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CHARACTERISTICS OF A LIGHT-DEPENDENT PROTON TRANSPORT IN CELLS OF *RHODOSPIRILLUM RUBRUM*

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SUMMARY

Suspensions of cells of *Rhodospirillum rubrum* harvested in the logarithmic phase of growth show a light-induced decrease of pH, which is dependent upon the presence of salts of Li^+, Na^+, K^+, Mn^{2+}, Mg^{2+}, or Ca^{2+}. NH_4Cl failed to increase the magnitude of the response. During the subsequent dark period, the pH of the medium increases. If the cells are suspended in 200 mM NaCl at pH 5.5 the decrease in the pH is complete in approx. 30 sec. The decrease in pH is inhibited if 3.3 μg/ml antimycin A or 10 μM carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) are added prior to illumination. If CCCP is added during the subsequent dark period there is a stimulation of the rise in pH. The effect of light intensity and initial pH on the pH response has been studied.

INTRODUCTION

In 1961 MITCHELL[1] proposed the 'chemi-osmotic hypothesis'. According to this hypothesis, the vectorial transport of H^+ across a membrane generates an electrochemical potential which drives ATP synthesis or the transport of cations. Support of the chemi-osmotic hypothesis has come from studies with cell organelles and with vesicles formed from membrane fragments. MITCHELL[2] has shown a substrate-dependent and an ATP-dependent proton transport in mitochondria. JAGENDORF AND HIND[3] and NEUMANN AND JAGENDORF[4] have demonstrated a light-dependent proton uptake by fragments of spinach chloroplasts. BALTSCHEFFSKY AND VON STEDINGK[5] and NISHIMURA and co-workers[6,7] have shown an increase in the pH during illumination of suspensions of chromatophores of *Rhodospirillum rubrum*. If the hypothesis is valid, it would be expected to apply to the intact cell. In this paper light-dependent pH changes with intact cells of *R. rubrum* are reported. The light-dependent decrease in the pH could be the result of an extrusion of protons or an accumulation of OH^- by the cell. We shall refer to the pH decrease as a proton extrusion.

METHODS

R. rubrum was grown anaerobically in the medium of COHEN-BAZIRE, SISTROM AND STANIER[8] in 250-ml reagent bottles in a water bath at 30°. Light was provided

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Abbreviation: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone.

by a 100-W incandescent lamp which gave an intensity of about 1500 foot candles at the surface of the submerged reagent bottle. Under these growth conditions, the stationary phase was reached after 5 days.

Stock cultures of the bacteria were maintained by transferring 10 ml of a 48-h culture to 15 ml of medium in a 25-ml screw-cap culture vial. After 48 h growth, the 25 ml was used as an inoculum for the 250-ml reagent bottle.

Cells were grown for 24 h for all experiments. They were harvested by centrifuging at 4200 × *g* for 10 min at room temperature and were washed three times with an amount of glass-distilled water equal to the starting volume. Unless otherwise indicated, the cells were resuspended in 2 mM Tris (pH 5.5). The absorbance was adjusted to 4 at 660 nm with a Beckman DB-G Grating Spectrophotometer. Prior to experimentation, the suspensions were bubbled with N_2 in the dark at 30° in a water bath to maintain anaerobiosis.

To measure pH changes, 3 ml of the cell suspension were added to a cuvette surrounded by a water jacket maintained at 30°. Additions of salts or inhibitors were made with syringes in volumes of 10 to 200 μl, while the cuvette was bubbled with N_2 to assure mixing and to maintain anaerobiosis. Carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) was added as a solution in acetonitrile, and antimycin A was added as a solution in ethanol. The solvents were tested, at the same concentrations, and found to have no effect on the parameters measured. The pH changes were determined with a glass microelectrode and a Radiometer pH meter, Model 26, and recorded with a Texas Instruments Servo/riter II recorder.

To determine the effect of light, the bacteria were illuminated by a 75-W incandescent lamp with an infrared filter (Wratten 88A) having a minimal band pass of 720 nm. The light intensity, which was controlled by a powerstat, was measured on the inside of the cuvette with a YSI-Kettering Model 65 Radiometer. Unless otherwise indicated the light intensity was $30 \cdot 10^4$ ergs $\cdot$ cm^{-2} $\cdot$ sec^{-1}. At the end of each experiment, HCl was added to a final concentration of 0.1 mM, and the change in pH was recorded. This addition of a known amount of acid permitted corrections for the buffering capacity of the medium and the cells.

