Light-Induced Membrane Potential and pH Gradient in *Halobacterium halobium* Envelope Vesicles†

Robert Renthal‡ and Janos K. Lanyi*

ABSTRACT: Illumination of envelope vesicles prepared from *Halobacterium halobium* cells causes translocation of protons from inside to outside, due to the light-induced cycling of bacteriorhodopsin. This process results in a pH gradient across the membranes, an electrical potential, and the movements of K^+ and Na^+. The electrical potential was estimated by following the fluorescence of a cyanine dye, 3,3′-dipentyloxadicarbocyanine. Illumination of *H. halobium* vesicles resulted in a rapid, reversible decrease of the dye fluorescence, by as much as 35%. This effect was not seen in nonvesicular patches of purple membrane. Observation of maximal fluorescence decreases upon illumination of vesicles required an optimal dye/membrane protein ratio. The pH optimum for the light-induced fluorescence decrease was 6.0. The decrease was linear with actinic light intensity up to about 4×10^5 ergs cm^{-2} s^{-1}. Valinomycin, gramicidin, and triphenylmethylphosphonium ion all abolished the fluorescence change. However, the light-induced pH change was enhanced by these agents. Conversely, buffered vesicles showed no pH change but gave the same or larger fluorescence changes. Thus, we have identified the fluorescence decrease with a light-induced membrane potential, inside negative. By using valinomycin-K^+-induced membrane potentials, we calibrated the fluorescence decrease with calculated Nernst diffusion potentials. We found a linear dependence between potential and fluorescence decrease of 3 mV/%, up to 90 mV. When the envelope vesicles were illuminated, the total proton-motive force generated was dependent on the presence of Na^+ and K^+ and their concentration gradients across the membrane. In general, K^+ appeared to be more permeable than Na^+ and, thus, permitted development of greater pH gradients and lower electrical potentials. By calculating the total proton-motive force from the sum of the pH and potential terms, we found that the vesicles can produce proton-motive forces near −200 mV.

Transmembrane electrical potentials are thought to be present in respiring mitochondria, photosynthesizing chloroplasts, and various bacteria. In contrast to the action potentials measured across synaptic membranes or the resting potentials detected across many types of cell membranes, the respiration- and photosynthesis-linked potentials are believed to arise primarily from movements of hydrogen ions (Mitchell, 1961). Mitchell's chemiosmotic hypothesis (for reviews see Mitchell, 1969; Greville, 1969) linked such proton translocation to ATP synthesis, the energy available for the formation of the phosphate bond being hypothetically equal to the sum of the electrical and chemical components of a proton gradient. Many experimental measurements support the idea of respiration- and photosynthesis-dependent transmembrane proton movements, and similarly, much evidence has accumulated to indicate the simultaneous development of an electrical potential (for review, see Skulachev, 1971).

The extremely halophilic bacterium *H. halobium,* which thrives in nearly saturated salt solutions (Larsen, 1962; Lanyi, 1974), develops differentiated regions, the purple membranes, on its cell envelope, when exposed to anaerobic conditions (Oesterhelt and Stoeckenius, 1971). This membrane is a two-dimensional crystalline array (Blaurock and Stoeckenius, 1971; Henderson, 1975; Blaurock, 1975; Henderson and Unwin, 1975) of the protein, bacteriorhodopsin, and various unusual lipids (Kushwaha et al., 1975). The purple color (absorbance peak at 570 nm) of the membranes is due to the protein-linked chromophore, *all-trans*-retinal. Upon illumination, the protein–chromophore complex rapidly passes through various photointermediates and returns to the initial state (Stoeckenius and Lozier, 1974). The net result of a single photoreaction cycle is the translocation of one proton from inside the cell to the extracellular medium (Bogomolni and Stoeckenius, 1974). Thus, the purple membrane appears to be a light-activated proton pump. Danon and Stoeckenius (1974) have shown that in whole cells of *H. halobium* the proton pump can replace the respiratory system as the source of energy for ATP synthesis, and Racker and Stoeckenius (1974) have demonstrated the activation of mitochondrial ATPase by the purple membrane in a reconstituted vesicle system. Subsequently, MacDonald and Lanyi (1975) found that vesicles prepared by sonicating *H. halobium* cells transported leucine in response to light and presented evidence to indicate that the driving force for this transport is electrical potential. Thus the purple membrane may be considered to be an ideal model system for studying the detailed mechanism of energy coupling.

The two components of the chemiosmotic proton-motive force have been documented in purple membranes incorporated into liposomes: the pH gradient by Racker and Stoeckenius (1974) and the electrical potential by Racker and Hinkle (1974) and by Kayushin and Skulachev (1974). We now report experiments in which we have been able to measure the magnitude of the light-induced electrical potential developed by the purple membrane in cell envelope vesicles, and we have compared the chemical and electrical components of the proton-motive force.

Microelectrodes have been successfully employed to detect cell electrical potentials. However, in small cell organelles and bacteria this is extremely difficult. Indirect methods have been used to demonstrate respiration- or photosynthesis-linked ion movements in mitochondria, chloroplasts, and bacteria, and

† From the Biological Adaptation Branch, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, California 94035. *Received September 10, 1975.* R.R. was supported by a National Research Council Postdoctoral Resident Research Associateship.

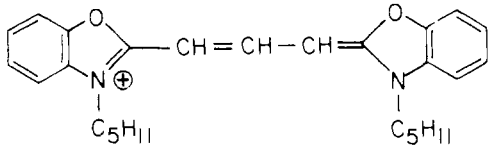
‡ Present address: Division of Earth and Physical Sciences, University of Texas at San Antonio, San Antonio, Texas 78285.

the existence of electrical potentials has been inferred from these studies. Fluorescence measurements have also provided evidence for membrane potentials in these systems; one in particular has been described in detail by Sims et al. (1974), utilizing cyanine dyes. We have used such changes in cyanine dye fluorescence to estimate the electrical potentials developed in *H. halobium* cell envelope vesicles. The following describes the determination of the transmembrane electrical potential, pH difference, and proton-motive force in this system.

