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# Action of Phenazine Methyl Sulfate, Inhibitors, and Uncouplers on the Light-Induced Proton Transport by Cells of *Rhodospirillum rubrum*

GERALD E. EDWARDS* and CARLTON R. BOVELL

Departments of Plant Science and Life Sciences, University of California, Riverside, California 92502

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*Summary.* Suspensions of log phase cells of *Rhodospirillum rubrum* at pH 5.5 show a light-induced decrease in the pH of the medium which is reversed during the subsequent dark period. The velocity and magnitude of the pH change were the same whether the cells were bubbled with air, $CO_2$-free air or $N_2$ during experimentation. The pH response is temperature dependent. Phenazine methyl sulfate (PMS) at concentrations above 0.05 mM stimulates the light-induced pH change. PMS at 1 mM gives a 2-fold increase in the initial rate upon illumination and a 1.5-fold increase in the total change in pH after 2 min of illumination. The inhibition of the proton transport by 10 μg/ml antimycin A or 20 μM 2-n-heptyl-4-hydroxyquinoline-N-oxide can be partially relieved by PMS. However, inhibition of the light-induced proton transport with 0.5 mM 2,4-dinitrophenol or 3 μM carbonylcyanide-m-chlorophenylhydrazone (CCCP) cannot be overcome by addition of PMS. Valinomycin, at a concentration of 3 μM, caused a slight stimulation of the light-induced proton transport in the presence of 200 mM KCl. The inhibition of proton transport by 3 μM CCCP was partially relieved with 3 μM valinomycin in the presence of 200 mM KCl, but the antibiotic was without effect when the cells were suspended in 200 mM NaCl. The results are discussed in terms of current theories of the action of PMS, antimycin A, valinomycin, and uncouplers on the light-induced electron flow and photophosphorylation in *R. rubrum*.

It was recently shown that a number of photosynthetic unicellular organisms cause pH changes in the medium when illuminated. In 1967 Mitchell [16] noted that cells of *Rhodospirillum rubrum* show a light-induced proton extrusion; this response was later described in more detail [8]. Scholes, Mitchell, and Moyle [26] have demonstrated light-induced pH fluxes by cells of *R. rubrum*, *Anabaena variabilis*, and *Rhodopseudomonas spheroides*. Cell suspensions of *Chlamydomonas reinhardti* cause an increase in the pH of the medium upon illumination and a decrease in pH during the dark period that follows [27].

* Present address: Department of Biochemistry, Graduate Studies Research Center, The University of Georgia, Athens, Georgia 30601.

It has been suggested that the proton transport by cells of *R. rubrum* is linked to photosynthetic electron transport and photophosphorylation since antimycin A and CCCP [1] inhibited the pH changes [8]. Apparently, antimycin A and HOQNO block the photosynthetic electron transport in the cells between cytochrome b and $c_2$ [19, 22]. These inhibitors also block photophosphorylation in chromatophores of *R. rubrum*, and the inhibition can be overcome by PMS [3, 9].

In the experiments reported here, we tested the action of PMS, antimycin A, HOQNO, DNP, CCCP, and valinomycin on the light-induced proton transport by cells of *R. rubrum*.

## Materials and Methods

*R. rubrum* was grown photoheterotrophically in the medium of Cohen-Bazire, Sistrom, and Stanier [6] for 24 hr with an illumination of 1,500 ft-c. The cells were harvested by centrifugation, washed twice and resuspended in glass-distilled water. NaCl or KCl was added to a concentration of 200 mM just prior to experimentation, because the light-induced pH change is very low in the absence of an added salt. Measurements of pH were made with a glass electrode as described previously [8]. The light intensity used during experimentation was $25 \times 10^4$ erg/cm$^2$ × sec. Experiments were run at 30 °C. Anaerobiosis during experimentation was maintained by bubbling with $N_2$ which had been passed through alkaline pyrogallol to remove any traces of oxygen. The pH of the cell suspension prior to illumination was 5.5. At the end of each experiment, a known amount of HCl was added in the absence of illumination in order to determine the buffering capacity of the cell suspension. [2] BChl concentration was determined spectrophotometrically after extraction with methanol-acetone [8].