To determine the concentration of bacteriochlorophyll, a 1-ml aliquot of the original suspension was centrifuged at 4200 × *g* and the supernatant fluid was discarded. The cells were resuspended in 0.1 ml of water and 4.9 ml of acetone–methanol (7:2, by vol.) was added. The mixture was centrifuged and the absorbance of the supernatant solution was determined at 772 nm. The absorptivity, according to CLAYTON[9], for bacteriochlorophyll in acetone–methanol, at 772 nm, is 75 mM^{-1} $\cdot$ cm^{-1}. An absorbance of 4 at 660 nm is equivalent to 18–20 μM bacteriochlorophyll.

CCCP and antimycin A (grade B) were purchased from Calbiochem, Los Angeles, Calif.

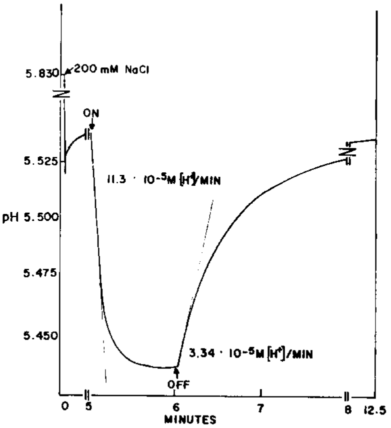
EXPERIMENTAL RESULTS

A typical example of the light-dependent pH change with *R. rubrum* is shown in Fig. 1. When the light is turned on, there is a rapid decrease in the pH which reaches a maximum in about 30 sec. The pH remains constant until the light is turned off. After several minutes, the pH returned to the original value of 5.5. The rate of pH decrease in the light is three times that of pH increase in the dark. All rates are determined from initial velocities after the light is turned on or off. The pH changes can be repeated through several cycles (Fig. 2).

At an absorbance of 4, the rate of proton extrusion approached a maximum when the radiant intensity was 50–60 · 10^4 ergs · cm^{-2} · sec^{-1} (Fig. 3). The maximum velocity of proton uptake in the subsequent dark period was achieved when the cells were preilluminated at 30 · 10^4 ergs · cm^{-2} · sec^{-1} (Fig. 3).

The amount of proton movement is a function of the concentration of NaCl in the medium (Fig. 4).

The typical pH response described for *R. rubrum* does not specifically require



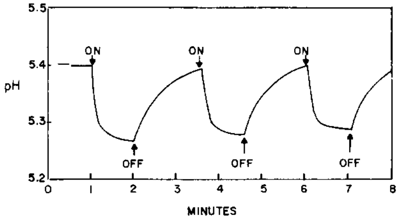
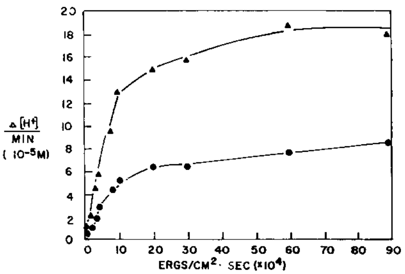


Fig. 1. Light-dependent pH changes with cells of *R. rubrum*. The cells were suspended in 2 mM Tris to an absorbance of 4.0 at 660 nm; the light intensity was 30 · 10^4 ergs · cm^{-2} · sec^{-1}. 0.1 mM HCl added at the end of the experiment caused a decrease in the pH of 0.63.

Fig. 2. Light–dark cyclic changes in the pH with cells of *R. rubrum*. The experimental conditions were the same as those described for Fig. 1 except that NaCl was added to a concentration of 200 mM 3 min prior to illumination. 0.1 mM HCl caused a pH change of 0.56.



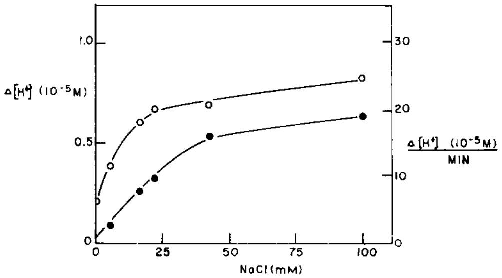


Fig. 3. The effect of light intensity on the rate of proton extrusion during the first illumination cycle of cells of *R. rubrum*. NaCl was added to a concentration of 200 mM 2 min prior to illumination; the pH of the suspension was 5.50. ▲—▲, light-on; ●—●, light-off.

