Light Energy Conversion in *Halobacterium halobium*

JANOS K. LANYI

Extraterrestrial Research Division, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, California 94035

**INTRODUCTION**

The genus *Halobacterium* includes a number of very similar species, *H. halobium*, *H. cutirubrum*, *H. salinarum*, *H. marismortui* (47, 110, 111), and *H. saccharovorum* (176). They are rod-shaped organisms, about 0.5 by 5 μm, motile, and contain a distinctive red pigment, bacterioruberin (85, 91). The unique characteristic of halobacteria is their habitat: highly concentrated or saturated saline solutions, such as the Dead Sea, the Great Salt Lake, and solar-evaporative ponds. The halobacteria have adapted to such an environment by accumulating salt intracellularly to at least the exterior concentration (28, 29, 52, 107). Growth is optimal between 3.5 and 5 M salt, which must be NaCl, although KCl is also required (53, 54). The potassium is concentrated by the cells and constitutes the major intracellular cation.

Lowering the NaCl concentration below 3 M is catastrophic for the cells, which then become round and lyse (1), and for various cellular components, which lose structural and functional properties. Activity for most enzymes is maximal above 2 M salt, and for some enzymes there is an absolute requirement for salt (for a review, see reference 100). All enzymes that have been investigated appear to be unstable below 1 M salt and some become inactivated in seconds. Some of these enzymes are reactivated when the salt is added back. The structural changes which must accompany such functional alterations have not been extensively investigated. Ribosomes are known to dissociate (5), and the cell envelopes have been found to lose proteins (98, 99, 146) and become fragmented (98, 170) at lowered salt concentrations.

Clues to the molecular bases of these salt-dependent phenomena have come from indirect sources. The presence of large excesses of acidic amino acids in proteins from halophiles suggested to Baxter (4), Brown (21), and others (89, 90) that the salt screens negative charges and is therefore needed to overcome electrostatic repulsion in these structures. Lanyi and Stevenson (108) pointed out that this explanation does not account for the anion specificities observed and for the requirement of several-molar salt concentrations, much higher than what ought to be sufficient for charge screening. Having explored the effects of various denaturants on reduced nicotinamide adenine dinucleotide (NADH)-menadione reductase activity, a membrane-bound enzyme, the latter authors proposed that NaCl or KCl above 1 M strengthens, by a salting-out effect, those hydrophobic interactions which are necessary for the native structure. This hypothesis was later extended to account for results with various other enzymes from halophilic bacteria (110, 113, 114). The effect of salt or the biochemistry of the halobacteria has been recently reviewed (40; S. T. Bayley and R. J. Morton, Crit. Rev. Microbiol., in press).

Unlike most bacteria, the halobacteria lack a rigid cell wall. The outside surface of the cytoplasmic membrane is covered with a hexagonal array of particles, 15 to 20 nm in diameter (170), composed of a glycoprotein (127, 129). The cytoplasmic membrane of the cells contains about 30% lipids, and the diverse variety of proteins found (22, 130) includes the usual complement of flavoproteins (70, 71) and cytochromes (25, 26, 95) of bacterial membranes. The lipids in these membranes are unlike those of other organisms, all containing ether derivatives of di-
hydrophytol (80–82), which is a C16 chain with four methyl branches, in addition to a few percent red and yellow carotenoids (85; G. I. King, R. A. Bogomolni, S.-B. Hwang, and W. Stoeckenius, Biophys. J. 17:97a, 1977) and squalenes. Some of the effects of the presence of these unusual lipids on bilayer structure have been considered (101, 105, 150). The fragmentation of the membranes of halophiles in the absence of salt and the subsequent release of membrane-bound proteins shows many analogies with the loss of native structure of enzymes from these organisms under the same conditions. A role for weak, salt-dependent, hydrophobic interactions in stabilizing the membranes was therefore proposed (98, 101).

When some species of halobacteria, such as H. halobium, are grown without shaking (low oxygen concentration), distinct regions ("patches") are formed in the cytoplasmic membrane. These lack the red carotenoids but contain a purple pigment and appear in freeze-fracture electron micrographs as sheets with ordered granular structure (15, 169). The patches may be isolated and contain only one kind of protein, which is linked to retinal. This "purple membrane" is now understood to constitute a unique light energy-transducing apparatus, which acts as an alternate system to the respiratory chain in generating an H+ gradient across the cell membrane. In demonstrating such a single specialized function for the purple membrane, conceptual and experimental advantages were gained. The elegance of this system has played a large role in dramatizing the validity of the principle of chemiosmotic energy coupling in bacterial and other membranes.

No obvious relationship between the salty habitat of the halobacteria and the appearance of this unique energy-transducing membrane has been found as yet. The physiology of these organisms and their means of coupling the light energy captured to energy-requiring processes is of interest not only because illumination affords an experimental advantage in studying the energized processes but also because energy transduction in these cells should reflect their adaptation to a highly saline growth medium and their reliance on two independent sources of energy. The objective of this review is therefore not only to describe the properties of the purple membrane but also to explore questions related to the coupling of the H+ gradient to other ionic gradients and to various energy-requiring processes, such as the transport of amino acids and the synthesis of adenosine 5'-triphosphate (ATP). Many of these questions, particularly those concerned with the structure and function of purple membrane, have been discussed recently in an extensive review by W. Stoeckenius, R. H. Lozier, and R. A. Bogomolni (Biochim. Biophys. Acta, in press).

Although all of the results discussed here have been obtained with H. halobium strains R1 or R'M1, evidence is available that other halobacteria also contain retinal and presumably purple membrane, and the conclusions reached can be probably extended to some degree to these other species as well.

**PURPLE MEMBRANE**

Purple membranes consist of patches, about 0.5 μm in diameter, covering under some conditions as much as half the surface of H. halobium cells. Freeze-fracture electron microscopy reveals that, unlike the red cytoplasmic membrane, which has no in-plane regularity, the purple membrane exhibits a hexagonal pattern of granularity (15). When salt is removed the red membrane fragments into small, slowly sedimenting pieces, whereas the purple membrane can be recovered in the form of sheets (144, 169, 170) of a size range corresponding to that of the original patches on the cell surface. There is no evidence for any alteration in the purple membrane during exposure to low ionic strengths. This resistance to disruption by the removal of salt provides a convenient method for purifying purple membranes, since after extensive dialysis against distilled water only the purple membranes remain intact and are recoverable by sedimentation. A preparation free of red membrane contamination can be obtained after sucrose gradient centrifugation (6, 144).

The purple membrane isolated in this way appears to have a fixed chemical composition: one protein only, of a molecular weight of 26,000 (20, 142), and specific lipids (92) constituting about 25% of the dry weight of the membranes (92, 135). The lipids in the purple membranes consist almost entirely of the diether analog of phosphatidylglycerol phosphate (which is also the main lipid in the red membrane) and a glycosulfolipid (92). Both lipids contain only dihydrophytanyl groups.

In freeze-fracture electron micrographs, the boundary between the cytoplasmic (i.e., red) membrane and the patches (i.e., purple membrane) is clearly visible. Although the two kinds of membranes appear to be contiguous, no structures which might separate them are evident at their boundary. Freeze-fracture treatment cleaves the membrane through its central hydrophobic core and leaves a protein-rich (granular) leaflet and a protein-poor (smooth) leaflet (15, 44), corresponding to the cytoplasmic and the external faces of the membrane, respectively.

The asymmetrical placement of protein in the
purple membrane sheets is suggested also by electron micrographs taken of dried, shadowed membranes (18), where about 50% of the patches appear smooth (one side up) whereas the other half exhibit a cracking pattern with 120° angles (other side up). X-ray diffraction patterns of purple membranes in oriented multilayers show that the membrane has a width of 4.9 nm and that the electron density profile across the width of the membrane is asymmetrical (14, 61). Low-angle X-ray diffraction patterns reveal that in the plane of the membrane the structure consists of a hexagonal lattice (6.3-nm repeating distance), with three proteins per unit cell of about 33.0-nm² area (14). Purple membrane sheets should be therefore considered two-dimensional crystals containing approximately 10² repeating units.

Henderson and Unwin (62) used a novel electron diffraction technique to obtain a three-dimensional image of the arrangement of polypeptide chains in the membrane. At 0.7-nm resolution, seven α-helices were detected; three of these were seen spanning the membrane at right angles, and four were positioned at angles 10 to 20° inclined from the perpendicular. Although the resolution of the method does not reveal the connections between the α-helices, it was suggested that the seven chains belong to one protein molecule. A threefold axis of symmetry in the plane of the membrane is obtained from three of these proteins facing one another, with the nine right-angle α-helices forming the sides of an equilateral triangle. The lipids are probably arranged into an interrupted bilayer, filling the gaps between the proteins.

The three-dimensional model for bacteriorhodopsin above (62) as well as X-ray-scattering data (14, 61) indicate that the protein contains extensive amounts of helical regions (up to 80%). Ultraviolet circular dichroism measurements give 73% α-helix content when corrected for light-scattering distortions (116) but much lower values when uncorrected (7). About 75% of the peptide bonds exchange H⁺ very slowly, as expected if they are internally H bonded (43, 87).

