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LIGHT-DRIVEN PROTON TRANSLOCATIONS IN *HALOBACTERIUM HALOBIUM*

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SUMMARY

The purple membrane of *Halobacterium halobium* acts as a light-driven proton pump, ejecting protons from the cell interior into the medium and generating an electrochemical proton gradient across the cell membrane. However, the typical response of cells to light as measured with a pH electrode in the medium consists of an initial net inflow of protons which subsides and is then replaced by a net outflow which exponentially approaches a new lower steady state pH level. When the light is turned off a small transient acidification occurs before the pH returns to the original dark level. We present experiments suggesting that the initial inflow of protons is triggered by the beginning ejection of protons through the purple membrane and that the initial inflow rate is larger than the continuing light-driven outflow. When the initial inflow has decreased exponentially to a small value, the outflow dominates and causes the net acidification of the medium.

The initial inflow is apparently driven by a pre-existing electrochemical gradient across the membrane, which the cells can maintain for extended times in the absence of light and oxygen. Treatments which collapse this gradient such as addition of small concentrations of uncouplers abolish the initial inflow.

The triggered inflow occurs through the ATPase and is accompanied by ATP synthesis. Inhibitors of the ATPase such as *N,N'*-dicyclohexylcarbodiimide (DCCD) inhibit ATP synthesis and abolish the inflow. They also abolish the transient light-off acidification, which is apparently caused by a short burst of ATP hydrolysis before the enzyme is blocked by its endogenous inhibitor.

Similar transient inflows and outflows of protons are also observed when anaerobic cells are exposed to short oxygen pulses.

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $TPMP^+$, triphenylmethylphosphonium.

INTRODUCTION

Several light-induced reactions have been observed in *Halobacterium halobium*. Phototactic responses [1], pH changes, inhibition of respiration [2] and ATP synthesis [3] occur when bacterial suspensions are irradiated with visible light. A purple chromoprotein, bacteriorhodopsin, forms a two-dimensional hexagonal lattice in patches of the plasma membrane. These patches have been termed the "purple membrane". They function as a light-driven proton pump which translocates protons out of the cells [2, 4].

Most of the light-induced effects observed in bacterial suspensions can be attributed to the presence of the purple membrane. Action spectra for ATP synthesis [3] and for inhibition of respiration and pH changes [5] indicate that bacteriorhodopsin is the pigment responsible for these effects. Purple membrane apparently converts light energy into an electrochemical gradient across the cell membrane. The cells can use the energy stored in the gradient to synthesize ATP through a reversible ATPase in the manner described for oxidative phosphorylation and chlorophyll-dependent photophosphorylation in Mitchell's chemiosmotic theory of energy coupling [6].

The recorded light-induced pH changes in cell suspensions are complex and may reflect control functions in the energy metabolism of the cells [7]. Proton fluxes in both directions are observed and their extent depends upon various external conditions such as light intensity, temperature, initial pH of the suspension and oxygen availability and also upon the initial state of the cells with respect to purple membrane content and presence of endogenous substrate. We report here a detailed study of the effect of these parameters on the pH changes and propose a consistent explanation.

METHODS

Bacterial suspensions

Halobacterium halobium R_1 is grown in a complex medium containing Oxoid peptone 1 % w/v in a basal salt solution containing 4.27 M NaCl, 0.027 M KCl, 0.081 M $MgSO_4$ and 0.0018 M CaCl, essentially as described previously [8]. Cells are harvested usually after 96 h at 37 °C when cell concentration of $2.5 \cdot 10^9$ cells/ml and purple membrane concentrations of approx. 10^{-14} g bacteriorhodopsin per cell are obtained. Bacteriorhodopsin concentration was determined following Danon and Stoeckenius [3]. Cells with low concentrations of purple membrane are obtained by increasing the aeration of the cultures.

The cells are centrifuged, resuspended in basal salt solution and washed at least once. The final pellet is resuspended in basal salt and adjusted to the appropriate cell concentration, typically $5 \cdot 10^9$ cells/ml. The cells are then aerated for approx. 1 h before use. Cell lysis is monitored by measuring the absorbance of the suspending medium at 260 and 280 nm. Suspensions with more than 5 % lysis are discarded. The determination of menadione reductase activity can serve as an alternative technique for determining cell lysis [9]. The pH of all cell suspensions is adjusted to the desired value by the addition of NaOH or HCl.

Cell concentrations are measured in a Petroff-Hauser counting chamber or by the apparent absorbance of the cell suspension at 600 nm in a Coleman Junior spectro-

photometer after calibration of the apparent absorbance versus cell number. Cell protein was determined in cell lysates with the biuret technique.

Chemicals

The following chemicals were used: KCN, sodium dithionite, NaCl and KCl from J. T. Baker; nigericin from Lilly Laboratories, Eli Lilly and Co.; Glycerol and $MgSO_4 \cdot 7H_2O$ from Mallinckrodt; Oxoid Bacteriological Peptone L37 from Oxoid Limited; FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) from Pierce Chemical Co.; DCCD (*N,N'*-dicyclohexylcarbodiimide) from Schwarz/Mann; phenylmercuric acetate and CCCP (carbonyl cyanide, *m*-chlorophenylhydrazone) from Sigma; valinomycin from Sigma Chemical Co. or Calbiochem; and $TPMP^+$ (triphenylmethylphosphonium) from K and K Laboratories.

Measuring chamber

The experiments are carried out in specially designed polymethacrylate or stainless steel chambers. Equilibration of the cell suspensions with air is provided through a hydrophobic Millipore membrane which forms the back wall of the chamber. Water-saturated air is blown continuously past the outer surface of this membrane. This measuring configuration will be referred to as an open chamber. Anaerobiosis is attained by replacing the Millipore membrane by a solid plug and sealing the top opening. This will be referred to as the closed chamber. The suspensions are illuminated through an acrylic window in the front wall of the chamber. The window area is 1.1 cm^2 for all chambers used. Specially designed top plugs permit the addition of reagents and the insertion of a thermocouple. Stirring is provided by a Teflon-coated magnetic spinfin and temperature control by a water jacket connected to a circulating thermostated bath. Proton concentrations are measured with a Beckman combination glass electrode (Beckman Instruments No. 39003) and an electrometer amplifier. Oxygen tension is followed with a Clark oxygen electrode (Yellow Springs Instruments Co., Inc., Yellow Springs, Ohio) with a measuring circuit of our own construction following the recommendation of the manufacturer.

Unless otherwise indicated, illumination is provided by a 300 W arc lamp (General Electric Co., Marc 300), filtered through 7 cm of water and a Corning 1-75 infrared filter. For broad band actinic illumination, the beam passes through a Corning 3-69 yellow cut-off filter. The highest light intensity available from this system is 10^7 ergs/cm^2 per s. Light intensities are measured with a radiometer (Model 68 Kettering radiometer, Laboratory Data Control Div., Milton Roy Co.) calibrated against a standard filament lamp (National Bureau of Standards). Monochromatic light at 575 nm is obtained with an interference filter (Baird Atomic) with a band width of 25 nm. The maximum light intensity available with this filter is approx. $8 \cdot 10^5$ ergs/cm^2 per s. Different light intensities are obtained with calibrated neutral density filters (Optics Technology, Redwood City, Calif.). Electromechanical rotatory solenoid shutters (Ledex Inc., Dayton, Ohio) initiate and terminate illumination.

Output signals are fed to an 8-pen Beckman Dynograph R or a two-channel Clevite-Brush high speed strip chart recorder. The slowest response time of the pH channel as measured by injection of pulses of standardized acid and alkali is approx. 1.0 s. The sensitivity of the measuring system is 0.001 pH unit, equal to the noise level at the highest amplification.

Absorption spectra are recorded with a Cary 14 R spectrophotometer equipped with the accessory for scattering samples. For the measurement of total incident radiation a chemical actinometer (ferrioxalate photolite solutions [10]) replaces the sample in the measuring chamber.