Antimycin A (grade B), CCCP, and valinomycin were purchased from Calbiochem (Los Angeles, Calif.). HOQNO and PMS were obtained from the Sigma Chemical Co. (St. Louis, Mo.). DNP was purchased from Matheson, Coleman and Bell (Cincinnati, Ohio). Stock solutions were made by dissolving the chemicals in the following solvents: DNP and PMS in water; antimycin A, HOQNO, and valinomycin in ethanol; and CCCP in acetonitrile.

## Results

Fig. 1 shows that the initial rate of proton extrusion and the total change in [$H^+$] after illumination are proportional to the cell density or to the

1 Abbreviations: OD, optical density; PMS, phenazine methyl sulfate; DNP, 2,4-dinitrophenol; CCCP, carbonylcyanide-m-chlorophenylhydrazone; BChl, bacteriochlorophyll; HOQNO, 2-n-heptyl-4-hydroxyquinoline-N-oxide; FCCP, carbonylcyanide-p-Trifluoromethoxyphenylhydrazone.

2 Poly and Jagendorf [24] have recently shown that the buffering capacity of chloroplast suspensions may be greater during illumination than during the dark period, depending on the initial pH of the suspension. At pH 5.5, they found no difference in the buffering capacity of the medium during or following illumination. In studies with *R. rubrum*, the buffering capacity of the medium may be different during measurements of the initial velocity of light-induced transport, during steady state proton transport with illumination, and during the dark period following illumination. Thus, the possibility for some error in determining the quanitative aspects of proton transport should be noted.

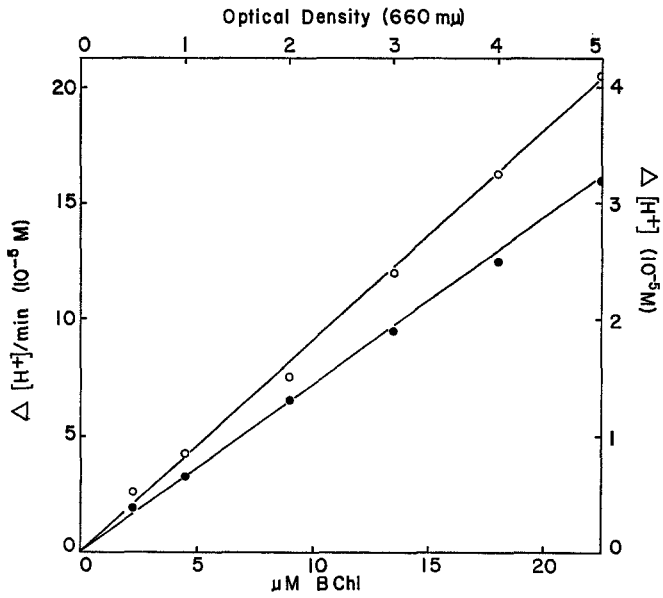


Fig. 1. The effect of varying OD or BChl concentration on the light-induced proton transport with cells of *R. rubrum*. Cell suspensions were diluted with glass-distilled water to give the optical densities tested. NaCl was added 2 min prior to illumination to give a final concentration of 200 mM, pH 5.50 ± 0.04. Cells were illuminated 2 min. Points represent averages of three replications. o——o, total change $[H^+]$ after 2-min illumination; •——•, rate of change $[H^+]$ upon illumination

concentration of BChl. The subsequent experiments were run with cells suspended at an OD of 4 at 660 nm measured with a Beckman DB-G Spectrophotometer.

Cells were used the same day they were harvested. However, they can be refrigerated at 4 °C for several days without a reduction in the magnitude of the light-induced pH change. There was an appreciable reduction of the initial rate of proton extrusion after storage.

The light-induced proton transport was the same whether the cells were bubbled with $N_2$, air, or $CO_2$-free air (Table 1).

Both the rate of proton extrusion and its dark reversal are temperature dependent with an apparent $Q_{10}$ of 1.8 for the "light-on" reaction and 2.18 for the "light-off" reaction between 10 and 30 °C as calculated from an Arrhenius plot (Fig. 2).