The regularity of the structure described above suggests that the motional freedom of its components is greatly restricted. Flash-induced dichroism studies (155, 162) indicate that the rotational relaxation time of the protein is far slower than those of proteins in other membranes. As expected, stearic acid-type spin labels are highly immobilized in purple membranes (27). Di-tert-butyl nitrooxide partitions very poorly into these membranes and, once incorporated, shows little temperature dependence for motion (W. Z. Plachy and J. K. Lanyi, unpublished data). These observations indicate that the purple membrane does not contain a fluid bilayer, such as it is commonly understood. The immobilization of the lipid phase must be the consequence of lipid/protein interaction but may be caused also by the inherently constrained motion of branched-chain lipid tails (101, 150). The fact that a difference in lipid composition between the contiguous red and purple membranes is observed (93) suggests that very little lateral motion of the lipids takes place across the perimeters of the patches.

The characteristic color of the purple membrane is derived from retinal (92, 142), which is covalently bound to a lysine residue via a protonated-Schiff base linkage (20, 142) and probably interacts noncovalently with neighboring aromatic amino acids. The 380-nm absorption peak of free retinal is thereby shifted to about 570 nm (142). Partial amino acid sequences of bacteriorhodopsin are now available (147), which, together with evidence from amino acid modifications (86; T. Konishi and L. Packer, in S. R. Caplan and M. Ginzburg, ed., *Halophilic Microorganisms*, in press). The molar extinction coefficient of the retinal/protein complex is 63,000 (139). The lysine bearing the chromophore is a neighbor, once removed, of 1 of 11 prolines in the molecule and in the present model of the structure is located near a bend between two α-helices (88, 147). Chemical similarities between the retinal/protein complex in the purple membrane and in the visual pigment, rhodopsin, prompted Oesterhelt and Stoeckenius (142) to name the bacterial protein *bacteriorhodopsin*.

The question of the position of the retinal chain in bacteriorhodopsin has been investigated by a variety of approaches. Linear dichroism at 570 nm of oriented purple membrane multilayers has yielded an angle for the chromophore of 23.5 ± 2°, inclined from the plane of the membrane (66; R. A. Bogomolni, S.-B. Hwang, Y.-W. Tseng, G. I. King, and W. Stoeckenius, Biophys. J. 17: 98a, 1977). Neutron diffraction of purple membranes has been carried out before and after the retinal was replaced with its deuterated analog (King et al., Biophys. J. 17:97a, 1977). The difference in the electron density map indicates that the retinal is located asymmetrically across the width of the membrane, the β-ionone ring occupying a position about one-third of the membrane width from the surface facing the external medium.

Exciton interaction between adjacent chromophores has been proposed on the basis of the observation that native purple membrane ex-
hibits positive and negative circular dichroism peaks near 570 nm, whereas membranes disorganized by detergent treatment show only a positive peak, as is expected for single chromophores (3, 8, 65). In bleached purple membrane reconstituted with added retinal, the biphasic circular dichroism peaks are regained nonlinearly but with a stoichiometry suggestive of pigment/pigment interaction (8). Since exciton interaction depends on the relative spatial orientation of the transition dipole moments, dichroism data are of great potential use in determining the positions of the three neighboring retinal chains relative to one another (66).

Native bacteriorhodopsin is only slightly bleached at normal light intensities. A cyclical light reaction was first observed with ether-treated purple membranes, where the recovery time of the bleached intermediate is slowed down sufficiently for the accumulation of the bleached species (139). This and other photointermediates have been observed also in samples illuminated at temperatures between −80 and −170°C (118, 143). The photochemistry of bacteriorhodopsin has been extensively studied, with flash spectroscopy (30, 36, 117, 118) and modulation excitation spectroscopy (166). It was first proposed that the results fit a single-photon-initiated linear reaction, with six intermediates, denoted by their absorption peaks (notation from references 117 and 118): bR570 (ground state), K590, L560, M412, N420, and O460. The reaction bR570 → K590 has a half-life of <10 ps (83), and it alone requires the absorption of a photon, although the other intermediates are also photoreactive. The reactions resulting in the formation and decay of L560, M412, N420, O460, and finally bR570 will proceed in the dark (with half-lives of 2 µs, 50 µs, 1 ms, 3 ms, and 5 ms, respectively) at room temperature (summarized in reference 118). Thus, after the absorption of a photon bacteriorhodopsin passes through all its intermediate states within 10 ms. The efficiency of photocycling varies with temperature and other conditions and is reported to be between 0.25 and 1 (9, 64, 118). Recently, branching reactions involving N420 or additional intermediates have been suggested (118, 161).

pH changes during illumination of ether-saturated purple membranes suspended in saline solution (139, 143) revealed that the photochemical cycle described above is accompanied by release and then uptake of protons. More recently, the H⁺ release was tentatively assigned, on the basis of kinetic evidence, to the L560 → M412 reaction, and the H⁺ uptake was assigned to one of the reactions leading from M412 to bR570 (24, 117, 119, 161). Resonance Raman spectra (112) of illuminated purple membranes indicate that the Schiff base is indeed deprotonated in M412. Of course, the release and uptake of the proton to and from the medium do not have to coincide with the changes in the protonation state of the Schiff base nitrogen. Tyrosine was suggested as a candidate for an intermediate proton donor (18; Konishi and Packer, in Caplan and Ginzburg, ed., Halophilic Microorganisms, in press). Very little else is known about the chemistry of the photon-initiated series of reactions. Some evidence is consistent with cis-trans isomerization of retinal (149).

Oesterhelt and Hess (139) reported that in ether-saturated bacteriorhodopsin the intrinsic fluorescence of the apoprotein undergoes cyclical changes during illumination. Since the fluorescence of proteins is generally thought to be dependent on the immediate environment of the aromatic residues, the changes in fluorescence were interpreted as evidence for a light-dependent conformational change. These results have been recently confirmed and extended, using untreated purple membranes (18). After a short flash, fluorescence in the ultraviolet spectrum was found to decrease on the same time scale as the formation of M412 and then to increase as this intermediate (plus O460, which is also formed at low pH) decays. The fluorescence changes are greatest at emission wavelengths of 350 nm and higher. Since added CsCl specifically quenches fluorescence at 350 nm and eliminates a large part of the light-induced fluorescence change, it was tentatively concluded that the changes originate primarily from tryptophan residue(s) exposed to the aqueous phase.

First circumstantial and later direct evidence (discussed in Bacteriorhodopsin: A Light-Driven Pump for Protons) had established that the cyclic deprotonation and reprotonation of bacteriorhodopsin during illumination occurs in a vectorial fashion: the net result is the translocation of H⁺ from one side of the membrane to the other. Figure 1 is a conceptual model for how this might happen. A detailed model of the molecular mechanism of the process was put forward by Konishi and Packer (in Caplan and Ginzburg, ed., Halophilic Microorganisms, in press). This model relies on many different kinds of evidence, particularly on the recent finding that the removal of retinal results in the establishment of a pathway for passive proton conduction through bacteriorhodopsin (Konishi and Packer, in press). The questions raised in the model relate to the migration of H⁺ in this channel during the light-induced cycle, the groups which act as donors and acceptors for H⁺, and the form in which the energy of the
FIG. 1. Conceptual model for the functioning of bacteriorhodopsin. The absorption of a photon (hv) is followed by changes in retinal-protein interaction, resulting in the state designated M_{412} (see text). In this state the Schiff base linkage between retinal and lysine is deprotonated, and the apoprotein has undergone a conformational change of an unknown nature. This conformation allows the unidirectional migration of a proton from the opposite side of the membrane, resulting in the reprotonation of the Schiff base. Uptake of a proton from the aqueous phase, together with conformational recovery, completes the cycle.

The photon is transferred to the immediate environment of the retinal molecule. Describing the molecular details of these processes and understanding how they relate to the structure of bacteriorhodopsin is one of the great opportunities and challenges of membrane biology, since this protein is a much simpler ion pump than any other known.

A second kind of light reaction observed with bacteriorhodopsin is light or dark adaptation. When the purple membrane is kept in the dark, its visible absorption peak slowly shifts from 570 to about 560 nm (7, 137, 141). Exposure to light results in a shift back to 570 nm. This transition is not accompanied by a gain or loss of H^+ (J. K. Lanyi, unpublished data). Light adaptation seems to consist entirely of the isomerization of retinal: light-adapted bacteriorhodopsin contains trans-retinal, but dark-adapted pigment contains a 1:1 mixture of trans- and 13-cis-retinals (75, 141, 167). The 13-cis pigment undergoes a photocycle different from that of the trans pigment (145, 167). No physiological function for light/dark adaptation has been proposed.

**BACTERIORHODOPSIN: A LIGHT-DRIVEN PUMP FOR PROTONS**

According to the chemiosmotic hypothesis (56, 57, 132-134) energy-transducing membrane enzymes are vectorial systems which take up some of their reactants and/or release some of their products on different sides of the membrane. Since during the course of such a reaction the products accumulate and/or the reactants are depleted from their compartments, the chemical potential differences across the membrane (and the electrical potential differences if the molecules involved carry a net charge) enter into the energetics of the overall process. Thus, additional energy is needed whenever gradients are produced, and energy is released whenever gradients are dissipated in the reaction. It follows, therefore, that the chemical and the electrical gradients can serve as reservoirs of energy when the appropriate molecular machinery is available for catalyzing the vectorial reaction. The Mg^{2+}-stimulated adenosine triphosphatase in mitochondria and bacterial membranes is an example of such machinery; here, the hydrolysis and synthesis of ATP is accompanied by the apparent movement of H^+ across the membrane.