RESULTS

The light-induced changes in pH of cell suspensions are usually complex and depend on the residual energy supply of the cells, the initial pH and the light intensity and wavelength. The first response to light after the cells have been kept for more than 2 h in the dark usually differs from subsequent reproducible responses. Therefore, in all experiments we preilluminated the cells. Fig. 1A (inset) shows a light-induced pH response of a freshly harvested cell suspension under anaerobic conditions. A cell suspension under these conditions has a large supply of endogenous substrate for respiration which lasts for several hours under aeration. After a short delay (or sometimes a small initial acidification, see Fig. 7) we observe transient net alkalinization, followed by a net acidification until a new lower pH level in the medium is reached. Under constant illumination the pH then remains at this level or may show a very slight and slow upward drift. When the light is shut off, a small transient acidification occurs and then the pH returns to a value close to the original dark level. The net acidification shown in Fig. 1A corresponds to about 45 protons for every bacteriorhodopsin molecule present. The transient net alkalinization is of comparable magnitude. These observations clearly indicate that the acidification reflects an outflow of protons from the cells and the alkalinization an inflow rather than reversible proton release and binding by the purple membrane (see also Figs. 2 and 4).

In order to analyze the fluxes we plot the light-induced pH transients on a semilogarithmic scale (Fig. 1B). The steady-state pH in the light is chosen as the baseline. The plot shows that the proton concentration approaches exponentially the baseline at long times, with half time $t_{\frac{1}{2}}^{\text{acid}}$. Therefore, we resolve an exponential acidification component and extrapolate this component to zero time to obtain the intercept $\Delta H_{\text{max}}{}^{+\text{acid}}$. Because the extrapolated segment lies above the original curve, subtraction of the extrapolated portion of this line from the early portion of the observed curve results in negative differences; the absolute value of these is plotted on a semilog scale resulting in another exponential component with $t_{\frac{1}{2}}^{\text{alk}}$ and intercept $\Delta H_{\text{max}}{}^{+\text{alk}}$. The data points at early times deviate from this second extrapolated line as would be expected from the fact that the alkaline transient occurs after a small delay. We therefore define a delay time t_{d} such that the observed net acidification $\Delta H_{\text{net}}{}^{+\text{acid}}$ is: $\Delta H_{\text{net}}{}^{+\text{acid}} = \Delta H_{\text{max}}{}^{+\text{acid}} - \Delta H_{t_{\text{d}}}{}^{+\text{alk}}$. We interpret the latter as a proton inflow and the positive extrapolated line as an outflow. The observed effect should then be given by:

$$\Delta H^{+}_{\text{observed}} \equiv \Delta H_{t_{\text{d}}}^{+\text{alk}} [1 - e^{-k_{\text{alk}} \cdot t}] - \Delta H_{\text{max}}^{+\text{acid}} [1 - e^{-k_{\text{acid}} \cdot t}]$$

where $k_{\text{alk}} = 0.69/t_{\frac{1}{2}}^{\text{alk}}$ and $k_{\text{acid}} = 0.69/t_{\frac{1}{2}}^{\text{acid}}$

For the obvious discrepancy at early times see Discussion. From this kind of analysis we may obtain calculated values for the initial rates of proton translocation in either direction, and the extent of the two effects as a function of light intensity or other parameters.

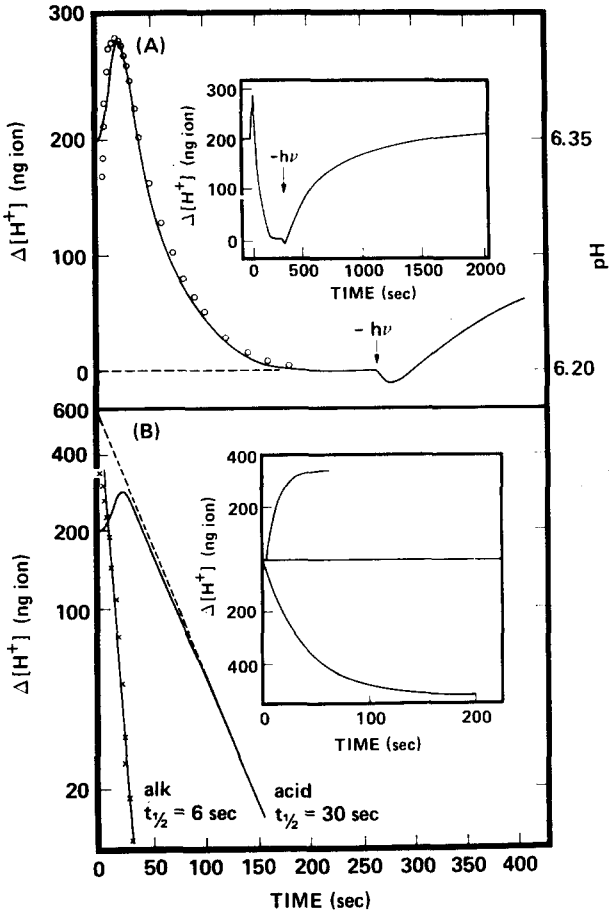

Fig. 1. Light-induced pH change in an anaerobic cell suspension. Bacteriorhodopsin concentration, $8 \cdot 10^{-15}$ g/cell; cell concentration, $5 \cdot 10^{9}$ cells/ml (1.3 mg protein/ml); light intensity, $6 \cdot 10^{5}$ ergs/cm^2 per s (effective bandwidth 530–680 nm; light source, 300 W tungsten-halogen filtered through 3 cm of water and Corning filters 3-69 and 1-75); temperature, 29 °C; volume, 3 ml. The light is turned on at time zero. (A) (—) Time-expanded plot of the pH change (full response is given in the inset); (– – –) baseline for the kinetic analysis shown in B. (B) (—) Semilogarithmic plot of the response shown in Fig. 1A obtained with a Hewlett-Packard logarithmic converter. The broken and solid straight lines are the kinetic components described in the text. The inset is a linear plot of the extrapolated exponential components where the outflow begins at time zero and the inflow is delayed by $t_d = 3$ s. (○) Reconstitution of the light response from the two exponential components (for discrepancies at early times see Discussion).

Action spectra [5, 7] show that both the alkalinization and the acidification, and by implication the influx and outflux, depend on the absorption of light by the purple membrane. The pH response curve varies considerably with experimental conditions. Only net alkalinizations or acidifications may be observed or larger transient acidifications may precede the initial alkalinization. These effects will be described in detail later. We shall first discuss experiments pertaining to the problem of how light absorption by the purple membrane, which is only known to cause a

translocation of protons from the cytoplasmic side to the external surface, can lead to an alkalinization of the medium, i.e. a net influx of protons.

The relative contribution of the alkaline and acid transients is markedly affected by the energy supply of the bacteria. The light-induced net proton uptake diminishes during starvation and addition of substrate (glycerol) restores it. It should be pointed out that such starvation experiments are difficult because of the extended time periods required to achieve starvation. Cell lysis has to be monitored and even proper control of relevant parameters such as temperature, light intensity and pH does not suffice because cell parameters such as purple membrane and respiratory enzyme content may change during these long term experiments. However, such experiments demonstrate a correlation between the alkalinization transient and the availability of an energy source.

The starvation experiments are consistent with the earlier suggestion that the initial inflow of protons represents the light-triggered relaxation of a pre-existing electrochemical potential [2]. Proton ionophores such as CCCP should dissipate such a preexisting protonmotive force generated by respiration. Fig. 2 shows the effect of adding CCCP to a respiring cell suspension after the light-induced steady-state pH level has been established. CCCP causes a rapid alkalinization of the medium and establishes a new pH level which is higher than the original dark level. The CCCP concentration is high enough to relax the gradient generated by the light completely. When the light is switched off no further pH changes are observed and a subsequent illumination period has only a negligible effect.

