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STEADY-STATE MEASUREMENTS OF ΔpH AND Δψ IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES BY TWO DIFFERENT METHODS

COMPARISON WITH PHOSPHORYLATION POTENTIAL

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The pH gradient, ΔpH, and the membrane potential, Δψ, formed during light-induced electron transport in *Rhodospirillum rubrum* chromatophores were measured by two independent methods: (a) using specific electrodes to monitor light-dependent uptake of NH_4Cl and SCN^- at chromatophore concentrations of about 0.1 mg bacteriochlorophyll/ml and (b) using 9-aminoacridine and 8-anilinonaphthalenesulfonic acid as fluorescent probes for ΔpH and Δψ, respectively, at chromatophore concentrations of about 0.01 mg bacteriochlorophyll/ml. The light intensity was measured and set at a level which saturated the highest bacteriochlorophyll concentration used. The steady-state values obtained with each method under phosphorylating conditions were compared with the phosphorylation potential maintained by the chromatophores under identical conditions. The results indicate that under all conditions employed the ratio H^+/ATP is greater than 2, and varies between 2.4 and 3.4 depending on the method used for estimation of the electrochemical proton gradient.

Introduction

Light-induced electron transport in *Rhodospirillum rubrum* chromatophores is accompanied by an uptake of protons [1], resulting in the formation of an electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) which is composed of both a pH gradient (ΔpH) and a membrane potential (Δψ) [2]. According to the chemiosmotic theory [3], these two components comprise the proton-motive force that drives ATP synthesis. A number of laboratories have therefore attempted to measure the size of both components, each employing a different method [4–9]. These methods fall into two general catagories: (a) those measuring the distribution of hydrophilic ions [10,11] and (b) those following changes in fluorescence or absorbance of optical probes [10,12].

The hydrophilic ions used thus far to monitor ΔpH and Δψ formed in *R. rubrum* chromatophores include permeating amines, such as CH_3NH_2 [13] and permeating anions, such as SCN^- [14]. Their distribution across the chromatophore membrane was assayed by a centrifugation procedure [4] or flow dialysis [7] using ^{14}C-labeled compounds. SCN^- distribution was also followed with an ion-selective electrode [8]. The optical probes include the fluorescent amine 9-aminoacridine. Its fluorescence is quenched during light-induced electron transport, as a consequence of its uptake, and the extent of the fluorescence quenching, reported to provide an estimate of ΔpH in chloroplasts [15], was also followed in *R. rubrum* [6]. The optical probes used to

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Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone; Tricine, *N*-tris(hydroxymethyl)methylglycine.

measure the $\Delta\psi$ in *R. rubrum* include the fluorescent dyes ANS [6] and 3,3-dipentyloxacarbocyanine [5]. Their fluorescence, which is enhanced during light-induced membrane energization [5,16], was shown to increase also by imposing an artificial diffusion potential across the membrane upon injection of KCl in the presence of valinomycin [5,6]. Recently, the decrease in absorbance of the oxonol dye OX-VI [17] has been used to monitor $\Delta\psi$ in *R. rubrum*.

The different methods used yielded widely different results. Thus, values reported for $\Delta\psi$ range from 60 to 110 mV [4–8] and up to about 260 mV [9] and the measured ΔpH values range from 0 mV [7] to 110–160 mV [4,6]. Consequently, in the three investigations in which the size of the overall $\Delta\tilde{\mu}_{H^+}$ was compared with the magnitude of the phosphorylation potential [6,7,9], the calculated stoichiometry (H^+/ATP) of the ATP synthase reaction in illuminated *R. rubrum* chromatophores at steady state varied between 2 and 6. The low H^+/ATP ratios were obtained when ΔpH and $\Delta\psi$ were measured with the optical probes [6,9], whereas the highest ratios were reported when these parameters were determined by the flow dialysis technique [7]. The optical probes are not universally accepted as quantitative indicators of ΔpH and $\Delta\psi$ [11] and the flow dialysis requires a very high concentration of particles which cannot be easily light saturated. Also, not in all cases has the phosphorylation potential been measured under conditions identical to those used for estimation of ΔpH and $\Delta\psi$.

In order to obtain reliable data it seems desirable (a) to compare measurements of ΔpH and $\Delta\psi$ by two independent methods in the same chromatophore preparation and (b) to carry out all measurements including those of the phosphorylation potential under similar conditions of reactant concentrations and light saturation. In this communication we have measured ΔpH and $\Delta\psi$ by specific electrodes and by fluorescent probes and compared the values obtained with the phosphorylation potential maintained under identical conditions.