Materials and Methods

All experiments were done in dim light at 23 ± 2 °C. Envelope vesicles were prepared from *H. halobium* strain R_1 by sonication, as described previously (MacDonald and Lanyi, 1975). In a typical preparation, vesicles in 4 M NaCl (membrane protein concentration 1 to 5 mg/ml) were concentrated by centrifugation and resuspended in a several-times smaller volume of salt, either 3 M NaCl or 3 M KCl. When the vesicles were to be buffered, the 4 M NaCl vesicles were diluted directly into the solution of 3 M salt and buffer to be used. As before (MacDonald and Lanyi, 1975), stock solutions were routinely characterized by assays for total protein (biuret), vesicle orientation (menadione reductase), and purple membrane (difference spectroscopy).

The cyanine dye used in these experiments, 3,3′-dipentyloxadicarbocyanine iodide, was a gift from Dr. Alan Waggoner, Amherst College. The structure of the dye is shown below:



According to the notation of Sims et al. (1974), it is referred to as diO-C_5-(3). Similar results to those reported here were obtained with the dye diI-C_1-(7). However, the latter was found to be unstable, undergoing a constant and relatively rapid decrease in fluorescence in the dark, which appeared to be accelerated by illumination with actinic light.

Valinomycin and gramicidin D were obtained from Calbiochem, and $TPMP^+$ [1] was from K & K Fine Chemicals. All other chemicals used were reagent grade and obtained from standard suppliers. Concentrated salt solutions were filtered through a 0.45-μm Millipore filter after preparation.

Fluorescence Measurements. A modified Schoeffel Instrument Company spectrofluorometer was used for fluorescence measurements. The fluorescence excitation source was a Hanovia 1-kW xenon lamp with a Princeton Applied Research Model 191 chopping motor between the lamp and the excitation monochromator. The chopping motor was operated at a frequency of 150 Hz. Excitation and emission wavelengths were isolated with Schoeffel 0.25-m grating monochromators and 4-mm slits. The fluorescence emission was detected by a thermoelectrically cooled Centronic Q4283B photomultiplier tube. The photomultiplier output passed through a Dymec 2460A preamplifier into a Princeton Applied Research Model 220 (PAR 220) lock-in amplifier, tuned to the chopping motor frequency. The output of the PAR 220 passed through a Tektronix 535A oscilloscope equipped with a 1A7A high gain differential amplifier into a Nicolet LAB-80 computer.

The actinic light source, a General Electric EJL 200-W quartzline lamp mounted 20 cm from the center of the fluorometer cuvette holder, was connected to a Trygon Electronics dc power supply operated at 20 V, 7 A. The light was filtered through 7 cm of water and an Optics Technology 700-nm short pass filter and was then focused through Corning cut-off filters 3-67 and 3-69 onto the sample. The actinic light source was directed at the sample 180° from the excitation light source, while the fluorescence was viewed at 90° to this axis. Illumination of the sample by actinic light was achieved by opening a manually operated shutter. The entire volume of the sample was illuminated by this light source, while the excitation light source covered only a small section at the center of the sample.

Between 0.05 and 0.1 ml of stock vesicles was diluted to 3.5 ml in the appropriate solution of 3 M salt (either NaCl or KCl). From 20 to 70 μl of the stock dye solution in ethanol was rapidly delivered to the vesicle suspension while shaking the tube. The dye concentration was estimated from the extinction coefficient given by Sims et al. (1974) for diO-C_2-(3) in ethanol since the alkyl chains do not influence the extinction coefficient.

Fluorescence data were recorded in three different ways. In some experiments full, corrected emission spectra were taken with the Schoeffel fluorometer unmodified by the chopping motor and lock-in amplifier. For these experiments, scattering baselines of vesicles without the dye were subtracted from the spectra. In other experiments, the modified Schoeffel instrument was used. The resulting spectra were uncorrected for photomultiplier sensitivity but did not require the light-scattering correction. Most of the measurements were made with the modified instrument by time-dependent dwelling on the 508-nm emission maximum of the dye. An excitation wavelength of 450 nm was used in all experiments.

The percent fluorescence change, ΔF, was calculated by measuring the fluorescence emission at 508 nm in the dark (F_D) and in the light (F_L) in the same arbitrary units:

$$\Delta F = 100[(F_L/F_D) - 1] \quad (1)$$

The limiting value of F_L was obtained after 3-5 min of illumination.

In the experiments in which concentration was a variable, increasing amounts of dye or vesicles were added to the same sample of either vesicles or dye. Increasing amounts of $TPMP^+$ were added to the cuvette containing the sample to test the $TPMP^+$ concentration dependence. Valinomcycin, $TPMP^+$, and gramicidin D were added directly to the cuvette, while the fluorescence was being monitored, and immediately mixed with a small plastic paddle. In the experiments in which pH was a variable, the same sample was titrated with 0.1 N NaOH or 0.1 N HCl using a syringe microburet. The light dependence was observed by inserting Optics Technology neutral density filters between the sample and the actinic light source. The light intensity was measured with a Kettering radiant power meter at 1.1-1.2 × 10^6 ergs cm^{-2} s^{-1}. (The intensity of the excitation light source at 450 nm was found to be less than 0.02% of the actinic light intensity.)

pH Measurements. Hydrogen ion concentration was measured with a Beckman semimicro combination pH electrode and an Instrumentation Laboratories meter. The output passed through an Instrumentation Laboratories DC-DC isolator, the 1A7A high gain amplifier, and a high frequency noise suppressor. The signal was recorded in the Nicolet computer.

The samples reached a reasonably stable pH value in about 15-20 min after preparation, after which time the pH showed only a small, upward drift. The pH, when measured on iden-

[1] Abbreviation: $TPMP^+$, triphenylmethylphosphonium ion. diO-C_5-(3), 3,3′-dipentyloxadicarbocyanine. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

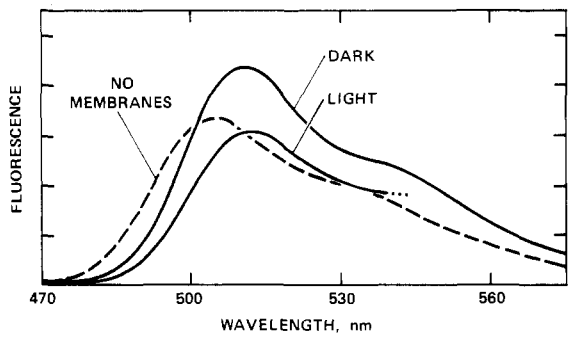


FIGURE 1: Fluorescence emission spectra of cyanine dye diO-C_5-(3). Excitation wavelength: 450 nm. Dashed line: 5×10^{-7} M dye in 3.0 M NaCl. Solid lines: 5×10^{-7} M dye in vesicles (0.16 mg/ml membrane protein), 3.0 M NaCl; upper line is fluorescence in the dark, and lower line is fluorescence while the vesicles are illuminated by actinic light above 540 nm. The decrease in fluorescence at 508 nm is 30%. The emission spectrum during illumination was not scanned above 540 nm to protect the photomultiplier tube from scattered light. The absorbance of the vesicle suspensions was below 0.08 in the wavelength region of interest. Scattering below 540 nm is significant but is not recorded by the chopped system (see Materials and Methods).

tical samples in 3 M KCl and 3 M NaCl, was several tenths of a pH unit more acidic in NaCl. We have not corrected the results for this apparent electrode error in KCl. The light sensitivity of the electrode was virtually completely eliminated by wrapping the electrode in aluminum foil. The remaining effect (a small downward deflection) was similar to the effect seen upon illumination of buffered vesicles (see Figure 6b). Samples were stirred with a magnetic bar continuously during pH measurements.