Another kind of energy-transducing machinery does not catalyze a chemical reaction, but couples the cross-membrane movement of two or more molecules. Systems which are thought to function in this manner include those for active transport, the transmembrane movements of amino acids, sugars, etc., being dependent on the coupled movements of H^+ or Na^+ (56, 57, 133, 164).

It follows from the concept described above that transmembrane gradients will serve as the means for energy transfer between two membrane components if these are involved in the movement of the same translocated molecules. Thus, the physiologically important consequence of the chemiosmotic hypothesis is that one membrane process may drive another through the generation and discharge of transmembrane gradients.

Two parameters are associated with the gradients which develop: the potential difference, or motive force, and the capacity of the system. Since the role of H^+ gradients in membrane energetics is widely recognized, the term "protonmotive force" has become familiar. It refers to the difference in electrochemical potential for protons in the two compartments separated by the membrane and is calculated from the relationship:

\[ \Delta p = \Delta \psi - Z \Delta p H \]  

where \( \Delta p \) is protonmotive force, \( \Delta \psi \) is electrical potential difference across the membrane, \( Z \) is a constant of ca. 60 mV at room temperature, and \( \Delta p H \) is the pH difference across the membrane. The motive force for the molecule, \( i \), in general is:

\[ \Delta \mu_i = m \Delta \psi - Z \log(a_i^{out}/a_i^{in}) \]  

where \( \Delta \mu_i \) is the electrochemical potential difference, \( m \) is the net charge of \( i \), and \( a_i^{out} \) and \( a_i^{in} \)
are the activities on the two sides of the membrane. The second term, $\Delta a_{\text{H}^+}$, corresponds to $\Delta \psi$, the chemical potential difference.

Equations 1 and 2 explicitly state that the electrical and chemical terms can alone or together provide the driving force for the transmembrane movement of the transported molecules.

The capacity of a compartmented system is given by the quantity of transported molecules which yields a given size of gradient (132). For the electrical gradient the capacity depends on the number of charges per molecule transported and on the electrical capacitance of the membrane (usually determined by the dielectric properties of the lipid bilayer). For the chemical gradient of $\text{H}^+$ ($\Delta \psi$) the capacity is given directly by the buffering power of the two compartments on either side of the membrane. It is evident that buffering increases the capacity for $\text{H}^+$, since strong buffering will result in the need to transfer more $\text{H}^+$ to produce the same pH difference across the membrane. The capacity of a system is greatly increased when the chemical entity transported is a normal intracellular ion, such as Na"+ or K"+. The higher the intracellular concentration of these ions, the higher the capacity for these ions and the more energy can be stored in their gradients.

Experimental evidence to suggest that the above-described principle applies to energy conservation in membranes has been obtained in a large number of systems. However, the simplicity and elegance of the Halobacterium system make it an ideal tool for the study of these effects.

That purified bacteriorhodopsin responds to illumination by translocating protons across the membrane has been shown by incorporating sheets of purple membranes into planar films (black lipid membrane), into small enclosed compartments (liposomes), or into a combination of both. It was found (74, 84, 153) that when purple membranes were dispersed with lipids the resulting preparation exhibited $\text{H}^+$ uptake during illumination. Electron microscopy confirmed that, for reasons probably having to do with charge asymmetry on the two sides of the membrane, the purple membrane is incorporated into the liposomes preferentially outside-in, i.e., in the direction opposite from the orientation in intact cells. In such experiments the quantity of $\text{H}^+$ lost from the medium is up to 20 times the number of bacteriorhodopsin molecules present (151). It may be thus concluded that the pH changes observed are not accounted for by ionization changes in the pigment molecules, but must be caused by net transport of $\text{H}^+$, corresponding to many photochemical cycles and vectorial deprotonations and reprotonations for each bacteriorhodopsin. On the other hand, Caplan and co-workers have found (42, 46) that the $\text{H}^+$ loss from the medium proceeded with biphasic kinetics. In these experiments the slower phase probably corresponds to the transported protons, but the authors attribute the faster phase to proton uptake by bacteriorhodopsin due to conformational changes.

The functional role of bacteriorhodopsin in establishing an electrochemical gradient for protons was demonstrated when Racker and Stoeckenius (153) incorporated mitochondrial adenosine triphosphatase and purple membranes into liposomes and detected light-dependent phosphorylation of adenosine 5'-diphosphate (ADP). Liposomes containing purple membrane have since served in many cases as easily energized systems for studying isolated and reconstituted proteins which catalyze energy-requiring membrane processes (151, 158, 182). Racker and Hinkle (152) and others (84) later showed that the $\text{H}^+$ uptake by the liposomes is accompanied by the uptake of a membrane-permeant anion, tetraphenylboron, thereby providing evidence for the development of an electrical potential across the liposome membrane (inside positive).

Thin lipid films (37-39, 63; P. K. Shieh, J. K. Lanyi, and L. Packer, Methods Enzymol., in press) and lipid-impregnated filters (Shieh et al., in press) separating two aqueous compartments where electrical measurements can be made have also been used to study light-induced $\text{H}^+$ translocation by bacteriorhodopsin. When purple membrane sheets or purple membrane-containing liposomes are incorporated into such partitions, photopotentials of 100 to 300 mV have been observed. The asymmetrical orientation of the purple membrane sheets probably comes from statistical chance (39, 63), since the number of membrane sheets involved is small. Adding $\text{La}^{3+}$ appears to produce a larger asymmetry of $\text{H}^-$ pumping, since it inhibits the $\text{H}^+$ uptake side of the membrane only (37). A more reliable way to incorporate preferentially oriented purple membranes into planar films consists of adding liposomes prepared as described above into one of the aqueous compartments together with $\text{CaCl}_2$. The ability of the system to produce a photopotential will increase with time (up to 1 h), presumably due to the association of the liposomes carrying the purple membranes with the planar films (38, 39; Shieh et al., in press).

These experiments have demonstrated the proton-translocating function of bacteriorho-
dopsin. Purified purple membranes not only appear to be structurally similar to purple membranes in intact cells but seem to be functionally equivalent, with no obvious impairment of activity resulting from exposure to the lowered salt concentration during isolation. On the other hand, more information about the way purple membranes function in the cytoplasmic membrane of *H. halobium* can be obtained by studying cell envelope vesicles. These can be prepared from cells by mechanical breakage (79, 121; J. K. Lanyi and R. E. MacDonald, Methods Enzymol., in press), and they contain both red and purple membranes. The membrane orientation in the envelope vesicles corresponds largely to that in whole cells (121; Lanyi and MacDonald, in press). The proportion of inside-in and inside-out red membrane surface in the vesicles can be estimated by determining NADH-menadione oxidoreductase activity in the presence and absence of Triton X-100. Since this enzyme is located on the surface of the red membrane normally facing the cytoplasmic side (99), the accessibility of NADH to the enzyme should give the percentage of misoriented red membrane surface. Typically, the vesicle preparations contain 85 to 90% inside-in red membrane (121). The similar degree of inside-in orientation of the purple membrane patches in the envelope vesicles is indicated by pH measurements after short light flashes (119). These experiments were carried out with envelope vesicles and with bacteriorhodopsin-containing liposomes to ascertain the vectorial nature of proton movement during the photochemical cycle. The pH changes occurring after single flashes of 1 ms were followed by measuring absorbance changes of a pH indicator dye. As expected from the direction of membrane orientation, envelope vesicles showed H* release, with a time constant of less than 1 ms, and liposomes showed H* uptake, with a time constant of about 10 ms. These results both confirmed the membrane orientation in the two systems and provided additional evidence for the scheme of vectorial proton loss and gain discussed in Purple Membrane.

A critical discussion of the methods used to measure the magnitudes of pH and electrical gradients is not the intention of this review. In *H. halobium* envelope vesicles, proton release can be conveniently followed by direct pH measurements in the extravesicular medium, since large and rapid pH changes will be caused by the illumination (103, 106, 121, 156). As in liposomes, the change in the number of protons in the medium is much larger than the number of pigment molecules, indicating that there is net translocation of H+. Heavily buffered vesicles release two to three times more H+ during illumination (103), suggesting that the H+ release is accompanied by a pH rise inside the vesicles. The pH differences between inside and outside have been determined under various conditions from the uptake of [14C]acetate (J. K. Lanyi, S. L. Helgerson, and M. P. Silverman, submitted for publication). The number of protons translocated at equilibrium is dependent on pH: the pH gradient is optimal at pH 3.5 to 4 and is greatly diminished above 7 (79; Lanyi et al., submitted for publication). A similar effect between pH 5.5 and 7 has been noted in *Escherichia coli* membrane vesicles, where proton translocation is caused by D-lactate oxidation (154). The pH dependence of *H. halobium* cell envelopes may be caused by direct pH effects on bacteriorhodopsin (17), by a mass action effect of the lowered H+ concentration inside the vesicles forcing the pump to work less efficiently, or by a possible lack of counterion permeability at higher pH, which would allow the development of higher electrical potentials but lower pH gradients. The latter does not seem to be the case, since at higher pH the pH difference becomes smaller even in the presence of the ionophore gramicidin (Lanyi et al., submitted for publication) and cation permeability rises rather than falls with pH (102). Drachev et al. (37) found that in planar membranes the photopotentials from bacteriorhodopsin were diminished when protons were translocated against a pH gradient.