Fig. 4. The effect of NaCl concentraton on the light-dependent extrusion of protons with cells of *R. rubrum*. Increasing amounts of NaCl were added to a suspension of cells. The points represent values of three replications which were corrected for the control by the addition of volumes of water, equal to the volume of NaCl. The illumination cycle was: 2 min light, 2 min dark. The total change in the H^+ concn. was calculated after 2 min of illumination. ○—○, total Δ H^+ concn. with NaCl; ●—●, Δ H^+ concn./min withNaCl.

TABLE I

EFFECT OF SALTS ON THE LIGHT-INDUCED pH CHANGES OF CELL SUSPENSIONS OF *R. rubrum*

Salt added 2 min prior to illumination; pH after adding salt 5.5 ± 0.2; total ΔH^+ concn. calculated after 2 min illumination; figures are averages of three replications.

Salt	*Cation (mequiv)*	$\Delta[H^+]/min\cdot 10^{-5}$ M	*Total* $[H^+]\cdot 10^{-5}$ M
None*	—	0.33	0.33
LiCl	188	12.7	1.71
KCl	188	12.9	2.30
NH_4Cl	188	2.1	0.39
NaCl	188	13.1	1.83
Na_2SO_4	194	11.2	1.56
$NaNO_3$	188	10.6	1.52
$MgCl_2$	194	19.0	3.04
$CaCl_2$	194	17.0	2.15
$MnCl_2$	194	22.1	3.57

* 200 μl of H_2O added instead of salt.

TABLE II

EFFECT OF THE INITIAL pH ON LIGHT-DEPENDENT PROTON TRANSPORT IN *R. rubrum*

In each illumination cycle, the light was on for 30 sec and off for 60 sec. Data represent averages of three replications; cells were in 200 mM NaCl (pH 5.0); the initial pH of each experiment was achieved by addition of HCl or NaOH; the absorbance of the cells was 4.0 at 660 nm.

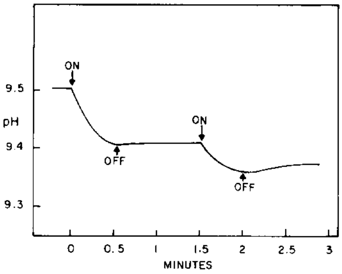
Initial pH	*Light-on*		*Light-off*		K_{on}/K_{off}*	Δ_{on}/Δ_{off}**
	Increase in $[H^+]\cdot 10^{-5}$ *M*		*Decrease in* $[H^+]\cdot 10^{-5}$ *M*			
	Δ/min	*Total* Δ	Δ/min	*Total* Δ		
4.5	3.91	0.57	2.09	1.21	1.87	0.47
5.5	13.4	1.68	3.70	1.25	3.62	1.34
6.5	18.4	3.65	3.51	0.96	5.95	3.81
7.5	12.5	2.51	2.11	0.26	5.95	9.65
8.5	25.5	4.06	3.80	0.62	6.70	6.60
9.5	74.4	15.3	3.90	1.69	19.0	9.0

* The ratio of the rate during illumination to the rate in the subsequent dark period.

** The ratio of the total change in H^+ concn. during illumination to the total change in H^+ concn. in the subsequent dark period.

NaCl. Chloride salts of Mg^{2+}, Ca^{2+}, K^+, Na^+, Li^+ and Mn^{2+} induce the light-dependent pH response (Table I). However, NH_4Cl has only a slight effect on the rates of pH change in an illumination cycle and no effect on the total decrease in pH when compared with the control (200 μl water added instead of cations).

The rates and magnitude of the light-induced proton movement were tested over a pH range of 4.5–9.5 (Table II). The greatest changes occurred at an initial pH of 9.5. At pH 4.5, the rates and magnitude of proton movement, during the illumination cycle, were less compared to those at pH 5.5 and 6.5. The observed changes were not as great during the second illumination cycle. A high initial pH selectively depressed the pH increase during the dark phase (Fig. 5).