Results

Fluorescence Spectra of the Dye, diO-C_5-(3). The envelope vesicle membrane is composed of patches of purple membrane (Oesterhelt and Stoeckenius, 1971) and regions of red membrane, containing a variety of proteins which include the respiratory enzymes (Lanyi, 1968), and lipids which include the red pigment, bacterioruberin (Baxter, 1960; Kelley et al., 1970). The absorption spectrum of an envelope vesicle suspension gave maxima at 470, 500, and 540 nm, corresponding to the red membrane carotenoid, and a shoulder at 570 nm corresponding to bacteriorhodopsin. In attempting to minimize the inner filter effect from absorbance and light scattering during the illumination of the vesicles with actinic light, we had to consider the different absorbance and fluorescence emission properties of the various electrical-potential-sensitive dyes available. The dye diO-C_5-(3) (Sims et al., 1974) proved to be ideal. Its fluorescence spectra under various conditions appear in Figure 1. In 3 M NaCl the emission maximum is at 500 nm. After the dye is added to the saline solution, the fluorescence intensity slowly decreases and requires several minutes to stabilize. When the envelope vesicles are added at this point, the fluorescence rapidly rises to a new constant level, and the emission maximum shifts 8 nm toward the red, indicating dye interaction with the membranes.

When the vesicles are illuminated with light above 540 nm, the fluorescence of the dye decreases as much as 35%, without a shift in the emission peak.

The fluorescence intensity rapidly returned to the initial level when the illumination was turned off. Repeated cycles of several minutes of actinic light followed by several minutes of dark showed nearly the same amount of light-induced fluorescence decrease followed by a return to the same initial level of fluorescence in the dark, for more than a dozen cycles.

Fluorescence emission was unchanged when actinic light was used on the dye in saline without the envelope vesicles. Similarly, no decrease in fluorescence occurred when purple membrane alone (in the form of nonvesicle sheets) was illuminated in the presence of the dye. In the latter case a slight increase (up to 5%) was observed, which may be due to the decrease in the 570-nm absorbance of the photoreacting bacteriorhodopsin (Stoeckenius and Lozier, 1974), either through a decreased inner filter effect or decreased Forster-mechanism quenching.

Mechanism of Fluorescence Changes. Sims et al. (1974) proposed a mechanism for the potential-dependent fluorescence changes, based on two effects: (1) upon binding to the membrane, there is a shift in the dye emission maximum toward the red; and (2) at high concentrations, the cyanine dyes tend to form nonfluorescent aggregates. They suggest that an increase in membrane potential (interior negative) will move the equilibrium between the membrane-bound and the free dye toward the membrane-bound state resulting in a fluorescence wavelength shift. The potential will also cause a redistribution of the positively charged dye between the inner and outer membrane surfaces, resulting in an increase in the extent of aggregation and thus a decrease in quantum yield. Combinations of these effects would give rise to a net decrease in fluorescence proportional to the increase in membrane potential (interior negative).

We therefore considered the possibility that one or both of these mechanisms was responsible for the fluorescence decrease observed with *H. halobium* envelope vesicles. The emission spectra in Figure 1 show a shift in the dye emission maximum when membranes are added to the dye. However, there is no further shift in the dye emission maximum upon illumination of the vesicles. When a vesicle suspension, containing an optimal amount of dye for fluorescence decrease (see below), was centrifuged in the dark, more than 90% of the dye was found to sediment with the membranes. This situation is in contrast to the results of Sims et al. (1974) who found that under these conditions only about half the dye was bound to red cell membranes. Thus, we conclude that in *H. halobium* vesicles the application of an electrical potential cannot cause any further binding of the dye to the membranes and most, if not all, of the light-induced fluorescence changes observed in this system are due only to the transmembrane redistribution of the dye. The higher affinity of the *H. halobium* membranes for the cyanine dye may be related to several factors: (1) the high polarity of 3 M salt may favor hydrophobic interactions between the dye and the membrane; (2) the *H. halobium* membrane is quite acidic in comparison with the red cell (Lanyi, 1974) and there may be greater electrostatic interaction between the positively charged dye and the membrane. One consequence of this high affinity of the dye for the membranes is a lower relative affinity of the dye for glass or quartz. We found, unlike Sims et al. (1974), that no correction for binding to the cuvette walls was necessary as long as the dye was added to the saline solution already containing vesicles.

Variation of Envelope Vesicle and Dye Concentration. Although different vesicle preparations contained different relative amounts of red and purple membrane, we considered the total protein concentration to be a parameter proportional to overall vesicle concentration. Figure 2a shows that the percent fluorescence decrease becomes larger with increasing vesicle concentration, up to an optimal value, above which the fluorescence decrease is diminished. These results are consistent with the mechanism proposed above for the observed fluorescence changes. At low vesicle concentrations the relatively high

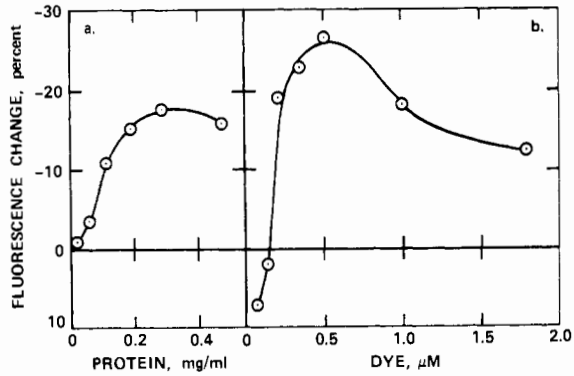


FIGURE 2: Concentration dependence of light-induced fluorescence change. Percent decrease in fluorescence at 508 nm upon illumination of vesicles by actinic light as a function of: (a) varying membrane concentration, 1×10^{-6} M dye; (b) varying dye concentration, 0.19 mg/ml membrane protein. Salt: 3.0 M NaCl.