PMS, which stimulates photophosphorylation in chromatophores from *R. rubrum* [9], caused a marked stimulation of the light-induced decrease in pH when added to a final concentration of 0.1 to 1 mM (Fig. 3). Antimycin A (10 μg/ml) inhibited the proton transport, whereas PMS in the pre-

Table 1. *Comparison of the effect of $N_2$, air, and $CO_2$-free air on the light-induced proton extrusion by R. rubrum*[a]

| Gas | $\Delta[H^+]$/min ($10^{-5}$ M) | Total $\Delta[H^+]$ ($10^{-5}$ M) |
|---|---|---|
| $N_2$ | 9.48 | 2.13 |
| Air | 9.45 | 1.98 |
| $CO_2$-free air | 9.05 | 1.92 |

[a] The cells were bubbled with the indicated gas. NaCl was added to a final concentration of 200 mM 4 min prior to illumination; the pH was 5.5. The values given are the averages of three replications. Total change in $[H^+]$ was calculated from the change in pH after 2 min of illumination.

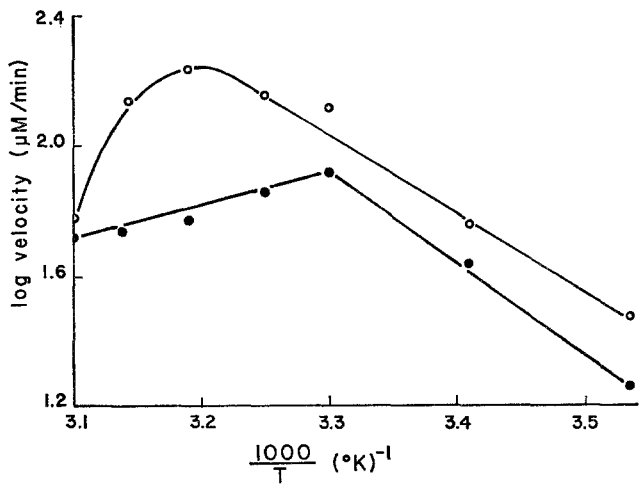


Fig. 2. The effect of temperature on light-induced proton transport by cells of *R. rubrum*. Cells were kept at 4 °C and the temperature was raised to the values tested just before experimentation; NaCl was added to give a concentration of 200 mM and pH of 5.5 4 min prior to illumination; cells were illuminated 4 min. Points represent averages of three replications; o——o, log of initial velocity of proton extrusion with "light-on"; •——•, log of initial velocity of proton uptake with "light-off" following illumination

sence of antimycin A stimulated the proton transport (Table 2). At a concentration of 20 μM, HOQNO inhibited the light-induced pH change, especially during the second illumination cycle. When HOQNO was added to the medium 2 min prior to illumination, there was a rise in pH (Fig. 4) which was not encountered after the addition of antimycin A. As shown in Fig. 4, 1 mM PMS stimulated the pH changes in the presence of HOQNO.

DNP had an effect on the light-induced proton transport similar to that previously noted with CCCP [8]. Addition of 0.1 or 0.5 mM DNP to a suspension of cells at pH 5.5 caused an immediate rise in the pH of the

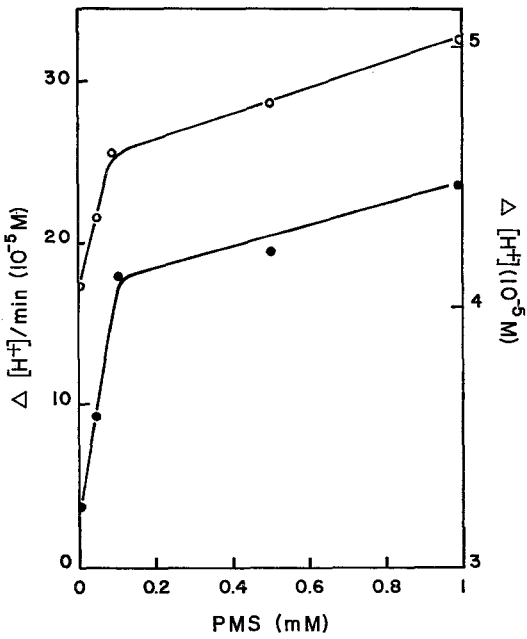


Fig. 3. Effect of PMS on the light-induced proton transport with cells of *R. rubrum*. NaCl was added to a final concentration of 200 mM, and PMS was added at the concentrations given, 2 min prior to illumination. Initial pH $5.5 \pm 0.05$. Points represent averages of three replications; o——o, rate of proton extrusion with illumination; •——•, total change $[H^+]$ after 2-min illumination