The electrical potential across the vesicle membrane during illumination has been measured by using the fluorescent dye 3,3'-dipentyloxadacarbocyanine (103, 106, 156). As shown earlier by Sims et al. (165), a membrane potential (negative inside) will cause this cationic dye to accumulate in the vesicle interior (in *H. halobium* vesicles, mainly on the interior membrane surface), resulting in aggregation-dependent fluorescence quenching. Diffusion potentials for K+ with added valinomycin were used to calibrate the observed changes in fluorescence, which were found to be linear with the Nernst potentials up to about -110 mV, with a slope of 0.33%/mV (156). Another method to determine electrical potential is to follow the uptake of [3H]-triphenylmethylphosphonium ion (TPMP+), a membrane-permeating cation (159). Both electrical potential (Δψ), measured in these ways, and pH gradient, determined from exterior pH changes and from acetate uptake, are dependent on the presence of K+. The changes in these two components of the photomotive force (see equation 1) are reciprocal. Thus, the pH change is greater (1.5-fold) and Δψ is smaller (3-fold) when KCl is the salt present rather than NaCl (156;
Lanyi et al., submitted for publication), consistent with the idea that the vesicle membrane is more permeable to K+ than to Na+, and thus in the presence of K+ a greater number of protons can be displaced. However, the interpretation of the differences observed between the results in KCl and those in NaCl must take into account the fact that whereas K+ is accumulated during illumination (79; Lanyi et al., submitted for publication), Na+ is extruded through a proton/sodium antiport mechanism (42, 103), as discussed below.

The measurement of the protonmotive force during illumination is considerably more difficult in whole cells of *H. halobium* than in envelope preparations, because of resting gradients of uncertain size for H+, K+, and Na+ and because of an unexpected complexity in the kinetics of the light-induced H+ movements. Both difficulties will be discussed in detail below. Steady-state values for ΔpH and Δψ have been obtained for cells, however, both in the dark and during illumination (2, 12, 131, 180). These studies used 5,5'-[14C]-dimethylthiazolidine-2,4-dione distribution for pH gradient determinations and [3H]TPMP+ distribution for electrical potential determinations (for a review of these methods, see reference 125). The values obtained are largely in agreement: at pH 6.5 to 6.6, in the dark the cells exhibit pH gradients of 0.45 to 0.6 units (inside alkaline), and during illumination the gradient increases by 0.12 to 0.16 units. Wagner and Hope (180) found considerably larger values for the pH gradients. The pH gradient before illumination, and to a smaller extent the light-induced increase, appears to be dependent on the external pH, showing a steep decrease between pH 5.5 and 7.5 (2). The electrical potential is much less pH dependent, similar to what was found with the envelope vesicles. Michel and Oesterhelt (131), Bakker et al. (2), and Bogomolni (16) estimate Δψ as −100 to −110 mV in the dark and −120 to −125 mV during illumination. These measurements are subject to some uncertainty, because the TPMP+ may be bound to the cells by mechanisms unrelated to the electrical potential. The data obtained for *H. halobium* are remarkably similar to those reported by Ramos et al. (154) for *E. coli* vesicles.

Various agents have been shown to increase membrane permeability to cations. The effects of these chemicals (ionophores) on energy coupling in membranes have been of major importance in establishing the validity of the chemiosmotic hypothesis (55, 56). Although the principle of the hypothesis is now well established, these agents still play a useful role in studies of membrane bioenergetics. The uncouplers carbonyl cyanide, p-trifluoromethoxy phenylhydrazine; carbonyl cyanide, m-chlorophenyl hydrazine; etc., which in many systems have been shown to function as proton carriers, discharge the light-induced protonmotive force both in whole cells of *H. halobium* (2, 12, 17) and in envelope vesicles (103, 121). Nigericin, which is thought to facilitate the electrically neutral exchange of K+ and H+, has also been shown to discharge ΔpH in envelope vesicles (79). Valinomycin- and gramicidin-induced cation influxes eliminate the light-dependent electrical potential in envelope vesicles (103, 156; Lanyi et al., submitted for publication), although gramicidin has no effect on intact cells (R. A. Bogomolni, personal communication). It appears from these reports that the effects of ionophores on the light-induced gradients in *H. halobium* are similar to those in other systems.

**SECONDARY GRADIENTS: TRANSMEMBRANE MOVEMENTS OF Na+, K+, AND Cl−**

Freezing point depression determinations with cell pastes (29) and chemical analyses of pelleted cells (30, 52, 107) have suggested that the internal salt concentration of extremely halophilic bacteria is sufficiently high to maintain osmotic equilibrium with the external medium. The intracellular concentration of K+ was found to be 3 to 5 M, and that for Na+ was found to be 0.5 to 1.2 M. A predominance of K+ inside the cells is a property of both procaryotes and eucaryotes, and the extracellular milieu usually contains more Na+ than K+. The gradients for these cations generally have to be achieved with large expenditures of energy, since the capacity of cells for these ions is high, and thus the quantity of cations moved across the cell membranes for a given size of gradient can be much greater than for H+. This energy problem is particularly serious for the halobacteria, since they contain 25 times more salt than do other organisms. The means by which Na+ and K+ gradients are developed thus become of major importance in the physiology of these bacteria. A related question is the passive permeability of the membranes to Na+ and K+, since the movements of these ions across the membranes determine both the capacity for osmotic work in nonmetabolizing cells and the relative contributions of ΔpH and Δψ during energization.

According to several reports (51, 52, 107), even starved, poisoned, or anaerobically maintained *Halobacterium* cells can retain K+ for several days against large gradients. Ginzburg and Ginzburg (49, 50) suggested that to account for this
phenomenon the intracellular binding of a considerable fraction of the K+ must be assumed. These authors also claim that the cell membrane is permeable to very large molecules (48), and their model of ion movements in these cells is based on cation exchange between cytoplasm and external medium due to binding rather than membrane transport. This model cannot be valid for H. halobium, since large gradients for cations have been demonstrated in cell envelope preparations from this organism, in spite of the fact that the envelopes are largely devoid of cytoplasmic contents. Furthermore, no binding of K+ by the cytoplasm of H. cutirubrum was detected by direct measurement (107). Lanyi and Hilliker (102) have reported that the slowness of K+ loss from H. halobium cells can be fully explained by the measured rate of passive K+/Na+ exchange in cell envelope vesicles, and there was no need to postulate intracellular binding for K+ to account for the retention of this ion by the cells.

In actively growing Halobacterium cells the K+ gradient can reach values up to several hundred-fold between inside and outside (53, 54). A simple model would have K+ in equilibrium with the electrical potential. In such a model K+ influx would be driven directly by Δψ, via either unfacilitated or facilitated transport or both. Garty and Caplan (45) have provided some support for this hypothesis, since they found that the influx of 86Rb was linearly dependent on the size of Δψ. Recently, on the basis of direct measurements of K+ fluxes in H. halobium cells, G. Wagner, R. Hartmann, and D. Oesterhelt (Eur. J. Biochem., in press) suggested that K+ permeability is about 200-fold greater than Na+ permeability. The high K+ permeability accounted both for the observed K+ diffusion potential in cells, amounting to about half of the theoretical Nernst potential, and for the dependence of the intracellular ATP/ADP ratio on the external K+ concentration.

Since the permeability of K+ in H. halobium cell membranes appears to be much greater than that of other ions, the K+ gradient, once established, represents a large reservoir of energy for the cells. Under such circumstances the concentration gradient of K+ maintains a diffusion potential of considerable size, up to about 30 mV per decade of concentration difference, determined by the relative permeating abilities of the ions present. The advantage of this strategy for the halophilic cells is that this diffusion potential is dissipated only by massive cation fluxes across the cell membrane, which are very slow, as discussed above. This kind of energy storage is, as suggested by Wagner et al. (in press), long-term.

Results with H. halobium cell envelope vesicles (Lanyi et al., submitted for publication) failed to show the high K+ permeability observed in intact cells or any evidence for a K+ diffusion potential without added valinomycin. Furthermore, the electrical potential during illumination showed the expected 1:1 relationship to the chemical potential difference of K+ only when K+ permeability was increased by adding valinomycin. Indeed, it was found that under many conditions Cl− movements predominated over K+ movements in illuminated vesicles. The influx of K+ is proportional to the external K+ concentration up to 1 M, precluding the existence of a significant saturable K+ permeation system (Lanyi et al., submitted for publication). It appears, therefore, that H. halobium envelope preparations are seriously impaired in their K+ permeability. The loss of K+ permeability during the preparation of the vesicles is surprising in view of the suggestion that K+ transport in cells is driven not by ATP hydrolysis, which is easily lost, but by the electrical potential (45; Wagner et al., in press).