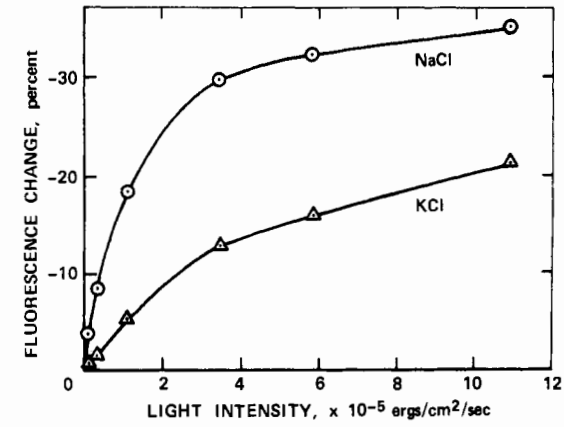


FIGURE 3: Light intensity dependence of fluorescence change. Amount of light-induced fluorescence decrease as a function of actinic light intensity. Full lamp intensity was attenuated with neutral density filters. Fluorescence changes measured at 508 nm. Conditions: 3×10^{-7} M dye, 0.048 mg/ml membrane protein. Upper curve: 3.0 M NaCl; lower curve: 3.0 M KCl. In both cases vesicles were loaded with 3.0 M NaCl.

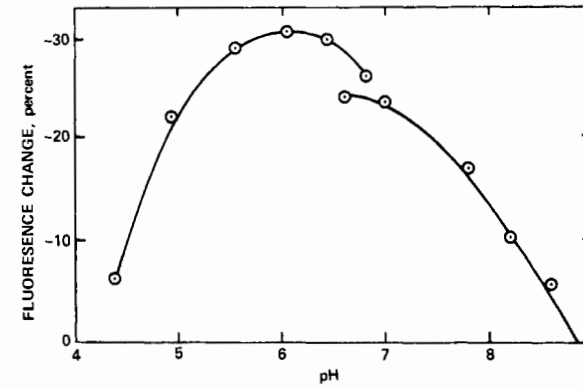


FIGURE 4: pH dependence of light-induced fluorescence change. Vesicles initially at pH 6.5. Left-hand curve: titrated with HCl to pH 4.4. Right-hand curve: titrated with NaOH to pH 9.0. Conditions: 0.13 mg/ml membrane protein, 5×10^{-7} M dye, 3.0 M NaCl.

dye concentration on the membrane surface results in aggregation even in the dark and therefore in a lessened light-induced effect. At higher vesicle concentrations the dye is more dilute on the membrane surface and light-induced redistribution does not cause large changes in the extent of aggregation. The decrease of the fluorescence change at the highest vesicle concentrations may be also due to increased light scattering, and an "inner filter" effect, which would decrease the fluorescence change by reducing the average intensity of the actinic light in the cuvette.

The amount of illumination-induced fluorescence change also varies with dye concentration, as shown in Figure 2b. The concentration dependence displays a maximum at about 5×10^{-7} M dye, for reasons similar to those considered for the vesicle concentration dependence. At very low dye concentrations the fluorescence change reverses sign, as is the case for the red blood cell membranes (Hoffman and Laris, 1974).

The above results show that, as expected from the proposed redistribution mechanism for the fluorescence change, maximal effects are dependent on an optimal dye/membrane protein ratio.

Effect of Light-Intensity Variation. The light-intensity dependence of the fluorescence change is shown in Figure 3. Saturating light levels appear to be reached in NaCl (upper curve) at about 4×10^5 ergs cm^{-2} s^{-1}. It is possible that the maximum fluorescence change obtainable in this system cannot be greater than about −35%, and thus the saturation is only apparent. If the extravesicle NaCl is replaced by KCl, as shown in the lower curve in Figure 3, the fluorescence changes are smaller while the light-intensity dependence remains similar.

Effect of pH Variation. The pH of the vesicle suspension influences the extent of the observed illumination-dependent fluorescence changes, a maximal response being found at pH 6.0. Vesicles titrated to more acid or alkaline pH give smaller fluorescence changes (Figure 4). The fluorescence change decreases to zero by pH 9.0. However, if the sample is back-titrated to pH 6.0, most of the fluorescence change is regained. But vesicles titrated to pH 4.5 are unable to recover on returning the pH to 6.0, even after 24 h. Thus, low pH appears either to irreversibly damage the vesicles, or to irreversibly affect the dye–vesicle interaction. The initial levels of dye fluorescence in the dark were found to be constant over a wide range of pH. Below about pH 5.5, the initial fluorescence increased considerably.

Effect of Salt Composition. As shown in Table I, vesicles with 3 M NaCl inside and outside give the largest fluorescence decrease upon illumination. If the external NaCl is replaced by 3 M KCl, the fluorescence decrease is about 30% less. With 3 M KCl inside and 3 M NaCl outside, the fluorescence decrease is half that found for the all NaCl system, and in all KCl, the fluorescence change is less than a third of that found for the all NaCl preparation.

We believe that these differences can best be understood in terms of differential permeability of the vesicle membrane to sodium and potassium ions.

Comparison of Fluorescence Change with Proton Translocation. Upon illumination, the purple membrane translocates protons from inside the *H. halobium* cell to the outside (Bogomolni and Stoeckenius, 1974). If this light-dependent proton gradient is electrogenic, then the rate of acidification of the extravesicle medium should lag behind the kinetics of the development of any membrane potential in a manner that depends largely on the back-permeability of the membrane to both protons and other cations.

The rate of fluorescence decrease upon illumination of NaCl-loaded vesicles in 3 M NaCl was rather slow compared with the rate of external pH decrease under the same conditions, as shown in Figure 5a (upper traces). It is not clear whether the rate of the fluorescence change under these conditions represents the kinetics of the development of a potential difference or that of the dye response. When 3 M KCl is

TABLE I: Measured Fluorescence and pH Changes and Calculated Proton Motive Forces.