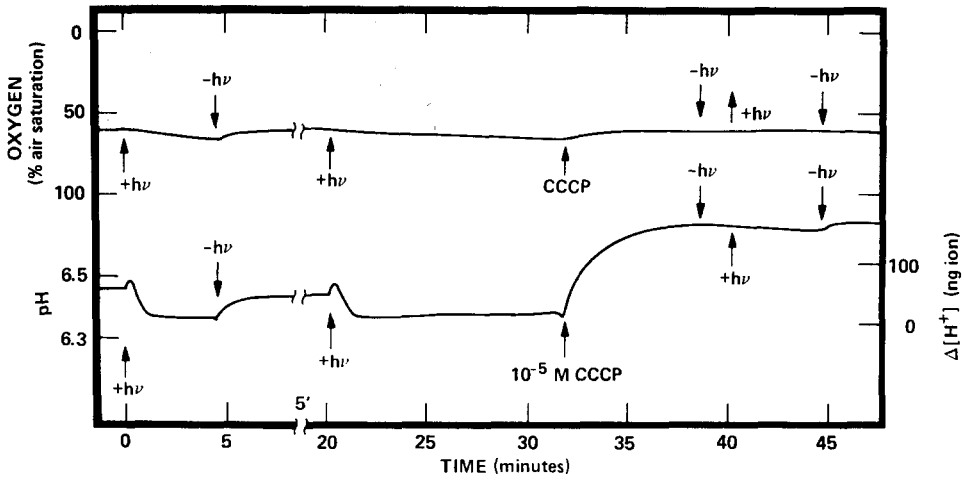

Fig. 2. Effect of CCCP at high concentrations on the light-induced pH changes of an aerobic suspension of freshly harvested bacteria. The inhibition of respiration by light is seen during the first and second illuminations as an increase in the oxygen tension in the air equilibrated measuring chamber (equilibration is obtained through a hydrophobic Millipore membrane which makes the back wall of the chamber). Addition of CCCP to a concentration of $1 \cdot 10^{-5}$ M during the light-on phase of the light-induced pH response results in a marked alkalinization of the suspension. No appreciable changes are observed when the illumination is discontinued. A subsequent illumination produces only a small acidification with no apparent effect on the respiration. Bacteriorhodopsin concentration, $6.5 \cdot 10^{-15}$ g/cell; cell concentration, $4.5 \cdot 10^{9}$ cells/ml (0.9 mg protein/ml); light intensity, 10^{6} erg/cm^2 per s (530–680 nm); temperature, 39 °C; volume, 2 ml.

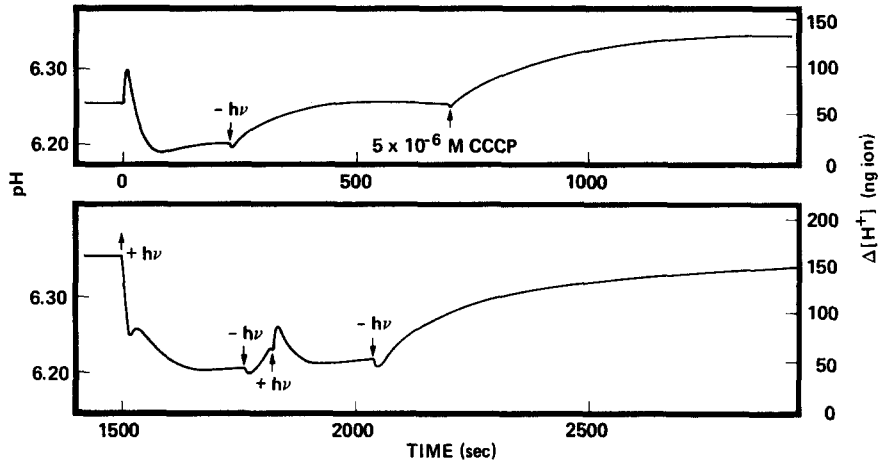

Fig. 3. Effect of CCCP at low concentration on an anaerobic suspension. Addition of CCCP at lower concentrations ($5 \cdot 10^{-6}$ M) in the dark results in a net alkalinization. Illumination of the suspension when a new dark steady state was reached results in a prompt acidification, and an inflow (alkalinization) is observed when an amount of protons equivalent to those driven inwards after addition of CCCP is pumped out by the action of light. Illumination is discontinued after a steady state is reached in the light. Resumption of illumination during the phase of dark alkalinization gives the typical biphasic response. Bacteriorhodopsin concentration, $7.5 \cdot 10^{-15}$ g/cell; cell concentration, $5 \cdot 10^9$ cells/ml (1.1 mg protein/ml); light intensity, $3 \cdot 10^5$ erg/cm^2 per s at 575 nm (25 nm bandwidth); temperature, 24 °C; volume, 3 ml.

That the pH level after addition of CCCP is higher than the original dark level suggests that even before the light caused an ejection of protons from the cells an electrochemical gradient of the same sign existed. This can be demonstrated by adding CCCP in the dark. Fig. 3 shows this for an anaerobic cell suspension. Illumination of the cells results in a transient alkalinization and sustained acidification of the medium with the same characteristics observed under aerobic conditions. After the light has been turned off and the original pH level reestablished, addition of CCCP results in an alkalinization which indicates a rather large inflow of protons into the cells. Illumination of the cells before CCCP is added in the dark is not necessary to obtain the effect. Apparently the cells can maintain a protonmotive force across the cell membrane for extended times in the absence of external energy sources.

The high uncoupler concentration used in Fig. 2 abolishes all the light-induced pH changes, because the back leakage of protons is so fast that even very large proton pumping rates obtained by a combination of high light intensity and high purple membrane content cannot develop a measurable steady-state pH difference across the membrane. At the lower concentration of uncoupler used in Fig. 3 this is not so. Illumination after addition of uncoupler in this case causes a prompt acidification of the medium with no indication of any alkalinization. However, a transient inflow is observed when an outside pH level equal to the original dark level has been reached; that is, when as many protons have been pumped out of the cells as had entered it after the addition of uncoupler. This transient inflow is followed by a further acidification. When the light has been turned off the pH increases and reillumination, before the pH has reached the level at which the transient inflow was observed, will again cause a transient inflow.

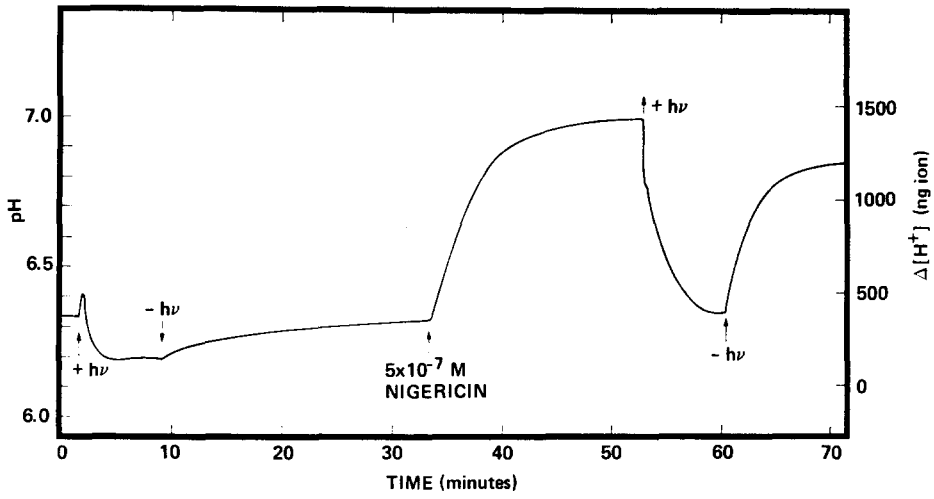

Fig. 4. Effect of nigericin on the light-induced pH changes of an anaerobic cell suspension. Features similar to those observed in Fig. 3 are observed following addition of nigericin. Freshly harvested cells, concentration $4.5 \cdot 10^9$ cells/ml (0.9 mg protein/ml); light intensity, 10^6 ergs/cm^2 per s (530–680 nm); temperature, 24 °C; volume, 3 ml.

Another agent that should abolish any inward directed proton gradient is the antibiotic nigericin, which can mediate the stoichiometric exchange or H^+ for K^+ across biological membranes [11]. Addition of nigericin to *H. halobium* cells at relatively low concentrations (Fig. 4) induces a very large alkalinization of the medium. Cells of halobacteria are known to contain high internal K^+ concentrations [12–14]. The observed alkalinization of the medium is consistent with the potassium proton antiport function attributed to nigericin. Illumination after addition of nigericin causes a prompt acidification of the medium with no trace of a transient alkalinization. Only later, after a considerable decrease in the pH of the medium has occurred, does a small transient inflow appear.