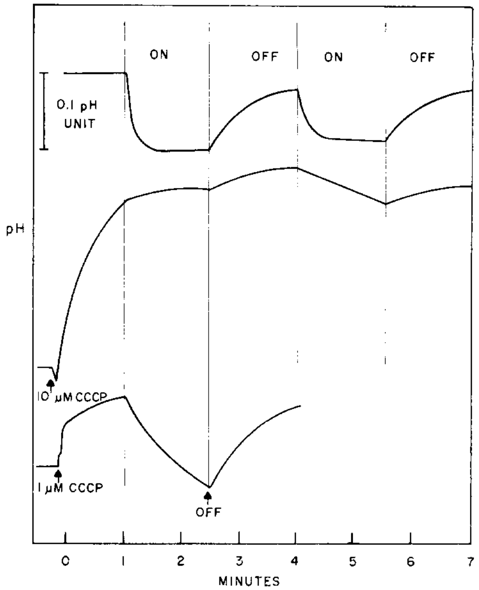
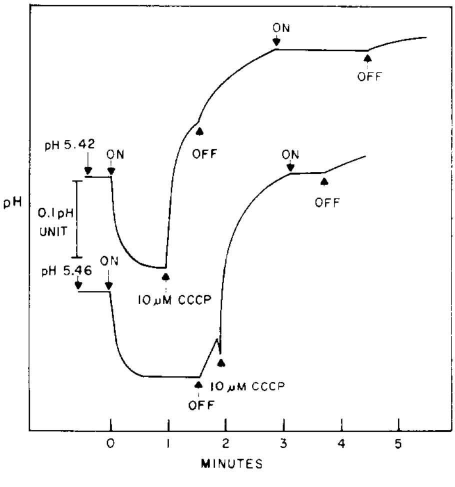


Fig. 5. Light-dependent pH changes with cells of *R. rubrum* at an initial pH of 9.5. The pH of the cell suspension, 5.0, was raised to 9.5 with NaOH. 0.1 mM HCl caused a pH change of 0.21. Other conditions were those given for Table II.

Fig. 6. The effect of the addition of CCCP prior to illumination on the light-dependent pH changes with cells of *R. rubrum*. NaCl was added to a concentration of 200 mM 4 min prior to illumination; the pH of the suspension was 5.50 ± 0.03. 0.1 mM HCl caused a pH change of 0.50 ± 0.06.



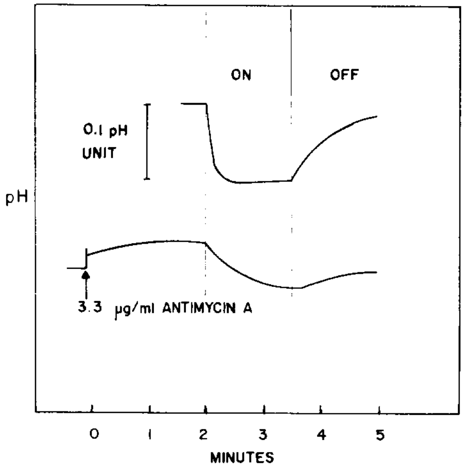


Fig. 7. The effect of the addition of CCCP during and after illumination on the light-dependent pH changes with cells of *R. rubrum*. NaCl was added to a concentration of 200 mM 2 min prior to illumination; the pH of the suspension was 5.50 ± 0.03. 0.1 mM caused a pH change of 0.50 ± 0.06.

Fig. 8. The effect of antimycin A, added prior to illumination, on the light-dependent pH changes with cells of *R. rubrum*. NaCl was added to a concentration of 200 mM 4 min prior to illumination; the pH of the suspension was 5.50 ± 0.04. 0.1 mM HCl caused a pH change of 0.52 ± 0.01.

Uncouplers of photosynthetic phosphorylation and inhibitors of electron flow were used to test further the nature of the pH response. The effects of CCCP at 1 and 10 μM and of 3.3 μg/ml of antimycin A were studied. When 1 μM CCCP was added 1 min prior to illumination, there was an immediate increase in the pH of the medium (Fig. 6). The rate of proton extrusion induced by illumination was much lower in the presence of CCCP. However, the total decrease in pH with or without 1 μM CCCP was about the same after 90 sec of illumination. If 10 μM CCCP were added during ilumination there was an increase in pH which was followed by another increase during the dark period (Fig. 7). With 10 μM CCCP, the light-dependent pH response was inhibited in the second illumination cycle. If 10 μM CCCP were added during the dark period following illumination, there was a stimulation of the increase in pH (Fig. 7). It is apparent that the CCCP rendered the cells permeable to protons and that the light-induced pH change was associated with photophosphorylation.

3.3 μg antimycin A per ml of cell suspension, added 2 min prior to illumination, partially inhibited the pH changes during the illumination cycles (Fig. 8). If the antimycin A was added during the light period there was an increase in the pH after a lag of about 15 sec. Antimycin A added during the dark period following illumination had no effect on the pH increase in the dark, but it partially inhibited the pH changes in the second illumination cycle.