Vesicle Salt Composition (3.0 M)							
External	Internal	Fluorescence Change at 508 nm (%)	pH Change, External	ΔpH^a	$-Z\Delta pH^b$ (mV)	$\Delta\psi^c$ (mV)	Δp^d (mV)
NaCl	NaCl	−35	0.0086	1.80	−106	−120	−229
KCl	NaCl	−21	0.0152	2.00	−118	−72	−190
NaCl	KCl	−18	0.0068	1.86	−110	−62	−172
KCl	KCl	−10	0.0129	2.02	−119	−34	−153

[a] See Discussion for explanation of calculation. [b] Z is 59 mV (see eq 3). [c] Potential difference across the membrane, determined from Figure 9. [d] Proton motive force, calculated from eq 3.

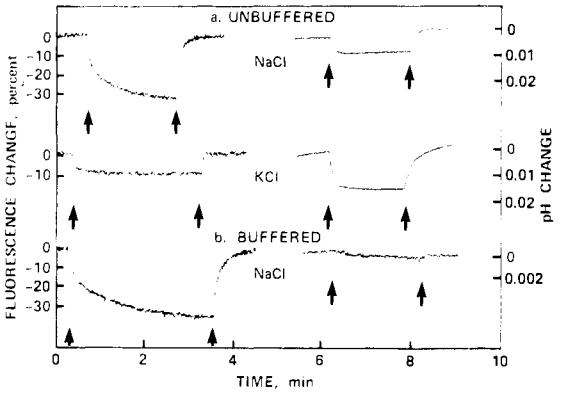


FIGURE 5: Comparison of light-induced fluorescence and external pH changes. Traces of the time course of fluorescence emission at 508 nm (left) and pH (right). (a) Unbuffered salt. Upper traces: 3.0 M NaCl. Lower traces: 3.0 M KCl. Fluorescence: 0.048 mg/ml membrane protein, 3×10^{-7} M dye. pH: 0.096 mg/ml membrane protein. Initial pH was 6.8. (b) Buffered both externally and internally with 0.05 M sodium phosphate buffer in 3.0 M NaCl, pH 5.7, 0.048 mg/ml membrane protein, 3×10^{-7} M dye. For each pair of arrows, the first indicates the beginning and the second the end of illumination.

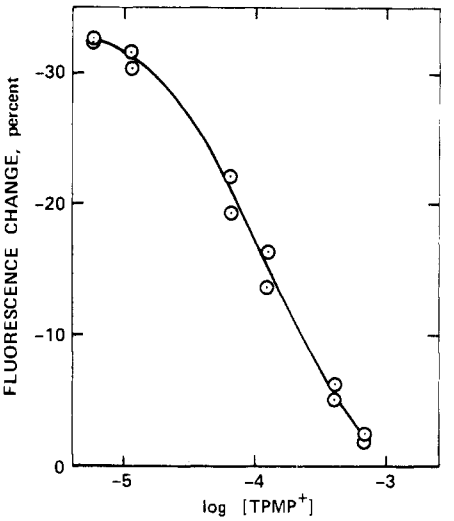


FIGURE 6: Inhibition of light-induced fluorescence change by $TPMP^+$. Fluorescence change at 508 nm after illumination by actinic light as a function of added triphenylmethylphosphonium ion ($TPMP^+$), a membrane permeable cation. Conditions: 0.11 mg/ml membrane protein, 3.6×10^{-7} M dye, 3.0 M NaCl.

present on either or both sides of the membrane, the kinetics of fluorescence change closely parallel the kinetics of the pH change measured with the glass electrode (Figure 5a, lower traces). Moreover, with 3 M potassium ion present outside the vesicles, the light-induced pH changes measured in the external medium were nearly twice those measured when 3 M sodium ion is present, as indicated in column 2 of Table I. At the same time, the fluorescence decrease developed upon illumination is substantially less in 3 M KCl than in 3 M NaCl. These results suggest that the *H. halobium* membrane must be substantially more permeable to K^+ than Na^+ and K^+ movements into the vesicles can partially replace the back-leaking of protons.

Fluorescence and pH Changes in Buffered Vesicles. Before relating the observed light-induced changes in cyanide dye fluorescence to membrane potential, we tested the possibility of direct interaction between the dye and the protons released by illumination.

Figure 5b shows the fluorescence and pH changes in vesicles buffered with phosphate both inside and outside. The external pH change is completely eliminated by the buffer and, very likely, therefore, a large part of the internal pH change as well, but any light-induced charge differences between the outside and the inside of the vesicles are expected to be preserved. As the traces illustrate, the fluorescence change in buffered NaCl is unaffected (although it develops more slowly). In buffered KCl the fluorescence change (not shown) is more than twice as large as the change observed with unbuffered vesicles, for reasons as yet unknown. These results indicate that the observed light-induced changes in fluorescence cannot be caused by a transmembrane pH difference. Unfortunately, uncouplers could not be used in the fluorescence experiments, because dye fluorescence was nearly completely quenched when the uncoupler was added.

Effect of a Membrane-Permeant Cation. Triphenylmethylphosphonium ion ($TPMP^+$) is an organic cation that is permeable in biological membranes and has been extensively used in studies of respiration (Skulachev, 1971). Except for fluorescence, $TPMP^+$ has many of the properties of a cyanine dye, and it has been used as an indicator of membrane potential in a variety of systems (Grinius et al., 1970). Figure 6 shows the effect of increasing $TPMP^+$ concentrations on the light-induced fluorescence changes. It is clear that at 7×10^{-4} M $TPMP^+$ the fluorescence decrease is nearly completely abolished. This result is not due to competition by $TPMP^+$ for any specific cyanine binding sites on the membranes, for even at the highest $TPMP^+$ concentration tested there was no $TPMP^+$-induced release of dye from the membranes, as measured by centrifugation studies similar to those described above. Moreover, the inhibition was not due to a disruption of proton pumping by the vesicles. Figure 7a compares the fluorescence and pH changes observed in vesicles to which $TPMP^+$ was added to 7×10^{-4} M concentration. Clearly, the light-induced acidification is enhanced by $TPMP^+$ (compare, for example, with Figure 5a), in a manner analogous to replacing