All the experiments discussed so far strongly suggest that the transient inflows observed are driven by gradients which may have been established either by respiration or by light and that the transient inflows are triggered when a certain critical value of the protonmotive force or some parameter associated with it has been reached. That respiration generates such gradients is expected from Mitchell's chemiosmotic hypothesis and has been amply demonstrated for mitochondria and some prokaryotic cells [15]. It is also true for halobacteria (Fig. 5). When a small amount of H_2O_2 is added to an anaerobic cell suspension, catalase activity in the cell suspension causes an immediate release of O_2 which is rapidly consumed by the cells (upper trace). A concomitant large acidification of the medium occurs comparable in extent to the acidification caused by light. When the O_2 is exhausted the pH begins to rise again and this rise is greatly accelerated by the addition of CCCP (not shown). Fig. 5 also shows that the O_2 but not the light effect is blocked by phenylmercuric acetate, which is an inhibitor of the electron transport chain in halobacteria [9]. The effectiveness of the respiratory inhibition is obvious from the slow utilization of the oxygen after addition of phenylmercuric acetate. It can be seen in Fig. 5 that the return from

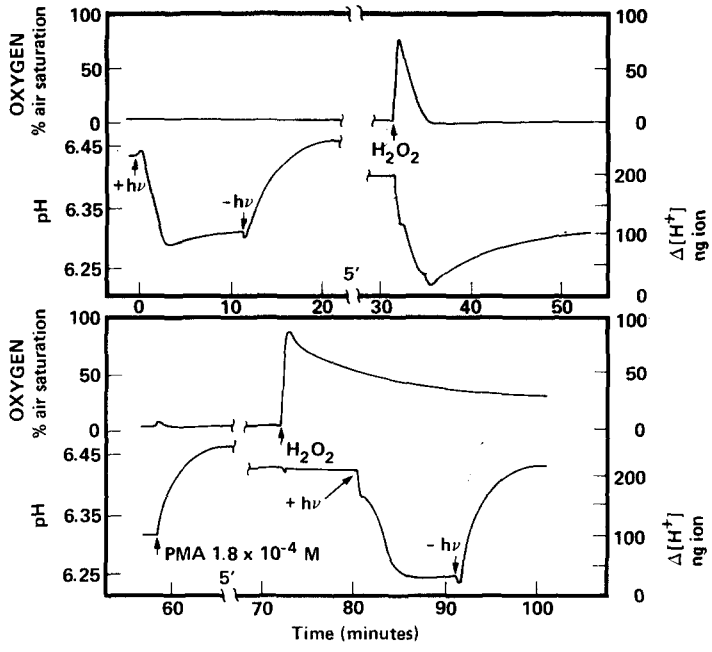

Fig. 5. Inhibition of the respiratory proton translocation by phenylmercuric acetate (PMA). Acidification of the anaerobic cell suspension is elicited by light and subsequently by oxygen from an H_2O_2 pulse. Addition of $1.8 \cdot 10^{-4}$ M phenylmercuric acetate causes an alkalinization of the suspension and completely inhibits the H_2O_2-induced acidification while the light-induced acidification is not affected. Bacteriorhodopsin concentration, $6 \cdot 10^{-15}$ g/cell; cell concentration, $4 \cdot 10^9$ cells/ml (0.88 mg protein/ml); light intensity, $2 \cdot 10^6$ erg/cm^2 per s (530–680 nm); temperature, 39 °C; volume, 3 ml.

the lower pH level after oxygen has been consumed is not as fast as after light is turned off. Also, it does not return to the original level even after CCCP has been added (not shown). We ascribe this to the production of acid during the oxidation of substrate.

Illumination of respiring bacterial cells reduces their rate of oxygen consumption [2, 16]. Illumination with $2 \cdot 10^6$ erg/cm^2 per s inhibits the rate of oxygen consumption by about 70%. The mechanism that links light absorption by the purple membrane to respiration has not been established. The simplest explanation is that the back pressure of the light-generated electrochemical gradient inhibits the rate of electron transport in the respiratory chain. To test this we add CCCP to a respiring cell suspension in a closed chamber (Figs. 6a and 6b); changes in oxygen concentration and pH are recorded simultaneously. In spite of the sloping baseline, the typical pH response to light can be seen and the decrease in pH in the medium coincides with the decrease in oxygen consumption. Addition of $2 \cdot 10^{-5}$ M CCCP causes the expected increase in pH and restores the original rate of respiration. Light now has no significant effect on pH or respiration. If, instead of CCCP, the lipophilic cation $TPMP^+$ is added, no effect on the respiration rate is observed; however, $TPMP^+$ causes a further decrease in the pH of the suspension (Fig. 6c and 6d). These observations substantiate our contention that both respiration and light absorption by the purple membrane

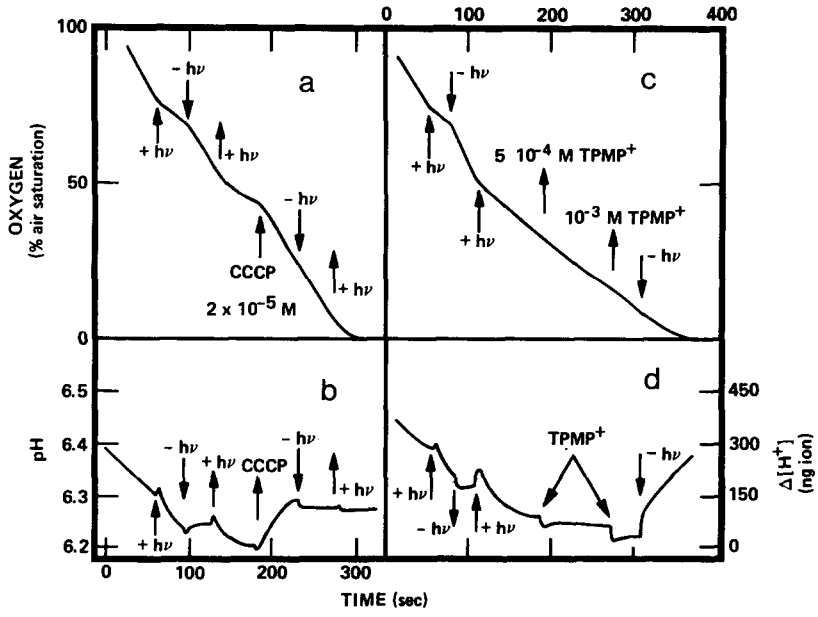

Fig. 6. Inhibition of respiration by light: effect of uncouplers and lipophilic cations. The experiments are conducted in a closed chamber and are initiated by a pulse of H_2O_2. Respiration-induced acidification is seen in the pH traces. Illumination of the suspension results in a decrease of the rate of oxygen uptake and the light-induced pH change can be observed in spite of a sloping baseline. The rate of oxygen uptake is restored when the light is shut off. In both traces a second illumination period is initiated, and while addition of CCCP to $2 \cdot 10^{-5}$ M in the light releases the inhibitory action of light (a, b), the lipophilic cation $TPMP^+$ at concentrations up to 10^{-3} M has no significant effect (c, d). Bacteriorhodopsin concentration, $7 \cdot 10^{-15}$ g/cell; cell concentration, $4.5 \cdot 10^9$ cells/ml (0.9 mg protein/ml); light intensity, $2 \cdot 10^6$ erg/cm^2 per s (530–680 nm); temperature, 39 °C; volume, 3 ml.

cause an ejection of protons from the cell and generate an electrochemical gradient. The membrane potential is collapsed by the high concentration of a permeant cation but its decreased contribution to the protonmotive force is compensated by the increase in the osmotic component of the electrochemical gradient.

The data presented so far indicate that the primary effect of light absorbed by the purple membrane is an ejection of protons from the cell increasing an existing electrochemical gradient, and that this process triggers a transient inflow of protons so that a transient net alkalinization of the medium is observed. This is followed by a sustained decrease in pH. The transient inflow is driven through energy stored by the cell before the beginning of the experiment in the form of an electrochemical gradient. If this explanation is correct, the initial rate of proton ejection should be proportional to the light intensity; the initial rate for the inflow cannot be predicted unless more is known about the triggering mechanism and the energy which drives the flow. Fig. 7 shows the light-on responses of a cell suspension under anaerobic conditions at various light intensities. It is obvious that the rate and extent of proton ejection increase with increasing light intensity as expected. The graphic analysis of the curves (see page 4) clearly indicates that the addition of the two exponential components does not fit the data at early times (Fig. 8). This is also apparent from a closer inspection of the original curves. Apparently there is a short delay before the inflow sets in. As far as can be