Table 2. *Effect of antimycin A and PMS on light-induced proton transport in R. rubrum*[a]

| Substance added | $\Delta[H^+]$/min ($10^{-5}$ M) | Total $\Delta[H^+]$ ($10^{-5}$ M) |
|---|---|---|
| None | 13.5 | 1.77 |
| 1 mM PMS | 35.7 | 2.48 |
| 10 μg/ml antimycin A | 4.6 | 1.14 |
| 1 mM PMS + 10 μg/ml antimycin A | 19.0 | 1.94 |

[a] NaCl was added to a concentration of 200 mM 4 min prior to illumination. pH, after addition of salt, was adjusted to 5.5. Where tested, PMS and antimycin A were added 2 min prior to illumination. Data represent averages of three replications. Total change $[H^+]$ was calculated 1 min after illumination.

medium. Both the initial rate of proton extrusion induced by illumination and the total decrease in pH were much lower in the presence of 0.1 and 0.5 mM DNP (Fig. 5). As shown in Fig. 5, the inhibition of the proton transport by DNP was not overcome by PMS. In the same manner, 1 mM

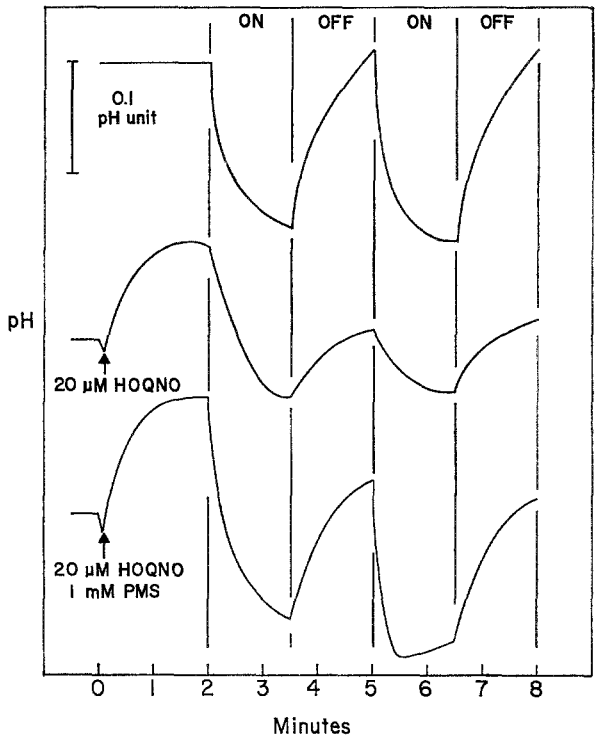


Fig. 4. Effect of HOQNO and PMS on the light-induced proton transport by cells of *R. rubrum*. NaCl was added to a concentration of 200 mM 4 min prior to illumination. The pH of the suspension was $5.5 \pm 0.04$. Addition of 0.02 mM HCl caused a change in pH of $0.21 \pm 0.01$

PMS added 2 min prior to illumination could not overcome the inhibition caused by 3 μM CCCP. Aerobically grown *R. rubrum* cells, which have only traces of BChl, did not show any light-induced pH change, nor did PMS cause a light-induced pH change with these cells.

Valinomycin, which stimulates the light-induced proton transport by chromatophores [12, 30], caused a slight stimulation of the light-dependent proton transport with cells of *R. rubrum* in the presence of $K^+$ (Fig. 6). Similarly, Scholes, Mitchell, and Moyle [26] showed that valinomycin stimulates the light-induced proton transport by cells of *R. spheroides* in the presence of 150 mM KCl at pH 6.0.

The inhibition of the proton transport by 3 μM CCCP was partially relieved by 3 μM valinomycin when the cells were suspended in 200 mM KCl (Fig. 6). However, when the cells were suspended in 200 mM NaCl, valinomycin did not stimulate the light-induced pH changes nor did it overcome the inhibition caused by CCCP.