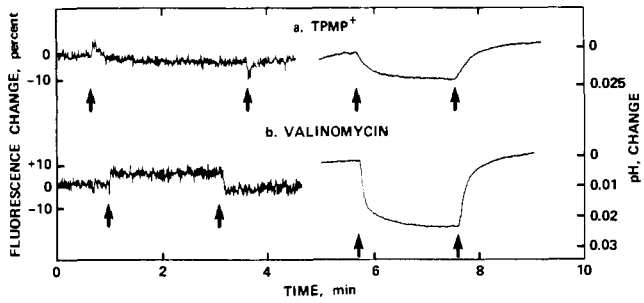


FIGURE 7: Inhibition of light-induced fluorescence change with concomitant preservation of proton gradient. Kinetics of changes in fluorescence (left) and external pH (right) induced by actinic illumination of vesicles. (a) Inhibition by $TPMP^+$. Conditions: 4.0×10^{-7} M $TPMP^+$, 0.11 mg/ml membrane protein, 3.6×10^{-7} M dye, 3.0 M NaCl. Initial pH: 6.95. (b) Inhibition by valinomycin. Vesicles in 3.0 M KCl. Fluorescence: 1.6×10^{-6} M valinomycin, 3.6×10^{-7} M dye, 0.075 mg/ml membrane protein. pH: 0.096 mg/ml membrane protein, 2.9×10^{-6} M valinomycin. Initial pH: 6.8. Illumination period occurred between arrows on each trace.

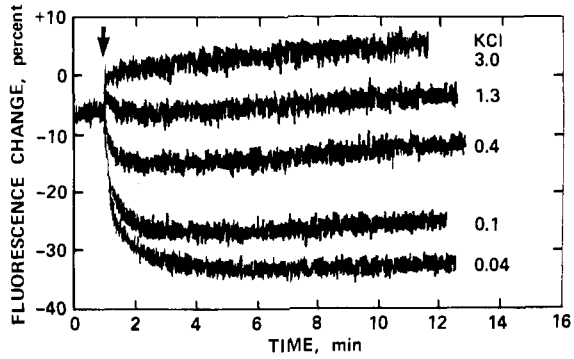


FIGURE 8: K^+-valinomycin-induced fluorescence changes. Kinetics of changes in fluorescence at 508 nm after addition of 10 μl of 1×10^{-4} M valinomycin in ethanol to vesicles (final valinomycin concentration: 2.9×10^{-7} M). Traces are of five separate experiments, each containing 3×10^{-7} M dye and 0.048 mg/ml membrane protein. The vesicles were all loaded with 3.0 M KCL. The external salt was a mixture of NaCl and KCl totaling 3.0 M. The external KCl concentrations were, from the top: 3.0, 1.29, 0.43, 0.13, and 0.043 M. External pH was 6.80 ± 0.05. The fluorescence changes were much less stable at higher valinomycin concentrations: when these were increased tenfold, the fluorescence decrease reversed within a few minutes after addition of valinomycin.

external NaCl with the more permeable KCl, consistent with the idea that $TPMP^+$ competes with the dye solely for charge transport.

Effect of Ionophores. We examined the effect of several cation carriers on the light-induced effects in the envelope vesicles. Gramicidin (a membrane pore) and valinomycin (a K^+ carrier) were each added to the vesicles in order to bring about cation movements across the membranes which may further test the relationship between fluorescence and membrane potential.

At a concentration of 1.4×10^{-7} M, gramicidin completely abolishes the fluorescence change. The trace is identical with Figure 7a and is consistent with the findings using $TPMP^+$, but in this case direct interference with the dye is ruled out. $TPMP^+$ increases membrane conductance and abolishes the electrical potential measured at concentrations over three orders of magnitude greater than the cyanine dye concentration used to detect the potential. By contrast, gramicidin is able to increase membrane conductance to the same point at one-third the concentration of the dye.

The potassium-ionophore, valinomycin, also abolishes the light-induced fluorescence change. With valinomycin concentrations of 1.6×10^{-6} M, the light-induced fluorescence change reversed and became an increase, as shown in Figure 7b. This increase was identical with the light-induced fluorescence changes seen with nonvesicular patches of isolated purple membrane, as discussed above, which cannot be due to transmembrane effects. Like $TPMP^+$, valinomycin enhances the light-induced pH gradient detected. For example, at a concentration of 3×10^{-6} M valinomycin in 3 M KCl, the external pH change observed increased from 0.0129 to 0.0216, as shown in Figure 7b. Addition of valinomycin in the dark caused a small, slow external pH rise. This effect was observed even in the absence of KCl. In the experiment in Figure 7b, this alkaline effect was about ⅕ the magnitude of the light-induced acidification.

Calibration of the Fluorescence Change with K^+ Diffusion Potential. By increasing the permeability to K^+ alone, valinomycin can give rise to a potential difference across membranes that have differing potassium ion concentrations on either side. This effect has been used by Hoffman and Laris (1974) and by Sims et al. (1974) to calculate the apparent membrane potential measured by a particular amount of fluorescence change. This calculation assumes that the valinomycin will carry potassium ions across the membranes to the maximum Nernst potential possible, that is

$$\Delta\psi = -\frac{RT}{F} \ln \frac{(K^+_{in})}{(K^+_{out})} \qquad (2)$$

where $\Delta\psi$ is the potential difference across the membrane in mV, R is the gas constant, T is the temperature in K, F is Faraday's constant, (K^+_{out}) is the potassium ion activity outside the vesicles, and (K^+_{in}) is the potassium ion activity inside the vesicles. It is implicit in these calculations that in the presence of valinomycin the permeability of all other ions is negligible relative to K^+.

The potential induced by valinomycin in the *H. halobium* system is entirely a diffusion potential. There can be no appreciable contribution to the total membrane potential from a difference in surface charge across the membrane at the high salt concentrations used in our experiments. However, an adverse consequence of the high ionic strength is that we do not know the actual activities of Na^+ and K^+. In 3 M KCl the activity coefficient of potassium is about 0.6 (Moore, 1962). We have assumed, in applying the Nernst equation, that in mixtures of highly concentrated NaCl and KCl, at a total of 3 M, the activity coefficient of KCl is approximately the same as that in 3 M KCl. This assumption is probably valid because the activity coefficient of Na^+ in 3 M NaCl is near 0.7. Thus, we have used potassium ion concentrations in the Nernst equation rather than activities, in calculating the potentials given below.