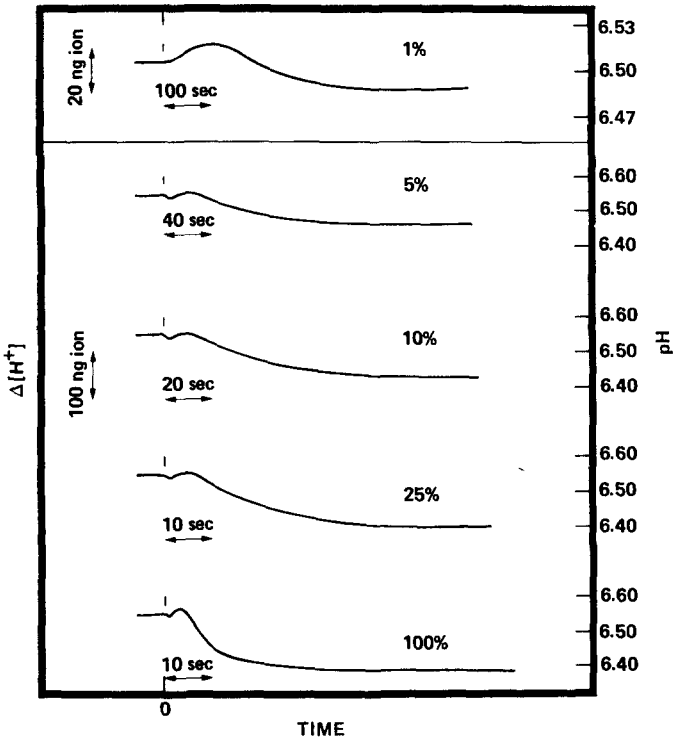

Fig. 7. Effect of light intensity on the pH response of an anaerobic cell suspension. Only the light-on portion of the traces is shown. Bacteriorhodopsin concentration, $8 \cdot 10^{-15}$ g/cell; cell concentration, $4.7 \cdot 10^9$ cells/ml (1.0 mg protein/ml); light intensity (100 %), $8 \cdot 10^5$ ergs/cm^2 per s; temperature, 28 °C; volume, 3ml. Light wavelength 575 nm (25 nm bandwidth). Illumination begins at time zero. Note differing time and proton concentration scales.

determined from the few data points obtained at early times, an initial proton ejection takes place and proceeds at a rate that increases with light intensity while the delay for the inflow decreases. This is consistent with our contention that the inflow is a response triggered by the ejection of protons.

The calculated rates of proton ejection are a linear function of light intensity and so are the initial rates of inflow (Fig. 9). The calculated total proton inflow changes relatively little with light intensity and saturates at a light intensity more than one order of magnitude lower than the proton outflow (Fig. 10). The data for proton ejection derived from the graphic analysis, therefore show the expected dependence on light intensity. The data for the inflow are not as consistent with the simple model proposed here and require further comment (see Discussion). The difference in light intensity required for saturation of inflow and outflow is also demonstrated by the experiment shown in Fig. 11. A cell suspension is illuminated at low intensity producing a net alkalinization of the medium. A sudden increase in light intensity results in prompt acidification without any further transient alkalinization.

The light-induced pH response also strongly depends on the initial pH of the cell suspension. All experiments discussed so far were carried out at pH values between 6 and 7, which is the typical pH range of our washed cell suspensions. The net alkalin-

ization increases at higher initial pH and decreases at lower initial pH until it disappears between pH 5.5 and pH 5.0 (Fig. 12); the net acidification shows the opposite behavior. This would appear to contradict our explanation for the light-induced pH

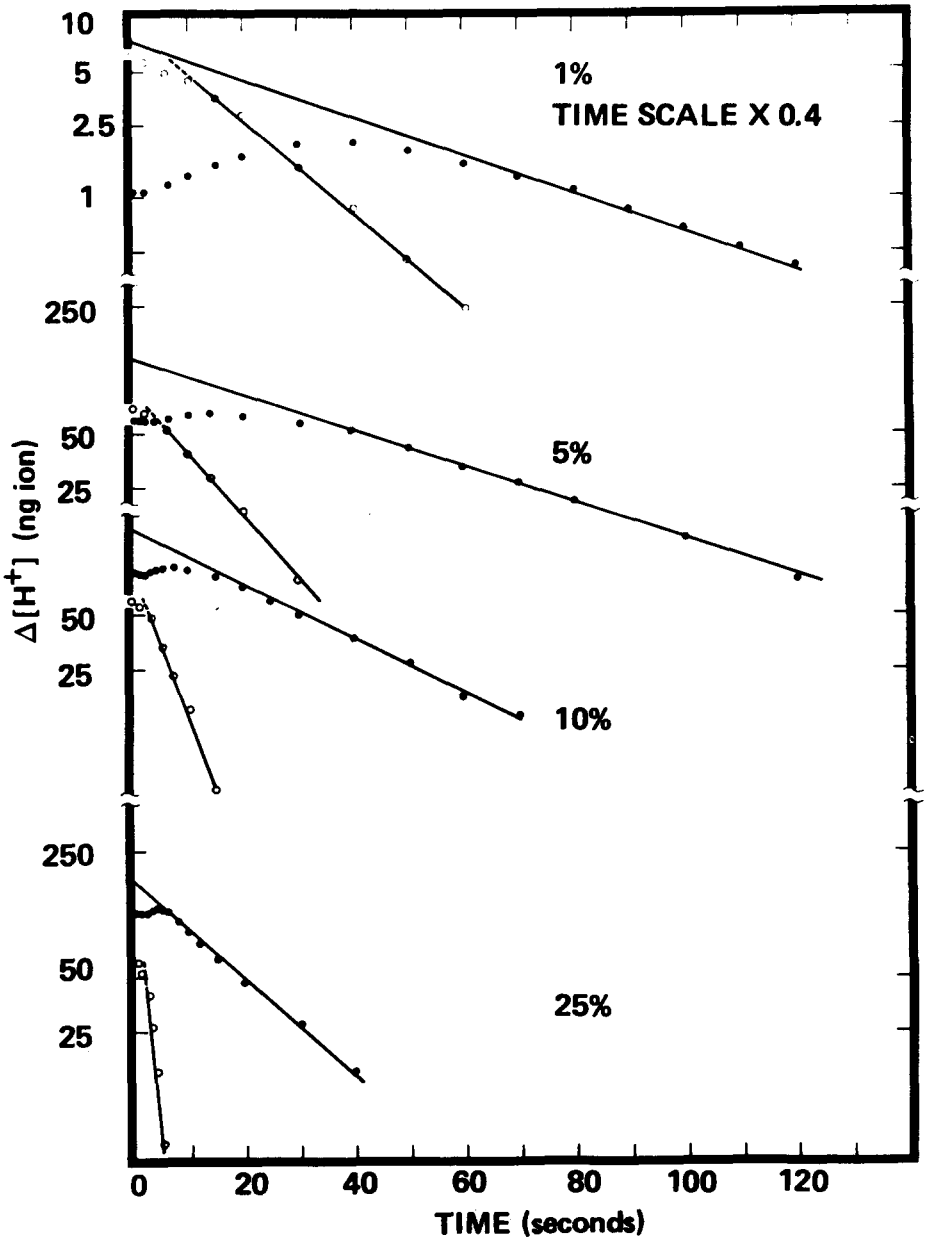

Fig. 8. Semilogarithmic plots of traces from Fig. 7. (●) Experimental points; (○) extrapolated $\Delta H_t{}^{+alk}$ points. The alkaline exponential component is discontinued at ordinate values such that would yield upon subtraction from $\Delta H_{max}{}^{+acid}$ the observed $\Delta H_{net}{}^{+acid}$ value. The corresponding time as before is t (delay). Light intensity (100 %), $8 \cdot 10^5$ ergs/cm^2 per s.