Lanyi and co-workers found (103, 106; Lanyi et al., submitted for publication) that, when H. halobium envelope vesicles or cells are illuminated, Na+ efflux occurs and high Na+ concentration gradients (out > in) develop. Since under these conditions Na+ moves against its electrochemical potential, the energetics of Na+ transport are very unlike those of K+ transport. Lanyi and MacDonald (103) observed that when both NaCl and KCl were included inside the envelope vesicles the pH changes measured during illumination were not as simple as when only KCl was present in the vesicle interior. Depending on the amount of NaCl inside the vesicles, there was an initial period of smaller pH change, followed by an increase in ΔpH to the value found in the absence of NaCl. The lengths of these initial periods during the illumination roughly coincided with the time required to deplete the vesicles of 22Na. These observations suggested that the efflux of Na+ is accompanied by an influx of H+, which decreases the size of the pH gradient. The simplest model to account for the results is H+/Na+ exchange, or (in the terminology of Mitchell), antiport (133). When the experiment was performed at higher pH values, between 6 and 7.5, the Na+−dependent influx of H+ during the illumination became more pronounced. At a pH of >7 the initial acidification of the external medium was followed by a rise in pH, leading to a transient reversal of the pH gradient. The reversal strongly suggests that the H+/Na+ antiport cannot proceed with a stoichiometry of 1:1, because such a process would be driven only by ΔpH and thus would be abolished.
as the pH difference approaches zero. On the other hand, at higher H+/Na+ stoichiometries the exchange would include net charge translocation, and the transport of Na+ would be driven also by the electrical potential. Δψ in this model could balance the system at a reversed pH gradient. A stoichiometry higher than 1 is suggested also by the kinetics of the pH changes in the experiments described above, which suggest that the H+ influx is not linearly dependent on ΔpH but with a higher power function.

Rapid changes in the electrical potential during H+/Na+ exchange could be followed with the fluorescent dye, 3,3'-diphenyloxadicarbocyanine, discussed in Bacteriorhodopsin: a Light-Driven Pump for Protons. It was found (103) that Na+ efflux is accompanied by greatly increased electrical potential, which decreases when the intravesicular Na+ is depleted. Since proton translocation by bacteriorhodopsin is almost certainly limited by the internal pH, the pump should be, in effect, reactivated by the H+ influx during Na+ efflux, and since the proton pumping becomes more rapid the electrical potential created is raised. The result of the various ion movements, according to this model, is the continued removal of Na+ from the vesicles and the recirculation of the same protons. The net loss of Na+, however, must be balanced by a counterion. In cells, Na+ is replaced by K+ (Wagner et al., Eur. J. Biochem., in press), whose influx is energized by Δψ. In vesicles, however, the Na+ efflux is only partially compensated for by the K+ influx (Lanyi et al., submitted for publication), so Cl- efflux needs to be postulated as well. Indeed, light-scattering measurements showed that illuminated vesicles collapse unless K+ and valinomycin are added or the external K+ concentration is set at very high values (Lanyi et al., submitted for publication). Figure 2 illustrates a qualitative model (103, 104; Lanyi et al., submitted for publication) which incorporates these ion movements. An interesting feature of this model is that it predicts positive feedback between active Na+ efflux and passive Na+ influx. Oscillations of the electrical potential are indeed observed when the initial Na+ concentration inside the vesicles is low (103). The light-induced efflux of Na+ from H. halobium envelopes and many of the associated effects described above have been confirmed by Caplan and co-workers (23, 42). These authors have shown ΔpH-induced Na+ flux and Na+ gradient-induced H+ flux in unenergized vesicles. Wagner et al. (in press) have demonstrated light-dependent Na+ efflux from intact H. halobium cells.

The characteristic biphasic pH changes which occur when envelope vesicles containing NaCl are illuminated are not seen during a second illumination unless an interval of several hours is allowed between the two light periods (103). The slow return of the Na+-dependent effect is consistent with the depletion of Na+ in the vesicles during the first illumination and the slow exchange of Na+ with K+ during the intervening dark time (102).

Another cation whose transport has been studied in H. halobium membranes is Ca2+. The transport of Ca2+ was shown to proceed by Na+/Ca2+ antiport (11), which is a eucaryotic mechanism, unexpected in a bacterium (163). In spite of the fact that illumination produces an Na+ gradient in these vesicles (out > in), no acceleration of Ca2+ efflux was observed under such energized conditions.

**AMINO ACID TRANSPORT**

In most bacteria the active (concentrative)
uptake of amino acids and sugars is energized by either terminal oxidation or ATP hydrolysis (13, 56). For transport in the first category, the source of energy has been shown to be the H⁺ gradient generated. In such transport the transmembrane movement of the metabolite is coupled to the movement of H⁺ (or Na⁺) down its electrochemical gradient. Mitchell termed such coupled translocation symport (133). Transport of this type is obtained not only in whole cells but also in cell envelope vesicles, which lack soluble components (76, 77). In at least one case the protein responsible for active transport has been solubilized with detergent, partially purified, and reconstituted with lipids (69). The transport of substrates by means of the second category, i.e., ATP hydrolysis but not proton-motive force, is more complex. In many of the ATP-dependent transport systems soluble factors have also been implicated, the "shockable proteins" (13), which bind the substrates of transport (148).

*H. halobium* cell envelope vesicles will actively accumulate 19 of the 20 commonly occurring amino acids (120, 122). Active transport in the vesicles will take place in response to illumination and the resulting chemical and electrical gradients and also in response to these gradients when they are provided by other means in the dark. In the envelope vesicles the involvement of ATP in energy coupling is minimal, since the envelopes neither contain ATP (or ADP and phosphate) nor produce ATP in measurable quantities when the appropriate substrates are present (106; J. S. Hubbard, personal communication).

As in other bacterial systems (for a review, see reference 19), transport groups common to several amino acids have been identified in *H. halobium* (120). Amino acids within such groups competitively inhibit the transport of one another, presumably owing to the existence of common transport carriers which bind and translocate the substrates. In *H. halobium* the groups are: (i) arginine, lysine, and histidine; (ii) glutamine and asparagine; (iii) aspartate; (iv) glutamate; (v) threonine, alanine, serine, and glycine; (vi) leucine, isoleucine, valine, and methionine; (vii) tryptophan, phenylalanine, and tyrosine; and (viii) proline (120). Kinetic resolution of the components of those transport groups which contain more than one amino acid is too complex to attempt at this time. A survey of maximal transport rates and substrate concentrations at half-maximal velocity (Km) in *H. halobium* envelope vesicles has been made (120).

The active transport of all amino acids, with the exception of glutamate, can be induced either by a concentration gradient of Na⁺ (out > in), artificially arranged in the dark by appropriately loading the vesicles or created during illumination as described above, or by an electrical potential, originating from K⁺ diffusion in the presence of valinomycin or from illumination (120). Since both chemical and electrical components of the Na⁺ gradient will drive the transport of these amino acids in the absence of other sources of energy, it has been concluded that the fluxes of Na⁺ and the amino acids are coupled to one another, and thus in *H. halobium* amino acid gradients are produced at the expense of the Na⁺ gradient. The existence of specific transporter proteins which facilitate the coupling of the two fluxes is implicit in this model. Any direct role played by H⁺ in these translocations is uncertain. Recent experiments have ruled out the direct participation of H⁺ in the transport of serine and aspartate (J. K. Lanyi,Biochemistry, in press).

Leucine is a representative amino acid, and its transport in *H. halobium* has been studied in detail. Figure 3 shows the course of [14C]leucine uptake during illumination. It is apparent that, whereas little transport occurs in the dark, illumination causes a rapid influx of the amino acid. At saturating light intensities (1 × 10⁸ to 2 × 10⁸ erg cm⁻² s⁻¹) leucine concentration gradients of 200 to 300 are obtained. The amino acid is not modified chemically during the transport. The action spectrum for leucine uptake closely resembles the absorption spectrum of bacteriorhodopsin (121). When the light is turned off or

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**Fig. 3.** Light-induced transport of leucine in *H. halobium* envelope vesicles. The vesicles contained approximately 2.9 M KCl and 0.1 M NaCl and were suspended in 2.5 M KCl-0.5 M NaCl, containing [14C]leucine. (A) No addition. (B) A 100-fold excess of unlabeled L-leucine added at 30 min; D-leucine, added similarly, had no effect. (C) TPMP⁺ (1 mM) added at 30 min, as indicated. Reproduced with permission from R. E. MacDonald and J. K. Lanyi, *Biochemistry* 14:2882-2889, 1975 (121).
the membrane potential is abolished by adding TPMP+, the leucine rapidly exits from the vesicles. The light-induced uptake is carrier mediated, since (i) the process is saturable, with a $K_r$ of about $1 \mu M$ for leucine, and (ii) the transport is stereospecific in favor of L-leucine. Under the conditions of this experiment the source of energy is primarily the electrical potential, since permeant cations, such as TPMP+, completely abolish transport; conversely, buffering the vesicles internally, which diminishes $\Delta$pH but increases $\Delta\psi$ (106), has an enhancing rather than inhibiting effect. A diffusion potential of K+, induced by adding valinomycin in the dark to appropriately loaded vesicles, will alone cause transient leucine transport (121).