We added 0.05-ml samples of vesicles, containing 3 M KCl inside, to 3.45 ml of 3 M salt of varying composition (from 3 M NaCl to 3 M KCl). The dye was added and a fluorescence baseline was recorded for about 2 min. At this time valinomycin was added to 3×10^{-7} M concentration. Typical traces showing the fluorescence changes are given in Figure 8, where the valinomycin-induced fluorescence decreases are seen to resemble the light-induced changes. The decreased fluorescence levels were stable for over 10 min. The initial fluorescence baselines have been superimposed in Figure 8. External 3 M NaCl gives slightly lower initial fluorescence (4%) than external 3 M KCl. This difference could be due to the presence of an intrinsic K^+-ionophore in the vesicles or to a different effect of K^+ and Na^+ on the dye-membrane interaction.

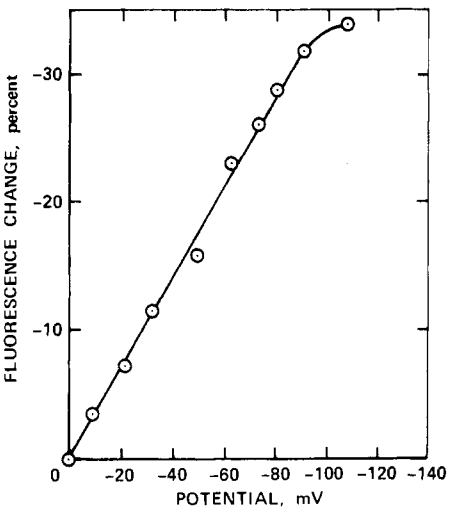


FIGURE 9: Calibration of fluorescence change to calculated diffusion potential. The maximum fluorescence changes from the experiments shown in Figure 8 (and additional points not shown there) are plotted against the electrical potential across the vesicle membrane (assuming valinomycin causes free outward diffusion of K^+).

With no potassium gradient (i.e., in either all sodium chloride or all potassium chloride) there was a small, rapid increase in fluorescence after addition of valinomycin. This may be due to an interaction between valinomycin and the dye or valinomycin and the membrane, for at higher concentrations of valinomycin the increase was greater. The results given below have been corrected for the small increase in fluorescence observed in the all KCl system.

Figure 9 shows the fluoresence decreases observed at varying Nernst potentials. At high potentials the fluorescence decrease begins to level off near −36%, but below this value the correlation between the measured and the calculated values is linear. In this region every percent fluorescence change corresponds to −2.8 mV membrane potential, compared with −1.3 mV, obtained by Hoffman and Laris (1974) in red blood cells. The maximum light-induced fluorescence change we observed (Table I) was −35%, in the all NaCl system. Thus, we conclude that in this case the light-induced potential is at least −120 mV. In the physiologically more pertinent case, with KCl inside and NaCl outside (Christian and Waltho, 1962; Ginzburg et al., 1970; Lanyi and Silverman, 1972), the membrane potential induced by saturating intensities of light appears to be about −62 mV.

Discussion

The results reported here give an estimate of the transmembrane electrical potential in the envelope vesicles from *H. halobium,* developed under saturating illumination. We find, by measuring changes in the fluorescence of a cyanine dye, that the potential is as much as −120 mV. Changes in cyanine dye fluorescence have been observed in studies of transmembrane electrical potentials of red blood cells (Sims et al., 1974; Hoffman and Laris, 1974), squid axons (Cohen et al., 1974), bacteria (Kashket and Wilson, 1974), and mitochondria (Tedeschi, 1974; Laris et al., 1975). In these studies the results indicate that the fluorescence changes observed upon inducing a membrane potential are approximately proportional to the membrane potential. The approximate nature of the correlation is due to uncertainties in (1) actual ion activities; (2) membrane permeabilities to all ions in the system; and (3) calibrations with other techniques, such as microelectrodes.

The *H. halobium* cell envelope vesicles we have studied are in several respects simpler than some of the other membrane systems in which cyanine dyes have been used. MacDonald and Lanyi (1975) described these vesicles previously and showed that they are uniform and largely right-side out, and that they contain functional patches of correctly oriented purple membrane. Like *H. halobium* cells, upon illumination, these vesicles pump protons out, acidifying the external medium. Thus, the effects we have studied may be elicited without adding any chemical perturbants to the system. Moreover, the composition of both the internal and external medium may be varied in this system. As a result, for example, we are able to infer that the *H. halobium* membrane is more permeable to K^+ than Na^+.

According to the chemiosmotic hypothesis of energy transduction (Mitchell, 1969), the synthesis of ATP is driven by the sum of the electrical potential and chemical gradient terms of a proton motive force (Δp). In units of millivolts, or chemical potential per equivalent of electrical charge, Δp was expressed by Mitchell as follows:

$$\Delta p = \Delta\psi - Z\Delta\text{pH} \qquad (3)$$

where $\Delta\psi$ is the transmembrane electrical potential, ΔpH is the difference in pH developed across the membrane, and Z is $2.3(RT/F)$ (about 59 mV at 23 °C). It has been argued that not all of this energy is available for ATP synthesis, and some workers have emphasized the importance of $\Delta\psi$ (Williams, 1974) or of ΔpH (Weber, 1974) to energy coupling. We have attempted to calculate the amount of energy that might be available for light-induced ATP synthesis, as implied in our measurements of light-induced changes in external pH and fluorescence. Table I gives the observed fluorescence changes and pH changes in columns 1 and 2. In column 3, the external acidification is converted to transmembrane pH difference (ΔpH) after the following considerations: (1) The internal pH was assumed to be equal to the external pH at the start of the experiment. This is likely because the internal medium is experimentally controlled and the vesicles are metabolically inactive under our experimental conditions. (2) The buffering capacity of the inside surface of the membrane nearly equals that of the outside surface (R. Bogomolni, unpublished experiments): vesicles were titrated between pH 6 and 8 in the presence and absence of 10^{-5} M carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, a transmembrane proton carrier); the buffering capacity of the vesicles in the presence of CCCP was almost exactly twice that of the vesicles alone. (3) We found the buffering capacity of the outside surface sharply increased above pH 8. The upturn was salt dependent, occurring at 8.2 in NaCl and 8.5 in KCl. The buffering capacity above these values increased by a factor of four. (4) The internal volume of the vesicles is 3 μl/mg protein (MacDonald and Lanyi, 1975).