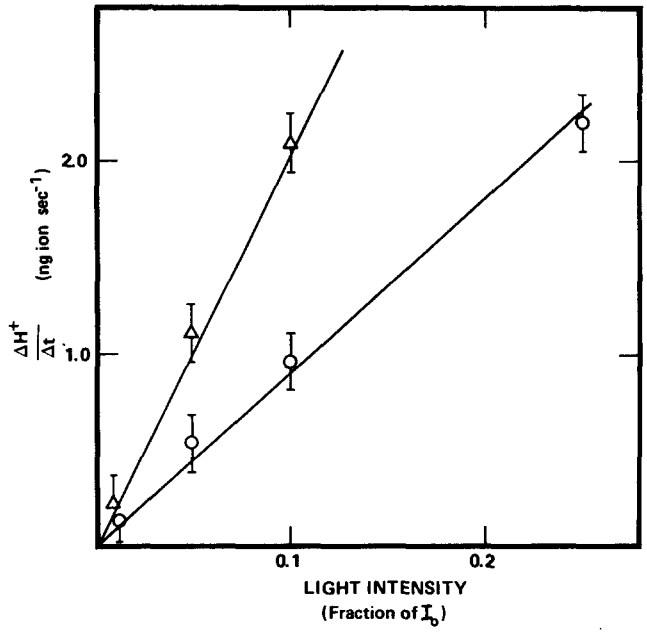

Fig. 9. Initial rates of proton translocation at pH 6.5 calculated from the resolved components of Fig. 8 as a function of light intensity. (The initial rates for $\Delta H_t{}^{+alk}$ are calculated at the time t (delay).) (△) Alkalinization component; (○) acidification component. I_0, $8 \cdot 10^5$ ergs/cm^2 per s.

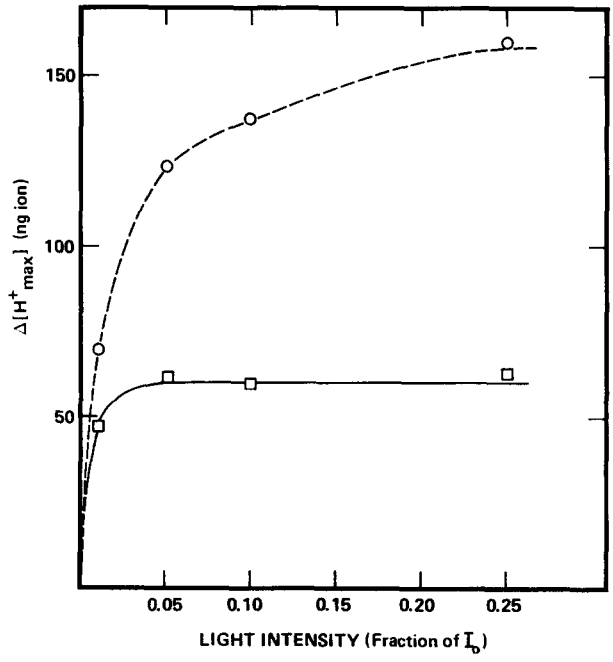

Fig. 10. Light intensity dependence of the extrapolated values of $\Delta H_{max}{}^{+acid}$ (○) and $\Delta H_{t_d}{}^{+alk}$ (□) from Fig. 8. I_0, $8 \cdot 10^5$ ergs/cm^2 per s.

changes, because if alkalinization represents only a transient inflow the same net steady state acidification determined only by the light intensity and purple membrane concentration should result at all pH values. Probable explanations for this apparent contradiction would be that a residual inflow remains which is larger at higher pH or

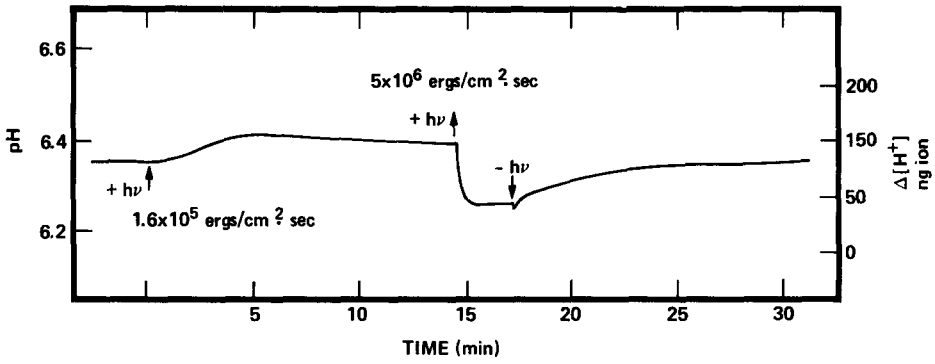

Fig. 11. Effect of increasing light intensity during illumination on the pH of an anaerobic bacterial suspension. Low light intensity elicits an alkalinization of the suspension. Light intensity is suddenly increased by removing a 1.7 *A* neutral density filter from the incident light beam, resulting in prompt acidification. Bacteriorhodopsin concentration, $8.5 \cdot 10^{-15}$ g/cell; cell concentration, $5 \cdot 10^9$ cells/ml (1.2 mg protein/ml); light bandwidth, (530–650) nm; temperature, 39 °C; volume, 3 ml.

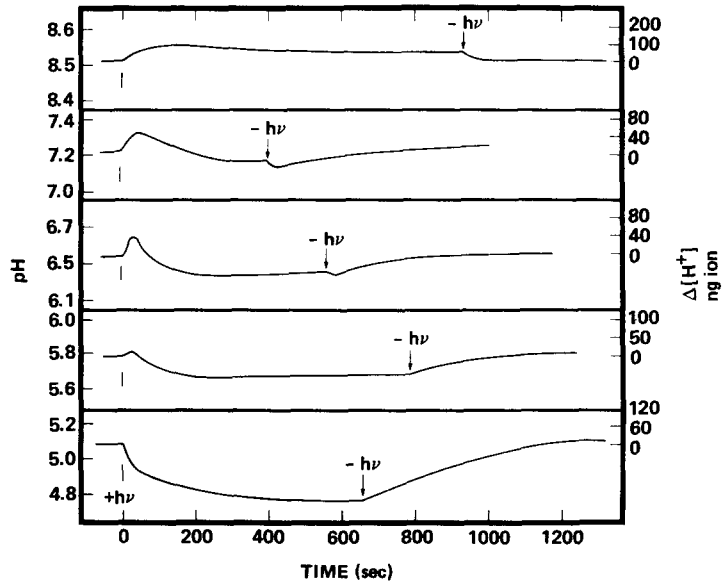

Fig. 12. pH dependence of the light-induced pH changes in an anaerobic cell suspension. Aliquots from a stock suspension at pH 6.5 are equilibrated at the desired pH by addition of 0.1 M HCl or NaOH 15 min before measurements. Bacteriorhodopsin concentration, $9.5 \cdot 10^{-15}$ g/cell; cell concentration, $4.7 \cdot 10^9$ cells/ml (1 mg protein/ml); light intensity, $2.7 \cdot 10^5$ erg/cm^2 per s at 575 nm (25 nm bandwidth); temperature, 24 °C; volume, 3 ml. The transient inflow at the onset of illumination, as well as the acidification transient after illumination is discontinued, gradually disappear as the suspension pH is lowered from 8.5 to 5.1. A small inflow can still be seen at pH 5.1.

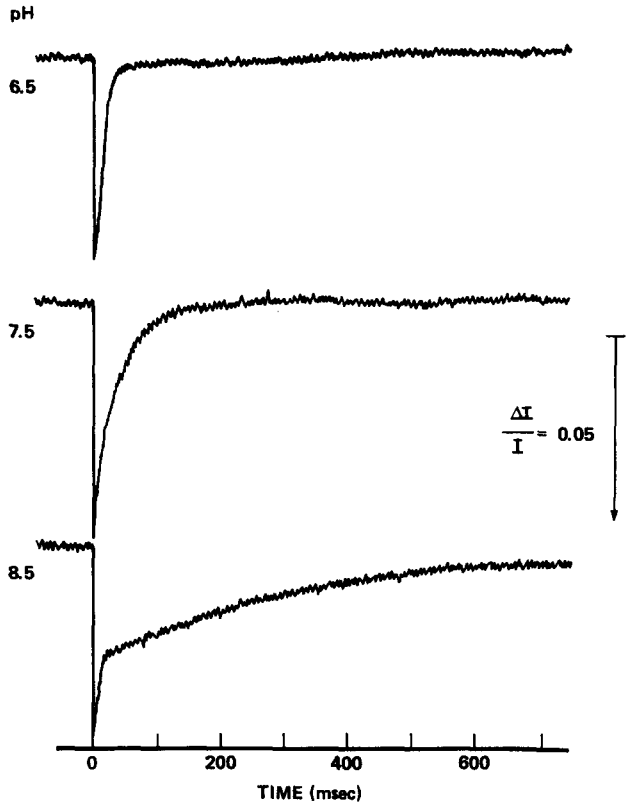

Fig. 13. Effect of pH on the light-induced transmission changes of intact bacteria at 22 °C measured at 540 nm. The cells are those used in Fig. 12. The transmission changes are elicited by xenon flashes of about 1 ms duration from an Ultrablitz Cornet 100 strobe (R. Bosch, W. Germany). Interference filters (Baird Atomic) of 575 nm (25 nm bandwidth) and 540 (10 nm bandwidth) select excitation wavelength and block the photomultiplier respectively. The traces are an average of 8 flashes (repetition rate 0.1 Hz) recorded in a Nicolet 1074 (Madison, Wisconsin) averager. The arrow indicates the magnitude and direction of the fractional increase in transmission.

that the pumping rate decreases with increasing pH. Both effects appear to contribute to the observed behavior. Fig. 13 shows flash-induced absorbance changes in cell suspensions at different pH values. In contrast to the behavior of purple membrane suspensions in water [17], the photoreaction cycle and therefore the light-saturated pumping rate is more than 30 times slower at pH 8.5 than at pH 6.5. For the possible contribution of an increased residual inflow at high pH and decreased inflow at low pH, see Discussion.