As found for the other amino acids (120), the active accumulation of leucine could be induced in the dark by arranging an Na+ concentration gradient (out > in) (121). Since such transport of leucine is much less sensitive to the proton ionophore carbonyl cyanide, $p$-trifluoromethoxy phenylhydrazone than is the light-induced uptake, it was concluded that the accumulation of leucine under these conditions is energized directly by the chemical potential difference for Na+. This point was reemphasized in later work (Lanyi, Biochemistry, in press; Lanyi et al., submitted for publication), when it was found that an Na+ concentration gradient caused no [$^3$H]TPMP+ uptake, i.e., $\Delta\psi$ was absent.

There is evidence to suggest that the Na+ gradient which is created during illumination is of sufficient size to play a role in energizing amino acid transport. Vesicles loaded with significant amounts of NaCl show biphasic uptake for leucine (104), the first phase corresponding to the effect of electrical potential alone and the second, larger phase corresponding to the effect of the concentration gradient for Na+, which arises only after several minutes. The first phase of transport is completely inhibited by valinomycin plus K+, but the second is only partially inhibited, as expected.

The transport of leucine requires Na+ outside the vesicles (121), as does the transport of all other amino acids (120). Maximal transport is observed when K+ is also present in the medium. The K+ requirement may reflect the fact that the concentration gradient of Na+ represents a large part of the driving force for transport, and this gradient could not arise if an appropriate counterion were not provided; however, other roles for K+ have been proposed (11, 120).

These results show that leucine accumulation is energized by both components of the electrochemical potential for Na+: $\Delta\psi$ and $\Delta$H$_{\text{m}}$. The role of $\Delta\psi$, at least, in driving leucine and proline transport in intact cells of _H. halobium_ has been confirmed by Hubbard et al. (73).

The study of the efflux of amino acids from deenergized vesicles is complicated by the fact that during illumination the vesicles lose most of their Na+ and acquire K+, and these cations do not exchange rapidly in the dark (102). The collapse of the pH difference and $\Delta\psi$ after the light is turned off does not reflect, therefore, the true state of energization for amino acid transport. It has been found that the efflux and exchange of [$^3$H]methionine from KCl-loaded vesicles is very slow but that that from NaCl-loaded vesicles is fast (60). This Na+ dependence for efflux suggests that the transport carrier couples the fluxes of amino acid and Na+ in both directions. However, the exchange of the radioactive amino acid during active uptake was slower than that observed after the light was turned off, indicating that the driving force for transport imposes a functional asymmetry on the system (60).

Glutamate transport in _H. halobium_ vesicles is exceptional in two respects: (i) the transport does not proceed at all unless a concentration gradient for Na+ is present, regardless of the existence of a membrane potential, and (ii) the translocation of this amino acid is predominantly unidirectional (106, 109). When NaCl is included in the vesicles, glutamate accumulation shows a lag, roughly proportional to the intravesicular concentration of Na+, and transport is seen only after the vesicles are depleted of Na+ (10, 106; Lanyi et al., submitted for publication). Glutamate transport under these conditions appears coincidentally with the second phase of leucine transport, described above (104). At lowered light intensities glutamate transport shows lags, followed by increasing rates of uptake, which also demonstrate the cumulative nature of the driving force for this transport. When the light is turned off, the pH difference and $\Delta\psi$ collapse within 30 to 40 s (103, 105, 156), but the concentration gradient for Na+ survives for much longer times (102, 106). As shown in Fig. 4, the ability of the vesicles to transport glutamate persists for 15 to 20 min. Figure 4 also shows that the rate of this post-illumination transport depends on the length of the illumination.

If glutamate transport is energized solely by the chemical component of the Na+ gradient, as suggested by the inability of $\Delta\psi$ to cause uptake, then the glutamate transport rates should provide information about the size of the Na+ concentration gradient produced during illumination. Lanyi et al. (109) found that a prearranged Na+ concentration gradient of $>500$ was required.
to energize glutamate transport in the dark at rates equivalent to those obtained during light-induced transport. Thus, at high light intensities at least, the Na\(^+\) concentration difference which arises through H\(^+\)/Na\(^+\) antiport must be very large.

The exit of glutamate is extremely slow when the light is turned off, even when a large excess of nonradioactive glutamate is added (109). This irreversibility of the glutamate movement is not caused by a requirement for Na\(^+\) inside the vesicles for exit, since adding gramicidin, which facilitates the exchange of Na\(^+\) and K\(^+\), or preloading the vesicles with Na\(^+\) and [\(^{3}H\)]glutamate does not result in efflux. Glutamate efflux from the envelope vesicles was observed only when ionic conditions opposite to those favoring influx were established: Na\(^+\) inside and K\(^+\) outside the vesicles (109). Thus, glutamate translocation seems to take place only toward the compartment containing K\(^+\) but not Na\(^+\). So, glutamate is trapped for kinetic reasons, for a time at least, inside the vesicles while the light-initiated gradients persist. The molecular details of such a transport carrier are very difficult to envisage.

The quantitative relationships between the chemical and electrical components of the Na\(^+\) gradient and the resulting amino acid gradients in *H. halobium* envelope vesicles have been recently explored (Lanyi, Biochemistry, in press). The ratios of coupling between the amino acid and Na\(^+\) gradients in these studies were expected to reveal the translocation stoichiometries. An experimental procedure was devised for imposing various magnitudes of $\Delta\psi$ on the vesicles without appreciable $\Delta\mu_{\text{Na}\text{H}}$ and $\Delta\mu_{\text{NaNa}}$ without $\Delta\psi$. Although these two driving forces are thermodynamically equivalent, the gradients of serine and aspartate produced during the stationary state did not agree. For $\Delta\psi$-driven transport Na\(^+\)/serine was 1 and Na\(^+\)/aspartate was 2, but for $\Delta\mu_{\text{NaNa}}$-driven transport these ratios were 2 and 4, respectively. These unexpected results suggest that the function of the transport carriers is modulated by the Na\(^+\) gradient, which supplies a regulatory effect, in addition to the energizing effect.

The initial rates of transport for serine and aspartate were not simple linear functions of the electrochemical potential difference for Na\(^+\). Rather, the transport of serine could be characterized with a Hill plot slope of 4 when $\Delta\psi$ was the driving force and of 2 when it occurred in response to $\Delta\mu_{\text{NaNa}}$ (Lanyi, Biochemistry, in press). Similarly, the Hill slopes for aspartate were 4 and 1, respectively, for $\Delta\psi$ and $\Delta\mu_{\text{NaNa}}$-driven transport. These kinetic complexities, together with the apparently variable stoichiometries discussed above, could be reconciled in a model which postulates that the transport carriers are multimeric structures with variable degrees of association (Lanyi, in press). Naturally,
this idea must be tested by more direct methods than transport kinetics. It seems unlikely that the properties which gave rise to this model are unique to *H. halobium*, and as other Na⁺-dependent bacterial transport systems are studied similar results may emerge.

Sodium gradient-dependent substrate transport is widely found in eucaryotic systems (for example, see reference 31), but until recently in procaryotes, with one exception (158), only H⁺ gradient-driven transport has been described. An Na⁺ requirement for transport has been often attributed to a cofactor role for this cation (for example, see references 78 and 157). It is easy to visualize how in extremely halophilic bacteria and perhaps in some marine microorganisms (173, 174) the presence of NaCl in the growth medium could favor the evolution of Na⁺ symport-based active transport systems. One may speculate, however, that the involvement of Na⁺ concentration gradients in energy metabolism may present advantages to other bacteria as well. The capacities of ΔΨ and Δι as energy reservoirs are inherently small, and these gradients collapse with the movement of a relatively small number of protons. A difference in Na⁺ concentration across the cell membrane, on the other hand, usually involves very large quantities of ions. Substantial Na⁺ concentration gradients (out > in) seem to exist in several bacteria during active metabolism (58), and Na⁺ symport systems have been recently described in *E. coli* (123, 178, 179) and *Salmonella typhimurium* (186).

**PHOTOPHOSPHORYLATION**

The formation of ATP from ADP and phosphate is a classical energy-requiring process, and in mitochondria and many bacterial membranes the energy is supplied by the oxidation of reduced substrates. In such systems, and in others where the energy is provided by light (e.g., in chloroplasts), the storage and transfer of energy implies the existence of an energized membrane state. As described above, the principle of chemiosmotic energy coupling identifies this intermediate state as the electrochemical gradient of H⁺ between the bulk phases across the membrane. Convincing evidence in favor of this concept was supplied by reconstitution experiments of Racker (151, 153) and others (158, 181). These authors have shown that liposomes containing purified purple membranes and adenosine triphosphatase from various sources will form ATP when illuminated. From such experiments one is led to expect that light would be a source of energy for phosphorylation in *H. halobium* cells as well.