In all KCl, for example, the external change in pH is a decrease of 0.0129 from the initial pH of 6.8. Thus, the net external increase in $[H^+]$ after buffering by the external membrane surface, is 4.8×10^{-9} M. At a protein concentration of 0.1 mg/ml, the internal vesicle volume is smaller than the volume of the external medium by a factor of 3.5×10^3. Thus, the decrease in internal proton concentration will be about 1.7×10^{-5} M. Of this amount, a decrease of 3×10^{-6} M H^+ brings the pH to 8.5. Above that level, the remaining 1.4×10^{-5} M is more strongly buffered and represents a decrease of only $(1.4/4) \times 10^{-5}$ M, or 3.5×10^{-6} M H^+. Thus, the total H^+ decrease inside the vesicles is about 6.5×10^{-6} M. This corresponds to a pOH of 5.2, or a final internal pH of 8.8. Therefore, the ΔpH across the membrane is 2.0.

In column 4, the ΔpH is converted to mV by multiplying by −59. In column 5, the fluorescence changes observed are converted to mV by consulting Figure 9. In column 6, the total proton-motive force implied by our measurements is summed from the $\Delta\psi$ and ΔpH contributions.

The calculated values for total proton-motive force range from 150 to 230 mV, near the values believed to be required for synthesis of ATP in vivo. At the physiologically relevant salt composition (internal KCl, external NaCl) we measured the proton-motive force as −172 mV. It should be emphasized that this is a minimal value, applying only to the vesicle system used in these studies, which are probably more permeable to protons than intact cells. Light-stimulated [$^{32}P_i$]ATP exchange has been observed in *H. halobium* envelope vesicles similar to ours (E. Racker, personal communication), but its relationship to any ion gradients is not yet clear. In *Streptococcus lactis,* Maloney et al. (1974) have measured valinomycin-induced ATP synthesis. In this bacterial cell system the production of ATP was studied as a function of proton-motive force, summed from valinomycin-K^+ diffusion potentials and pH indicator measurements (Maloney and Wilson, 1975). It was found that, while no net ATP synthesis could be detected below about 200 mV of proton-motive force, above this level ATP production proceeded regardless of the relative contributions of $\Delta\psi$ and ΔpH. The relative contributions of these two components of the proton-motive force and the magnitude of the latter are determined in *H. halobium* vesicles largely by the movements of K^+, the more permeant of the cations present. It is apparent, however, that in this system the light-dependent proton pump should be able to energize the synthesis of ATP entirely through chemiosmotic gradients.

Acknowledgments

We thank Dr. Alan Waggoner for his generous gift of the cyanine dyes. We are grateful to Drs. Waggoner and Roberto Bogomolni for suggesting these experiments and offering their advice and encouragement. We also thank Vernon Yearwood-Drayton for his expert technical assistance.

References

Baxter, R. M. (1960), *Can. J. Microbiol. 6,* 417.
Blaurock, A. E. (1975), *J. Mol. Biol. 93,* 139.
Blaurock, A. E., and Stoeckenius, W. (1971), *Nature (London), New Biol. 233,* 152.
Bogomolni, R., and Stoeckenius, W. (1974), *J. Supramol. Struct. 2,* 775.
Christian, J. H. B., and Waltho, J. A. (1962), *Biochim. Biophys. Acta 65,* 506.
Cohen, L. B., Salzberg, B. M., Davila, H. V., Ross, W. N., Landowne, D., Waggoner, A. S., and Wang, C. H. (1974), *J. Membr. Biol. 19,* 1.
Danon, A., and Stoeckenius, W. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1234.
Ginzburg, M., Sachs, L., and Ginzburg, B. Z. (1970), *J. Gen. Physiol. 55,* 187.
Greville, G. D. (1969), *Curr. Top. Bioenerg. 3,* 1.
Grinius, L. L., Jasaitis, A. A., Kadzyauskas, Yu. P., Liberman, E. A., Skulachev, V. P., Topali, V. P., Tsofina, L. M., and Vladimirova, M. A. (1970), *Biochim. Biophys. Acta 216,* 1.
Henderson, R. (1975), *J. Mol. Biol. 93,* 123.
Henderson, R., and Unwin, P. N. T. (1975), *Nature (London) 257,* 28.
Hoffman, J. F., and Laris, P. C. (1974), *J. Physiol. 239,* 519.
Kashket, E. R., and Wilson, T. H. (1974), *Biochem. Biophys. Res. Commun. 59,* 879.
Kayushin, L. P., and Skulachev, V. P. (1974), *FEBS Lett. 39,* 39.
Kelly, M., Norgard, S., and Liaaen-Jensen, S. (1970), *Acta Chem. Scand. 24,* 2169.
Kushwaha, S., Kates, M., and Martin, W. (1975), *Can. J. Biochem. 53,* 284.
Lanyi, J. K. (1968), *Arch. Biochem. Biophys. 128,* 716.
Lanyi, J. K. (1974), *Bacteriol. Rev. 38,* 272.
Lanyi, J. K., and Silverman, M. P. (1972), *Can. J. Microbiol. 18,* 993.
Laris, P. C., Bahr, D. P., and Chaffee, R. R. J. (1975), *Biochim. Biophys. Acta 376,* 415.
Larsen, H. (1962), *Bacteria 4,* 29.
MacDonald, R., and Lanyi, J. K. (1975), *Biochemistry 14,* 2882.
Maloney, P. C., Kashket, E. R., and Wilson, T. H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3896.
Maloney, P. C., and Wilson, T. H. (1975), *J. Membr. Biol. 25,* 285.
Mitchell, P. (1961), *Nature (London) 191,* 144.
Mitchell, P. (1969), in Theoretical and Experimental Biophysics, Vol. 2, Cole, A., Ed., New York, N.Y., Marcel Dekker, pp 159–216.
Moore, W. (1962), Physical Chemistry, Englewood Cliffs, N.J., Prentice Hall, p 351.
Oesterhelt, D., and Stoeckenius, W. (1971), *Nature (London), New Biol. 233,* 149.
Racker, E., and Hinkle, P. C. (1974), *J. Membr. Biol. 17,* 181.
Racker, E., and Stoeckenius, W. (1974), *J. Biol. Chem. 249,* 662.
Sims, P. J., Waggoner, A. S., Wang, C. H., and Hoffman, J. F. (1974), *Biochemistry 13,* 3315.
Skulachev, V. P. (1971), *Curr. Top. Bioenerg. 4,* 127.
Stoeckenius, W., and Lozier, R. (1974), *J. Supramol. Struct. 2,* 769.
Tedeschi, H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 583.
Weber, G. (1974), *Ann. N.Y. Acad. Sci. 227,* 486.
Williams, R. J. P. (1974), *Ann. N.Y. Acad. Sci. 227,* 98.