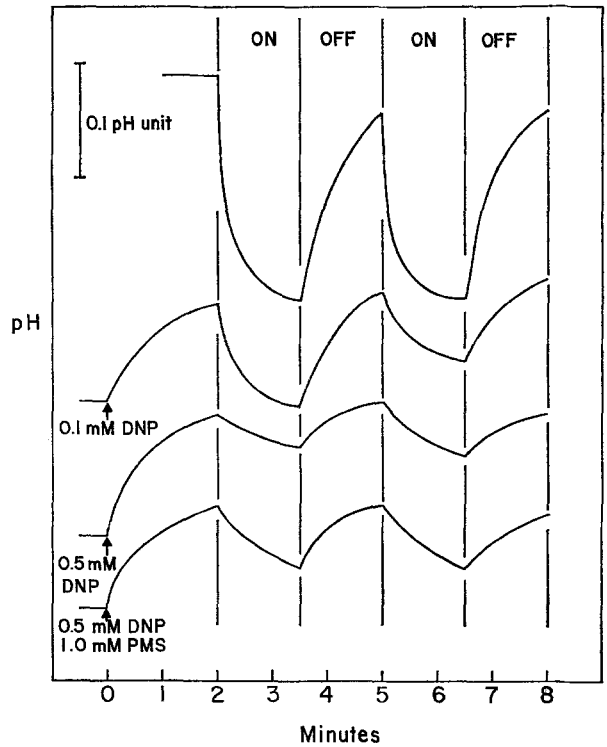


Fig. 5. The effect of DNP and PMS on the light-induced proton transport by cells of *R. rubrum*. Conditions were the same as in Fig. 4. Addition of 0.02 mM HCl caused a change in pH of $0.15 \pm 0.02$

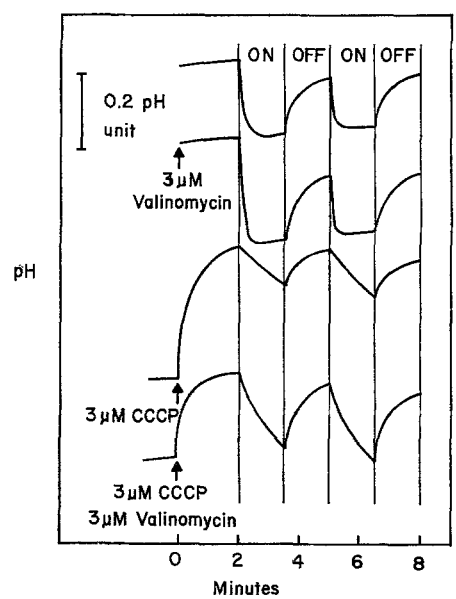


Fig. 6. The effect of valinomycin and CCCP on the light-induced proton transport with cells of *R. rubrum* in the presence of KCl. KCl was added 4 min prior to illumination to give a final concentration of 200 mM. The initial pH was $5.5 \pm 0.04$. Addition of 0.02 mM HCl gave a change in pH of $0.18 \pm 0.02$

## Discussion

Photoheterotrophically grown cells of *R. rubrum*, in the presence of 200 mM NaCl at pH 5.5, show a total proton extrusion of 1.8 moles $H^+$/mole BChl (Fig. 1). The light-induced proton consumption by isolated spinach chloroplasts is 0.5 to 0.8 moles/mole chlorophyll [7, 13]. When calculated on a mole/mole basis, the light-induced proton consumption by chromatophores varied from 0.5 to 1.0 mole/mole BChl [28, 29]. In comparison, the *R. rubrum* cells have an equivalent capacity for proton transport under the conditions studied. However, variables such as ionic strength, pH, membrane integrity, and specific activity of the chlorophyll (μg chlorophyll/mg protein) would control the degree of proton transport in any system.

Since the light-induced pH change with cells of *R. rubrum* occurs in the absence of $CO_2$ (Table 1) and without the addition of a substrate, it appears that the proton transport may be linked to a cyclic electron flow.

