions, in a parallel manner over a 20-min incubation period.

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