Danon and Stoeckenius (35) first described photophosphorylation in *H. halobium* cells containing bacteriorhodopsin. The cells maintained an ATP level at a constant value during respiration. When the medium was made anaerobic or respiratory poisons were added, the levels of intracellular ATP dropped by about 70%. Illumination under these conditions restored the ATP level. The synthesis of ATP, energized either by respiration or by illumination, is sensitive to the usual inhibitors of phosphorylation, e.g., dicyclohexylcarbodiimide, Dio-9, and phlorizin (33, 35, 73), as well as to uncouplers (35, 136). The energy for ATP in these experiments is clearly provided by the H⁺ gradient developed during illumination. As in other bacterial systems (124, 126), ATP synthesis could be induced in *H. halobium* cells by a combination of K⁺ diffusion potential and pH gradient in the absence of other sources of energy (33; Wagner et al., Eur. J. Biochem., in press).

When intact *H. halobium* cells are illuminated, the pH changes in the medium are complex. The light-induced changes in pH reflect both the expected extrusion of H⁺ by bacteriorhodopsin and at least two kinds of transient influx of H⁺. The first of these reverse fluxes is observed only after long periods (hours) of incubation in the dark between illuminations and resembles the pH changes seen with NaCl-containing envelope vesicles (16, 17). This kind of pH rise during illumination, first reported as an anomalous response (17, 136), is probably due to H⁺/Na⁺ exchange, which only occurs when large quantities of Na⁺ are accumulated inside the cells before the illumination. The second type of light-induced pH rise occurs repeatedly, with every illumination, and this effect has been linked to ATP synthesis. The sequence of events associated with illuminating *H. halobium* cells has been examined closely in a number of laboratories. When intact cells are illuminated above pH 4.5, the external pH first rises, indicating net H⁺ influx, and then drops below the initial value, indicating subsequent net H⁺ efflux (16, 17, 33, 136). At saturating light intensities the duration of the pH rise is less than 1 min, although at lower intensities it is longer and does not completely reverse (17; Wagner et al., in press). Concurrently with the pH rise, ATP is synthesized (17, 33, 136). The kinetics of the pH change and ATP increase are reported to correspond well by some investigators (16, 17) but poorly or not at all by others (59, 138).

The pH changes during illumination were resolved graphically by Bogomolni et al. (17) into a rapid alkalinization component and a slower acidification component. These components
were proposed to represent H⁺ influx through the proton channel of the adenosine triphosphatase and H⁺ efflux through bacteriorhodopsin, respectively. Dicyclohexylcarbodimide, an inhibitor of ATP synthesis, was indeed found to abolish the alkalinization component. The method of graphic resolution is shown in Fig. 5, which relates pH changes to ATP levels. The rate of H⁺ influx was calculated as 2.9 H⁺/ATP synthesized. Because the light intensity dependence of the H⁺ influx was much lower than that of H⁺ efflux, it was proposed that the H⁺ influx is not a primary, driven process but that it represents the relaxation of a preexisting resting gradient for H⁺. Such a gradient was indeed demonstrated by Bogomolni et al. (17), who were able to show its collapse with uncouplers or nigericin, resulting in the loss of the light-induced alkalinization. Starvation of the cells also caused loss of the H⁺ influx, whereas adding glycerol, an oxidizable substrate, restored it. The existence of a resting gradient for H⁺ in *H. halobium* cells, maintained by respiration, is supported by measurements of ΔpH and ΔΨ (2, 131). According to this view, illumination of bacteriorhodopsin causes further H⁺ ejection from the cells, but the observed H⁺ influx occurs primarily in response to the preexisting H⁺ gradient. The model in this form would not provide for a reversed net flow of protons, however. Since the main part of the energy for ATP formation is provided by the preexisting H⁺ gradient, poised presumably below a threshold value, the additional protonmotive force during

![Graph](http://mmbr.asm.org/)

Fig. 5. Resolution of light-induced pH changes into two components during the illumination of *H. halobium* cells and their relationship to ATP synthesis. The cells were kept anaerobic at pH 7.2, and at zero time they were illuminated at a light intensity of 2.5 \times 10^8 \text{erg} \text{cm}^{-2} \text{s}^{-1} (equivalent to 25 mW/cm²). Symbols: ---, measured pH trace; ○, time course of the H⁺ extrusion component, calculated by curve peeling; □, calculated time course of the H⁺ influx component; ○, sum of the two calculated pH change components, superimposed on the measured trace to demonstrate the validity of the graphic resolution; △ and ·····, ATP synthesis. In the inset the time course of the pH changes and the pH levels are shown also after the illumination is ended. Reproduced with permission from R. A. Bogomolni, R. A. Baker, R. H. Lozier, and W. Stoeckenius, *Biochim. Biophys. Acta* 440:68-88, 1976 (17).
illumination must act in a nonlinear manner, as a gating potential. Furthermore, ATP synthesis within the first minute of illumination continues in spite of the fact that the ΔpH across the cell membranes is now smaller than it had been before the illumination. Measurement of membrane potential under these conditions (16) indicates that ΔΨ initially increases, but only by 10 to 15 mV. The total protonotive force during the light-induced synthesis of ATP thus may decrease below the dark (and threshold) value. Because of this possibility it is probably necessary to propose a hysteretic effect as well. Nonlinearity of the ATP/ADP ratio with protonotive force follows from calculations based on the chemiosmotic hypothesis, and in bacterial cells a threshold value of about -200 mV was indeed found for ATP synthesis (124, 126). In explaining the hysteretic effect, Bogomolni (16) favors the possibility that the adenosine triphosphatase complex of *H. halobium* contains a regulatory subunit which would be activated by the rise in internal pH or by the increase in ΔΨ++. When the illumination is ended, the levels of intracellular ATP return to their previous lower values. After the light is turned off, the external pH changes are the reverse of those at the beginning of the illumination: first a decrease in pH and then a rise until the pre-illumination pH is reestablished. Since the initial decrease in pH is abolished by dicyclohexylcarbodiimide, Bogomolni et al. (17) attributed it to H⁺ efflux produced by the hydrolysis of ATP.

Oesterhelt and co-workers have a different view on why there is an initial reversal in the light-induced H⁺ flow in *H. halobium* cells. They have proposed that the reversal of H⁺ flux is a consequence of the simultaneous activation of the electronogenic proton/sodium antiport and ATP synthesis by the light-induced H⁺ extrusion (59; Wagner et al., Eur. J. Biochem., in press). The crucial element of this hypothesis is the electrogenicity of the antiporter, which will return to the cell more than one proton per charge, resulting in a net flux of H⁺ into the cells with little or no change in ΔΨ. This behavior would resemble the observations with *H. halobium* cell envelopes, where net reversal of H⁺ flux above pH 7 was also obtained (103). Oesterhelt’s view is supported by recent results, which indicate that in cells the net H⁺ uptake during the first 2 min of illumination is virtually completely balanced by Na⁺ efflux (Wagner et al., in press). Also, Hartmann and Oesterhelt have reported experiments in which they could dissociate the light-induced H⁺ uptake from ATP synthesis through their different responses to external pH and light intensity (59). One of these experiments, in which the alkalinization effect but not ATP synthesis is induced by increasing the light intensity in a pre-illuminated culture, is shown in Fig. 6.

An important question in analyzing the possible role of the proton/sodium antiporter in the light-induced pH changes is the amount of free Na⁺ inside the cells after repeated illuminations. The Na⁺ content of the vesicles is virtually completely depleted during illumination (23, 42, 103, 106; Lanyi et al., submitted for publication), and the Na⁺ is regained very slowly (102). In contrast, recent experiments show that even prolonged illumination of cells (>60 min) at high light intensities will leave a large internal Na⁺ concentration (Wagner et al., Eur. J. Biochem., in press). If so, the cellular Na⁺ levels are never very low, regardless of the illumination history of the sample, and the operation of the proton/sodium antiporter must always be included in the balancing of ion movements.

An extreme view is that H⁺ influx and H⁺ efflux are independent energized processes. Matsuno-Yagi and Mokuhata (128) found that an *H. halobium* strain which contained much-decreased amounts of purple membrane responded to illumination with only the alkaline effect. The acidification in the normal strain and the alkalinization in the mutant showed different sensitivities to heating and hydroxylamine treatment. ATP synthesis was correlated with the alkaline effect only, and the authors proposed that bacteriorhodopsin may have two distinct roles.

**RELATIONSHIP BETWEEN BACTERIORHODOPSIN AND THE RESPIRATORY CHAIN**

The halobacteria are obligately aerobic, with the exception of *H. marismortui*, which will reduce nitrate (181), and contain cytochromes of the *b* and *c* types, as well as an *α*-type cytochrome oxidase (25, 26, 95, 96, 113), and respiratory chain-linked NADH dehydrogenases (70, 71, 97, 108). NADH and *α*-glycerophosphate are rapidly oxidized by membrane fragments, but succinate is not (96).