PMS evidently stimulates photophosphorylation and proton transport in chromatophores [29] by stimulating the cyclic photosynthetic electron flow. In the present studies, 1 mM PMS caused a 2-fold stimulation of the initial rate of light-induced proton transport and a 1.5-fold increase in the total change in pH with illumination (Fig. 3). These results were obtained in the absence of $CO_2$ and exogenous substrates. It is probable that PMS stimulates a cyclic electron flow which is linked to a vectorial proton transport.

Antimycin A and HOQNO caused an inhibition of proton transport which could be relieved with 1 mM PMS. Several workers [3, 12] have shown that the inhibition of photophosphorylation in chromatophores by HOQNO and by antimycin A could be overcome with PMS. It has been suggested, on the basis of spectrophotometric studies with *R. rubrum* cells, that HOQNO and antimycin A block the flow of electrons between cytochrome b and cytochrome $c_2$ [19, 22]. PMS apparently provides a "bypass" for electron flow around the inhibited site. Since PMS causes a stimulation of the proton transport in intact cells, it may indicate that the bypassed reactions are the rate-limiting steps to electron flow.

Nishimura, who showed that there was a light and dark reaction in cyclic electron flow in chromatophores, found that PMS did not affect the primary light-induced step in photophosphorylation, but that it did enhance electron flow reactions in the dark [18]. He also showed that the dark reactions of photo-induced electron flow in chromatophores are temperature dependent even in the presence of PMS. The $Q_{10}$ for the initial rate of the light-induced proton transport in *R. rubrum* cells is 1.8, which indicates

that the reaction is temperature dependent. In the presence of 0.5 mM PMS, the $Q_{10}$ was 2.63 for the initial rate of the light-induced proton transport between 10 and 45 °C, which suggests that PMS does not bypass all of the enzymes necessary to catalyze proton transport.

DNP, which has been shown to inhibit photophosphorylation in chromatophores of *R. rubrum* [9], inhibited the light-induced proton transport at a concentration of 0.5 mM. DNP, CCCP, and other uncouplers have been shown to facilitate proton diffusion across membranes [4, 10, 23]. PMS did not overcome the inhibition caused by DNP or CCCP. Although PMS may enhance the rate of proton extrusion in the presence of an uncoupler, a pH differential would not be established due to the permeability of the membrane to protons.

Valinomycin, an antibiotic which specifically increases membrane conductance of $K^+$, $Rb^+$, and $Cs^+$ [5, 17], gave a slight enhancement of the light-induced proton transport by *R. rubrum* cells in the presence of 200 mM KCl. Such enhancement by valinomycin might be expected if the light-induced pH changes observed are due to a $H^+/K^+$ exchange across the membrane, and if this exchange reaction is limited by the membrane permeability to $K^+$.

Jackson, Crofts, and von Stedingk [12] showed that the inhibition of the light-induced proton transport in chromatophores by FCCP in the presence of 0.4 mM KCl could be overcome by valinomycin. With cells of *R. rubrum*, the inhibition of light-dependent proton transport by the uncoupler CCCP in the presence of 200 mM KCl could be partially relieved by 3 μM valinomycin. These results can be explained in terms of the chemiosmotic hypothesis. Initially, a light-induced charge separation across the membrane by the extrusion of a $H^+$ would allow an electrical potential to develop. In the presence of a salt like KCl, the electrical potential could be partially discharged in favor of a pH differential by a $H^+/K^+$ exchange at a rate limited by the uptake of the metallic cation. However, an uncoupler such as CCCP could discharge the electrical potential by making the membrane permeable to protons; hence, there would be a decreased $H^+/K^+$ exchange. In the presence of CCCP and valinomycin, the membrane would be permeable to $H^+$ and $K^+$. The charge separation by extrusion of a $H^+$ from the cells could be readily released by uptake of $K^+$ or $H^+$. Only the uptake of $K^+$ in exchange for $H^+$ would allow a pH change, but this process would occur slowly since the uptake of $H^+$ could also release the membrane potential (Fig. 6). Since valinomycin allows the penetration of $K^+$ specifically, it is significant that the antibiotic has no effect on the light-induced proton transport in the presence of 200 mM NaCl, with or without