DISCUSSION

The data presented in this paper support the existence of a proton transport mechanism in *R. rubrum* which is dependent upon illumination. Proton extrusion measured by pH changes, requires a cation such as Na^+, Mg^{2+}, Mn^{2+}, Li^+, Ca^{2+} or K^+, usually provided as the chloride salt. $NaNO_3$ and Na_2SO_4 were as effective in stimulating the pH change as was NaCl (Table I). NH_4Cl is without effect. NH_4^+ may act as an inhibitor of electron flow or as an uncoupler of photosynthetic phosphorylation as found by JAGENDORF[10] with chloroplasts. The rate and the magnitude of pH change in the presence of 200 mM NaCl were inhibited 19 and 32 %, respectively, by 10 mM NH_4Cl.

The dependence of proton extrusion on the presence of cations in the medium suggests a proton pump that is coupled to the uptake of cations. Such a proton–cation exchange has been demonstrated in *Escherichia coli* by EPSTEIN AND SCHULTZ[11].

At pH 5.5 in the presence of 200 mM NaCl, there is a rapid decrease in the pH during illumination which reaches a steady state after approx. 30 sec. During the steady state it is assumed that the extrusion of protons is in equilibrium with the uptake of protons. After several illumination cycles, the pH returns to its original value (Fig. 2). It is not clear whether the decrease in the H^+ concentration in the dark period following illumination is accompanied by an extrusion of Na^+ and/or other cations or is attributable to diffusion of H^+ and Cl^- into the cell.

With cell suspensions at pH 7.5, 8.5 and 9.5, the pH did not return to the original value after several illumination cycles; this might represent an accumulation of Na^+ in exchange for protons which process is not reversed in the subsequent dark period.

Both the rates of proton production and the total amount of protons produced increased markedly with increasing initial pH (Table II). It may be that the initial adjustment of the pH, in the range 4.5–9.5, resulted in the establishment of a potential imposed by the pH differential across the membrane. Such a pH differential has been used by JAGENDORF[10] to explain the generation of ATP in the dark with spinach chloroplasts. Now, if the external pH is less than that of the cell, the light-induced proton extrusion would tend to increase the pH differential and proton production would be self-inhibitory. If the external pH is greater than that of the cell, proton extrusion would tend to decrease the pH differential and thereby discharge the potential. The ratios of the rates of proton extrusion in the light to the rates of proton uptake in the dark (Table II) support this interpretation. However, a clearer understanding of these data depends on a quantitative determination of cation movement during the illumination cycle.

NISHIMURA[6] and VON STEDINGK[12] have described a light-dependent increase in the pH of suspensions of chromatophores from *R. rubrum,* whereas we have observed a decrease in the pH with illumination when using intact cells. This reversed direction of proton transport comparing intact cells and chromatophores may be explained by assuming that the photosynthetic apparatus of *R. rubrum* is located in or on invaginations of the cytoplasmic membrane, as has been shown by BOATMAN[13] and by HOLT AND MARR[14] from observations of electron micrographs. The delimiting membrane of vesicles formed from such invaginations, as a result of sonication, would be expected to be inverted relative to the plasma membrane of the intact cell. MITCHELL AND MOYLE[15] have observed a similar reversal of the direction of proton movement in vesicles formed from fragments of mitochondria prepared by sonication, which they attribute to an inversion of the cristae membranes relative to the membrane of the intact mitochondrion.

In applying the chemi-osmotic hypothesis for photophosphorylation to the observed proton movement with intact cells of *R. rubrum* it can be proposed that the light-dependent proton extrusion creates an electrochemical potential which drives phosphorylation.

As other workers have suggested, CCCP and other uncouplers could discharge the potential by facilitating the diffusion of protons across the membrane[16]. At 10 μM CCCP the light-dependent pH responses with *R. rubrum* were inhibited, which indicates that proton transport is linked to phosphorylation. If 10 μM CCCP were added to cells at pH 5.5 in the dark, during illumination, or during the subsequent dark period, there was an immediate rise in the pH which indicates that the protons were free to move across the membrane. Similarly, VON STEDINGK AND BALTSCHEFFSKY have shown that the uncoupler gramicidin inhibits the pH responses in chromatophores if added prior to illumination, and it decreases the pH if added during illumination[17].

Antimycin A inhibits phosphorylation by blocking electron transport between cytochrome *b* and cytochrome c_2 (refs. 18, 19). With cells of *R. rubrum,* 3.3 μg/ml antimycin A inhibited the pH changes when added prior to illumination, which shows that the proton transport is dependent upon the electron transport system.

As of now, we have no basis for distinguishing between proton extrusion as a necessary intermediate between electron transport and ATP formation, or proton extrusion as a consequence of the expenditure of ATP or some high-energy intermediate(s).

Future studies will include measurements of the transport of other cations and their relationship to the proton transport.

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