Fig. 12 shows very clearly another effect upon which we have not commented so far, even though it is present in most of the other pH traces shown. When the light is turned off, a brief additional acidification is observed before the pH returns to the original dark value. The same transient acidification is seen at the end of short oxygen pulses when the cells become anaerobic (Fig. 5). In general this acidification appears to be related to the transient early proton inflow, because it decreases with decreasing initial pH values and disappears approximately when the inflow disappears. At high

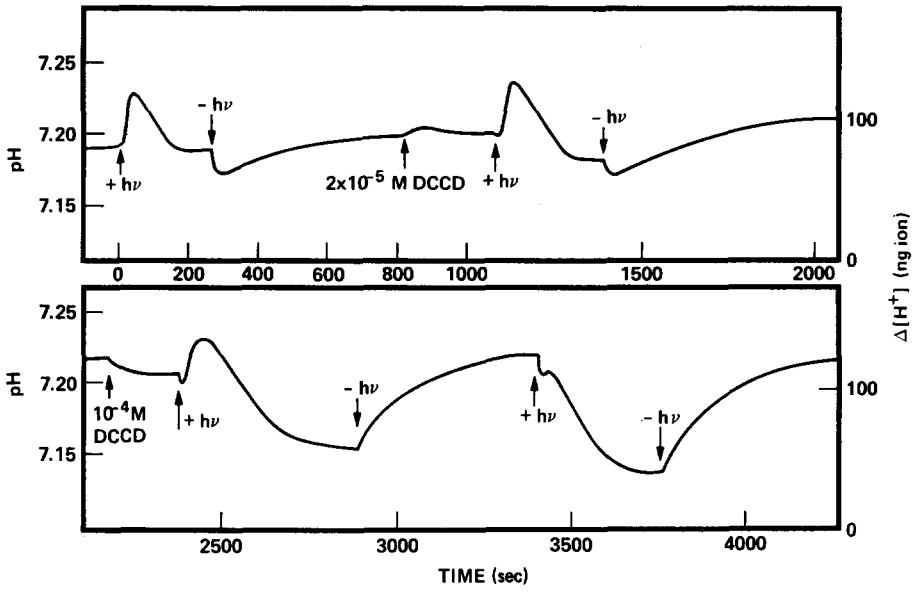

Fig. 14. Effect of DCCD on the light-induced pH changes in an anaerobic bacterial suspension. Intermediate stages of inhibition of the various components to the pH changes show the appearance of an initial acidification, the disappearance of the acidification transient at the light-off times and finally an inhibition of the alkalinization transient. Bacteriorhodopsin concentration, $7.9 \cdot 10^{-15}$ g/cell; cell concentration, $5 \cdot 10^9$ cells/ml (1.2 mg protein/ml); light intensity, $1.8 \cdot 10^5$ erg/cm^2 per s, 575 nm bandwidth; temperature, 39 °C; volume, 3 ml.

pH, when the final pH level in the light is higher than the dark level, the transient acidification changes to a sustained acidification, which returns the pH of the suspension to the original dark level.

The correlation between the light-on alkalinization and the light-off acidification is further emphasized by their common susceptibility to inhibitors. Fig. 14 shows a cell suspension at slightly alkaline pH where both transient components are rather pronounced. When DCCD is added and the light turned on, a small acidification appears before the transient alkalinization and the transient acidification is diminished, although the sustained acidification has become larger. The reaction of DCCD with the cells is slow. To speed it up a larger dose was added at 2400 s. The light response now shows more pronounced changes in the same direction, the transient acidification has already disappeared and as the reaction proceeds further, the transient inflow is further delayed and becomes smaller and the sustained outflow increases. Finally, the transient inflow also disappears completely (not shown).

Even though DCCD is a rather reactive molecule, it has been shown to inhibit selectively the membrane-bound energy-tranducing ATPase of mitochondria, chloroplasts and prokaryotic cells [18, 15]; it also inhibits photophosphorylation as well as oxidative phosphorylation in *H. halobium* [3]. The inhibition by DCCD of the transient inflow and the light-off proton ejection suggests that both are mediated by the membrane-bound ATPase of *H. halobium*. Other inhibitors of the ATPase, e.g. peliomycin and ossamycin, at 10^{-4} M concentrations showed similar effects. If the transient inflow occurs through the ATPase, it should be accompanied by ATP

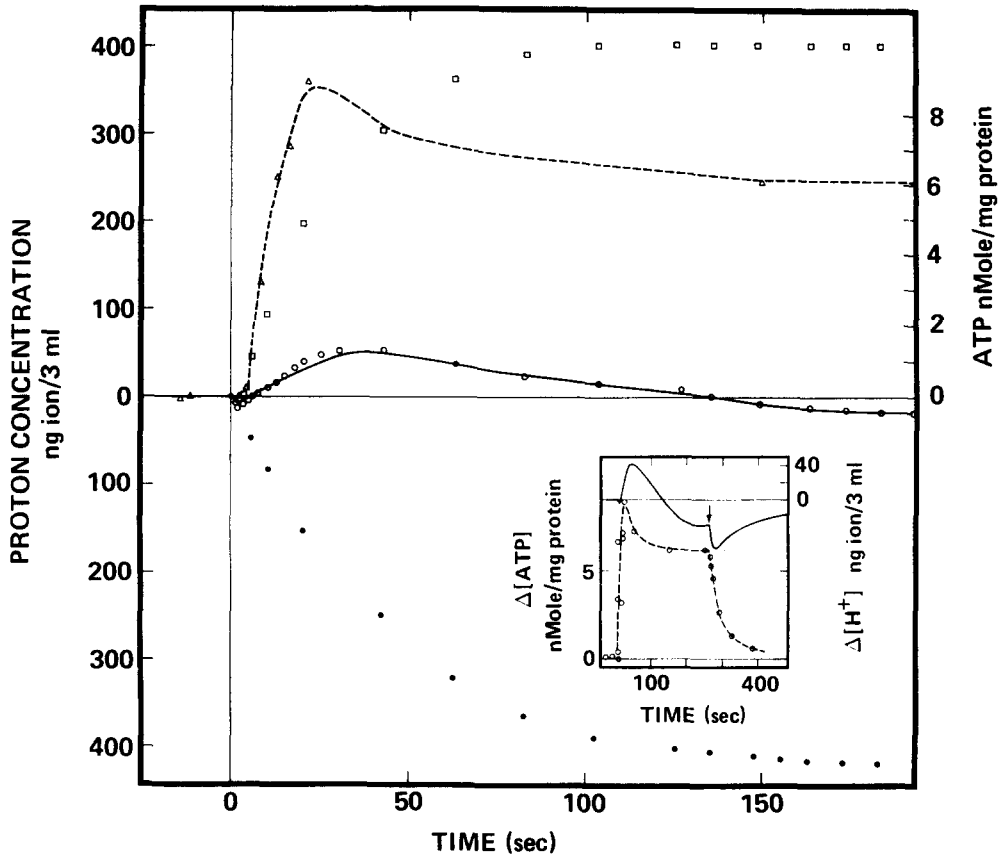

Fig. 15. Light-induced proton concentration change and photophosphorylation in an anaerobic cell suspension. The inset shows the whole time course of the ATP changes and the pH change induced by light in the bacterial suspension; the light is turned on at zero time and turned off at the arrow. The figure depicts the light-on portion of the responses; illumination begins at zero time. (—) Light-induced pH change; (○) fitting of the experimental curve obtained by addition of the extrapolated kinetic components; (△) ATP changes; (□) extrapolated inflow component; (●) extrapolated outflow component. Bacteriorhodopsin concentration, $7.5 \cdot 10^{-15}$ g/cell; cell concentration, $5 \cdot 10^9$ cell/ml (1.2 mg protein/ml); light intensity, $2.5 \cdot 10^5$ ergs/cm^2 per s; 575 nm (25 nm bandwidth); temperature, 39 °C; volume, 3 ml; initial pH, 7.20.

synthesis and this appears to be borne out by experiments. Fig. 15 shows an experiment where intracellular ATP concentrations have been monitored during a light response in anaerobic cells. The pH changes in the medium are recorded and the pH response has been graphically resolved into inflow and outflow components. Intracellular ATP increase and inflow show a short delay and rise together. The ATP level remains high after the inflow subsides. Preliminary estimates of the H^+/ATP ratio for the graphically resolved influx give a minimal value of 2.9.