Since the membranes are not permeable to NADH and the dehydrogenase is located on the inside membrane surface (99), the envelope vesicles, which are normally inside-in, do not oxidize NADH. On the other hand, dimethylphenylenediamine (10), tetramethylphenylenediamine (13; K. Andersen, Ph.D. thesis, University of Trondheim, Trondheim, Norway, 1975) and ascorbate, with and without phenazine methosulfate or ferricyanide (Andersen, Ph.D. thesis), are rapidly oxidized by the vesicles. Oxygen uptake and the formation of a colored oxidation
product from the phenylene diamines are inhibited by cyanide and azide, indicating that the electron transfer involves cytochrome oxidase.

Bogomolni et al. (17) have shown that the pH changes which occur when oxygen is introduced into an anaerobic suspension of *H. halobium* are similar to those occurring during illumination. It seems reasonable, therefore, that respiration and light absorption by bacteriorhodopsin might be alternate sources of protonmotive force in *H. halobium*. Cell envelope vesicles will transport glutamate when various respiratory substrates are added (10; Andersen, Ph.D. thesis). The dependence of this transport on Na⁺ outside the vesicles, its inhibition by Na⁺ inside the vesicles, and other properties are so similar to the light-driven process that Belliveau and Lanyi (10) concluded that the entire sequence of events leading to the formation of the concentration gradient for Na⁺ is induced by respiration in much the same way as it is by the action of light on bacteriorhodopsin.

Oxygen uptake by *H. halobium* cells is inhibited up to 30% during illumination (140). This effect is similar to respiratory control observed in other membrane systems capable of oxidative phosphorylation, but it does not seem to necessarily involve ADP and phosphate, since the nonphosphorylating envelope vesicles show it also (J. W. Belliveau, R. A. Bogomolni, and J. K. Lanyi, unpublished data). At least 24 quanta of light are absorbed by bacteriorhodopsin when the consumption of one molecule of oxygen is prevented, and since the respiratory chain would translocate 12 protons per molecule of oxygen, Oesterhelt and Krippahl (140) argued that the quantum yield of light-induced proton transport is about 0.5. This value is in remarkable agreement with estimates from direct measurements.

Bogomolni et al. (17) reported that uncouplers diminish the photoinhibition of respiration, but TPMP⁺, which abolishes the electrical potential while increasing ΔpH, has no effect. Whether respiration is controlled through tight coupling with proton translocation, so that the gradient of H⁺ developed during illumination reverses electron transport, or through changes in intracellular pH cannot be decided yet. It is interesting that Litvin et al. (115) relate the photoinhibition of respiration entirely to ATP synthesis. These authors report also on a photostimulation of respiration below pH 5.5, which they attribute to the light-induced rise in internal pH.

Danon and Caplan found (34) that ¹⁴CO₂ uptake by starved *H. halobium* cells is greatly increased during illumination. Since the label is

![Graph](Image)

**Fig. 6.** Changes in pH and ATP levels during illumination of *H. halobium* cells. The cells were kept anaerobic at pH 7.2 and were illuminated at the intensities shown. Symbols: -----, pH trace; x and ------, ATP level. The increase in light intensity at 17 min resulted in alkalinization but not in an increase in ATP. Reproduced with permission from R. Hartmann and D. Oesterhelt, Eur. J. Biochem. 77:325–335, 1977 (59).
found in sugars, it must have been fixed through reductive pathways, and hence it is probable that NADH was produced during the illumination by reversal of electron transport. This would be consistent with the inhibition of oxygen consumption by the illumination.

PHOTOTACTIC RESPONSES

The halobacteria are motile, and it is likely that the energy requirements for flagellar motion can be supplied by illumination. Besides this kind of response to light, the organisms exhibit a complex light-regulated behavioral pattern. The cells swim in a straight line at constant light intensity but tumble and reverse direction when the light intensity is changed (68). At higher wavelengths the reversal of direction occurs in response to a decrease in light intensity. The action spectrum for this effect resembles the absorption spectrum of bacteriorhodopsin, with a peak at 565 nm. At lower wavelengths the reversal of swimming occurs in response to an increase in light intensity. The action spectrum for this effect shows peaks at 280 and 370 nm, with a long tail up to about 530 nm (68). The result of these phototactic responses is that the cells tend to avoid regions illuminated at wavelengths below 530 nm but seek out regions illuminated above this wavelength.

The pigments responsible for the two photoreponses have not been directly identified, although the 565-nm response certainly seems to be due to bacteriorhodopsin or a very similar chromoprotein. However, when _H. halobium_ was grown with nicotine, which inhibits retinal (and carotenoid) synthesis, both 565- and 280/370-nm responses were absent, whereas both effects could be restored by adding retinal (67). Thus, the 280/370-nm pigment may also be a retinal–protein or at least contain a chromophore derived from retinal.

BIOSYNTHESIS OF PURPLE MEMBRANE

The principal physiological role of purple membranes in _H. halobium_ is clearly the generation of protonotive force upon illumination. In spite of this, for reasons which are not yet clear, _H. halobium_ has not been successfully grown with illumination as the only source of energy. That the purple membrane serves only as an auxiliary energy conversion device, in addition to the respiratory chain, is evident from the fact that significant quantities of purple membranes are synthesized by the cells only when required, i.e., at low oxygenation and when light is available (72, 143). The induction of purple membrane synthesis under these conditions takes place without substantial increases in cell mass.

The regulation of bacteriorhodopsin biosynthesis is complex. It appears that it is the retinal-free protein, bacterio-opsin, whose synthesis is accelerated in response to low oxygen tension (171). When the retinal production in the cells is inhibited by nicotine, this protein will accumulate and can be converted to bacteriorhodopsin upon addition of retinal (171). The crystalline structure of bacteriorhodopsin does not appear, however, unless protein synthesis can take place. The presence of bacterio-opsin, in turn, regulates retinal synthesis. When bacterio-opsin is not made or is allowed to react with the retinal analog 4-ketoretinal, retinal synthesis is inhibited (172).

The bacterio-opsin in aerated or nicotine-treated cells is found in a distinct membrane fraction called “brown membrane.” These constitute patches on the cell surface, as the purple membranes do, but do not show crystalline structure. The brown membrane contains cytochromes and other proteins besides bacterio-opsin and bands at a lower buoyant density than that of the purple membrane (32). When the proteins of the brown membranes in nicotine-grown cells are pulse-labeled with radioactive amino acids, the label is first recovered in the brown membrane fraction but later shifts to the purple membrane fraction (32). Thus, the brown membrane is the in vivo precursor of the purple membrane. The steps leading from brown membrane to purple membrane formation have not been described.

CONCLUSIONS

From the discussion above it is evident that energy is conserved in _H. halobium_ in various forms: in the conformation of bacteriorhodopsin, in the electrochemical gradients of H⁺, Na⁺, and K⁺, as well as in the gradients of transport substrates, and in the energy-rich phosphate bond of ATP. These have different time constants for formation and decay and represent energy reservoirs of different sizes. (i) After the absorption of a photon, the photochemical states of bacteriorhodopsin store sufficient free energy to translocate a proton within 10 ms. The mechanism of the translocation and the way energy is transferred to the proton is not yet known, but it is likely that a channel for H⁺ exists in the protein. (ii) The translocation of protons results in a gradient for H⁺ across the membrane, comprising both electrical and chemical components. The total size of the gradient is at least −180 mV, but its capacity must be relatively small. Thus, when illumination is ended, various cou-
pled processes and passive leaks cause it to collapse within 1 min in envelope vesicles and within a few minutes in intact cells. (iii) Antiport of H⁺ and Na⁺ results in an Na⁺ gradient, poised in the same direction as the protonmotive force. The complete collapse of the concentration gradient for Na⁺ takes several hours, and even its initial high value after the illumination will persist for 15 to 20 min. (iv) During the active extrusion of Na⁺, a larger fraction of the protonmotive force is shifted to electrical potential, since with the recirculation of H⁺ the net effect is the removal of Na⁺. This electrical potential drives passive K⁺ influx until the gradients of H⁺, Na⁺, and K⁺ are in equilibrium with the proton pump. Under physiological conditions this results in a large K⁺ concentration gradient in addition to the Na⁺ gradient, poised in the opposite direction from the gradients of H⁺ and Na⁺. The capacities of the gradients of K⁺ and Na⁺ are very large in these cells, which contain high salt concentrations, and must represent the major form of energy storage. (v) Coupled to the electrical and chemical components of the Na⁺ gradient, but not directly to the H⁺ gradient, is the active transport of various amino acids. (vi) Finally, illumination is accompanied by the synthesis of ATP. The formation of ATP has been linked to H⁺ influx, and the energy required for the phosphorylation is no doubt provided by the gradient of H⁺.

For many years halobacteria have been considered exotic creatures, with their own unique rules of conduct. It is becoming evident, however, that these species represent only a minor variation on the single theme of life. Investigations of the photophysics of halobacteria have thus helped to better define questions, and in some cases to provide some answers, which are of general interest to students of membrane bioenergetics. The chemiosmotic hypothesis, which is now generally accepted, was first formulated by Mitchell about a decade before the discovery of bacteriorhodopsin. By the end of that decade an impressive amount of evidence had been already accumulated in favor of this concept of energy coupling. Nevertheless, the simplicity of the light-transducing system in Halobacterium halobium and its experimental advantages have played a role in removing some objections to the hypothesis and thus must have served to convince many students of membrane energetics of its validity.

LITERATURE CITED