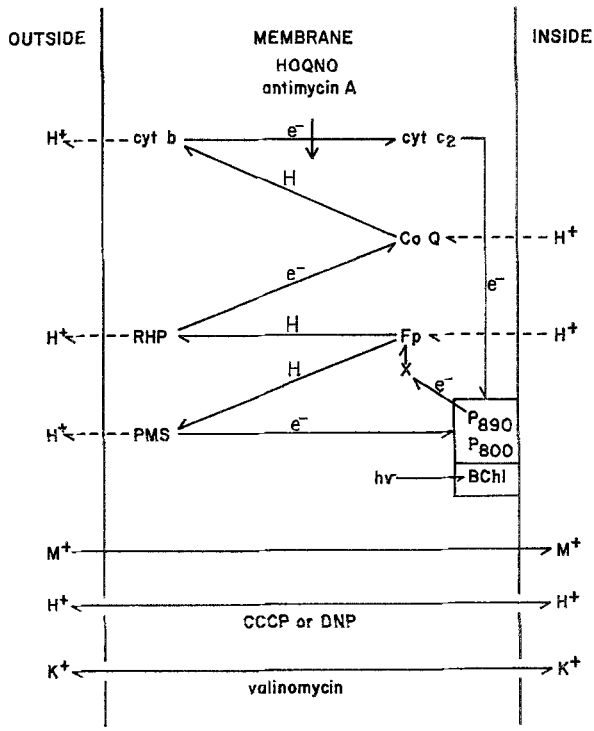


Fig. 7. Hypothetical scheme of the light-induced proton and electron transport in photoheterotrophically grown cells of *R. rubrum*. OUTSIDE refers to the extracellular space, INSIDE to the intracellular. Abbreviations: *Fp*, flavoprotein; *RHP*, rhodospirillum heme protein; *Co Q*, ubiquinone-10; *cyt b*, cytochrome b; *cyt c*, cytochrome $c_2$; *X*, unidentified primary reductant; $M^+$, metallic cation; $P_{890}$, $P_{800}$, special photoactive bacteriochlorophyll; *H*, hydrogen atom; $e^-$, electron

CCCP. From these observations, it appears that the pH differential which may develop depends on the rate of proton extrusion by the cells and on the permeability of the membrane to protons and exchangeable metallic cations.

A hypothetical scheme which presents a mechanism for proton transport by cells of *R. rubrum* based on the results obtained is shown in Fig. 7. It has been suggested that PMS may bypass a phosphorylating site in chromatophores as it mediates a cyclic electron flow [2, 20]. At low light intensities, the quantum efficiency for phosphorylation is lower in the PMS system than in the physiological system. At high light intensities, the PMS-mediated phosphorylation is greater than in the physiological system, which may be because of an enhanced electron flow through a single phosphorylating site [20].

PMS is capable of accepting electrons from succinate in the presence of succinic dehydrogenase [14]. In the cyclic photosynthetic electron transport of *R. rubrum*, PMS could accept electrons from an early reductant such as flavoprotein since o-phenanthroline, which inhibits the cyclic photophosphorylation in the physiological system, does not inhibit the PMS-mediated phosphorylation [25]. Although ubiquinone-10, flavoprotein, and rhodospirillum heme protein have been shown to be involved in cyclic electron transport and photophosphorylation in *R. rubrum*, the exact sequence of these electron transport carriers is not known [1, 11, 21].

According to the chemiosmotic hypothesis for ATP synthesis, each phosphorylating site consists of an oxidation-reduction loop which contains a vectorial proton-transporting mechanism. The scheme in Fig. 7 has two sites for proton transport in the physiological system, and hence two sites for phosphorylation according to the chemiosmotic hypothesis. The PMS could bypass one of the proton-transporting oxidation-reduction loops. At the relatively high light intensities used in this research ($25 \times 10^4$ erg/cm$^2$ $\times$ sec), the PMS-mediated cyclic electron flow may be enhanced which could maintain phosphorylation by stimulating proton transport at the single phosphorylating site.

Others have shown that proton transport and photo- or oxidative phosphorylation are closely linked to electron transport in chromatophores [12], mitochondria [15], and chloroplasts [13]. The action of PMS, inhibitors, and uncouplers on the light-induced proton transport in *R. rubrum* cells indicates that the proton transport is linked to electron flow coupled with phosphorylation.

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