DISCUSSION

The experiments presented here are consistent with earlier observations [2, 4] and show that light absorption by the purple membrane causes an ejection of protons

from *H. halobium* cells. Under physiologic conditions the observed pH changes in the suspension are apparently complicated by a transient inflow of protons shortly after the light has been turned on and a brief increased ejection of protons when the light is turned off. These two transient effects can become the dominant features of the light response at alkaline pH values or low light intensities.

The inflow is accompanied by ATP synthesis, and this observation offers an explanation for the transient nature of the inflow. As protons enter the cell through the ATPase, the intracellular level of ATP rises and will finally be limited by the availability of $ADP+P_i$. Then this inflow should be reduced to a small value determined by the intracellular hydrolysis of ATP. Now the continued proton ejection begins to dominate the response and under typical conditions causes the net acidification which exponentially approaches a steady state. Its value will be determined by the rate of proton ejection, the residual flow through the ATPase and other processes diminishing the proton gradient including a passive leak. The residual flow through the ATPase is now sufficient to maintain a high steady-state ATP level in the cells.

DCCD inhibition of the light-off acidification and its correlation with the light-on alkalinization link it as well to the ATPase. It has been shown in mitochondria that respiration under phosphorylating conditions activates the ATP hydrolysis function of the membrane-bound ATPase, apparently by dissociation of the inhibitor protein from F_1 [19–21]. Mitochondrial ATPases are very similar to prokaryotic energy-coupling ATPases [22, 15, 6] and it appears reasonable to assume that the same activation of the ATP hydrolysis function occurs during phosphorylation in *H. halobium*. The short burst of proton ejection, occurring when the light is turned off, could therefore be accounted for by the same mechanisms, i.e. activation of the ATP hydrolysis function during phosphorylation and a short delay of the inactivation when phosphorylation stops. Note that the same small burst of proton ejection also occurs when respiration ceases after an oxygen pulse (Fig. 5). The link of the light-off acidification with the initial transient alkalinization can also be understood on the same basis. The inflow indicates rapid ATP synthesis in the light, which is required to obtain the near maximal ATP levels necessary for a rapid ejection of protons by the ATPase when the light is turned off or oxygen is exhausted. This would also explain the only apparent lack of this correlation seen so far: whenever we obtain only a prompt sustained acidification of the medium, e.g. in the presence of low concentrations of the uncoupler, at low pH or in the presence of DCCD, we see no transient acidification at the end of the illumination period. Only when we obtain a clean acidification by using a low light intensity first, and then increasing the light intensity suddenly, is the burst of additional acidification at the end of the illumination period present (Fig. 11). Apparently here the lower light intensity is already sufficient to reach a near maximal internal ATP concentration, so that only an additional outflow of protons is observed when the light intensity is increased, but a short additional ejection through ATP hydrolysis occurs when the light is turned off.

We assume that the major driving force for the inward flow of protons at the beginning of the light response is a pre-existing electrochemical potential. Evidence to support this assumption is based mainly on the observations that adding uncouplers in the dark causes an inflow of protons, exhaustive depletion of energy resources abolishes the light-induced inflow and regeneration of an electrochemical gradient restores it. As pointed out earlier [3], *H. halobium*, when deprived of energy sources,

is able to preserve its energy reserves for relatively long times. It maintains intracellular ATP at about 30 % of the maximal level and its high intracellular K^+ concentration in a low K^+ medium without nutrients and in the dark is maintained for up to two days [14]. How this high K^+ concentration gradient is coupled to the energy metabolism of the cell is presently not understood; however, it represents a very substantial potential source of stored energy which should not be overlooked. It is possible that the cells are relatively permeable to K^+ and impermeable to other ions and that the potential of the resting cell is close to the K^+ potential. The coupling may, of course, be less direct. Nevertheless, it seems reasonable to assume a membrane potential in harvested and washed cells of 130–180 mV. We do not know what the contribution of the pH gradient to the total protonmotive force is; however, it does not appear to be large (ref. 23 and Bakker and Caplan, personal communication).

The resting cells in our experiments therefore may maintain a protonmotive force close to that necessary for ATP synthesis. A small additional contribution from light-driven proton ejection would explain the short delay and small pH changes seen in our experiments before proton inflow begins and ATP synthesis sets in. P. C. Maloney, Harvard Medical School (personal communication) in similar experiments with *Escherichia coli* has estimated the necessary protonmotive force to be approx. 200 mV. We have to assume a certain hysteresis in the activation and deactivation of the ATPase to explain the net alkalinization observed. A further contribution to the observed inflow may be the consumption of protons in the synthesis of ATP, due to changes in protonation of the reaction partners. This should not amount to more than one H^+/ATP at pH 8.0 and our preliminary estimates show that this is insufficient to explain more than a small part of the observed net inflow. Moreover, the inflow is still large at pH 5.7 where proton consumption by ATP synthesis becomes negligible [24, 25]. The proton consumption depends, of course, on the intracellular pH which we did not measure; however, the inside pH does not appear to be much higher than the outside pH (ref. 23 and Bakker and Caplan, personal communication). A further complication for estimates of the H^+ consumption is the unknown pK_a change of the reactants due to the high ionic strength.

It is at present not clear which parameter activates the ATPase. It could be the total protonmotive force. However, the experiments with cells suspended at low pH showing no initial alkalinization suggest that it may be either inside pH or the pH difference across the membrane. Our interpretation of the results also requires that there is a certain spread in the sensitivity of the ATPase molecules to activation, and this may contribute to the apparent linear dependence of the inflow on light intensity (Fig. 9). It is also clear that other ions must be moving to at least partly neutralize the membrane potential generated by the proton pump, and their permeabilities probably also change with pH. All these problems will make it necessary to determine intracellular pH and membrane potential changes during the light response. These are difficult experiments and will require further work. They should, however, provide us with interesting data about the functioning of the ATPase, as well as the purple membrane.

In the proceedings of two conferences, Oesterhelt [26, 27] has discussed an experiment similar to the one shown in Fig. 15. His interpretation, however, differs from that given here. He assumes no pre-existing potential and does not resolve the net pH changes into fluxes in opposite directions but rather postulates that light-induced ejection of protons gives rise to a membrane potential of 300 mV in less than 1 ms.

Therefore, most protons would be driven back into the cell immediately through the ATPase, without causing a measurable pH change in the outside medium. The net alkalinization is then explained by proton consumption during ATP synthesis. We have shown here that a pre-existing membrane potential does exist. We have also pointed out that the proton consumption during ATP synthesis in our opinion contributes but cannot qualitatively account for the large inflow occurring at neutral and slightly acid pH. Note that our resolved fluxes at pH 6.5 give an initial rate for the influx more than twice as large as the initial rate for the efflux and at the maximal net alkalinization we find outside a deficit of 2.4 H^+/ATP. In addition, Oesterhelt would have to assume an electroneutral exchange mechanism for the additional inflow of protons. Also, his value for the membrane potential rise appears too high, because the photoreaction cycle of bacteriorhodopsin needs several milliseconds for completion and the necessary compensatory movement of other charges, which must exist to allow the large steady state acidification to occur, would tend to diminish the potential. To decide the relative contribution of proton consumption and preexisting membrane potential to the influx, it will be necessary to measure inside pH, membrane potential with the necessary time resolution and to actually determine the proton consumption during ATP synthesis under the high ionic strength condition prevailing inside the cell.

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