Materials and Methods

Materials. Growth of *R. rubrum* cells and isolation and storage of chromatophores were performed as previously described [13,16]. Their bacteriochlorophyll concentration was determined from the absorbance at 880 nm using the in vivo extinction coefficient given by Clayton [18].

Determination of proton uptake. Proton uptake was assayed with a Radiometer GK2321C combination electrode and a Radiometer PHM 64 pH meter connected to a strip recorder. The chromatophores were suspended in a thermostatically controlled, magnetically stirred vessel at 25°C in a final volume of 2.2 ml containing 20 mM NaCl, 6 mM $MgCl_2$ 0.5 mM diaminodurene, 100 mM KCl, 2 μM valinomycin and 6 mM Tricine-maleate (pH 6.0). The samples were illuminated by a slide projector (with heat filter removed) fitted with a 250 W, 24 V halogen lamp. The light was filtered through 9 cm of water and a combination of a Corning C.S. 3-69 and a Schott RG 715 filter. Light intensities were changed by varying the voltage supplied to the projector lamp and were measured at the center of the vessel with a Radiometer light meter, model 65 (Yellow Springs Instrument Co., OH).

Measurements with selective electrodes. The determination of ΔpH and $\Delta\psi$ from the distribution of either a permeant amine or anion across the chromatophore membrane was done by following the extent of their light-induced uptake with two specific electrodes. ΔpH was measured with an Orion gas-sensing ammonia electrode (model 95-10, Orion Research, Sussex) which senses free ammonia in the micromolar range. The potential developed by the addition of calibrating pulses of NH_4Cl and the change in potential resulting from the uptake of ammonia upon illumination were recorded with a Photovolt OmniScribe recorder connected in parallel with a variable resistor to an Orion model 701 pH meter set on the millivolt mode. Since the electrode senses free ammonia and the p*K*a of NH_4^+ is 9.25, the sensitivity of the electrode decreases significantly below pH 9.0. Therefore, ammonia uptake with this electrode cannot be measured below pH 9.0. Consequently, all measurements described below, including $\Delta\psi$ and phosphorylation potential measurements, were carried out at pH 9.0.

The extent of ammonia uptake was calculated from the decrease in its concentration in the suspension medium, taking the internal osmotic volume of the chromatophores to be 50 μl/mg bacteriochloro-

phyll [4]. Assuming that only the unprotonated ammonia species is fully permeable across the cell membrane, the distribution of ammonia across the chromatophore membrane is related to the proton distribution according to the following expression [15]:

$$\frac{[A_T]_{in}}{[A_T]_{out}} = \frac{[AH^+]_{in} + [A]_{in}}{[AH^+]_{out} + [A]_{out}} = \frac{K_a + [H^+]_{in}}{K_a + [H^+]_{out}} \quad (1)$$

where $[A_T]$ is the total ammonia concentration, $[AH^+]$ and $[A]$ are the protonated and unprotonated forms of ammonia, respectively, $[H^+]$ is the proton concentration (or activity), and K_a is the ammonia dissociation constant ($K_a = [A][H^+]/[AH^+]$). Since the value of $K_a = 10^{-9.25}$ (i.e., $pK_a = 9.25$) when $[H^+]_{out} = 10^{-9}$ (i.e., $pH_{out} = 9.0$) and $[H^+]_{in} = 10^{-8}$ or greater (i.e., $pH_{in} = 8$ or below), Eqn. 1 can be written as:

$$\frac{[A_T]_{in}}{[A_T]_{out}} = \frac{[H^+]_{in}}{K_a + [H^+]_{out}} \quad (2)$$

This equation can be solved for pH_{in} by rearrangement and taking the –log of both sides as shown:

$$-\log[H^+]_{in} = -\log[K_a + [H^+]_{out}] - \log\frac{[A_T]_{in}}{[A_T]_{out}} \quad (3)$$

Since the value of $-\log(K_a + [H^+]_{out})$ at pH 9.0 is 8.8, Eqn. 3 becomes:

$$pH_{in} = 8.8 - \log\frac{[A_T]_{in}}{[A_T]_{out}} \quad (4)$$

ΔpH, defined as $pH_{out} - pH_{in}$, at $pH_{out} = 9.0$ is therefore:

$$\Delta pH = 9 - \left(8.8 - \log\frac{[A_T]_{in}}{[A_T]_{out}}\right) = 0.2 + \log\frac{[A_T]_{in}}{[A_T]_{out}} \quad (5)$$

$\Delta\psi$ was obtained from the uptake of SCN^- measured with the Orion liquid membrane NO_3^- electrode (model 93-07) for reasons described by Kell et al. [8], assuming that SCN^- equilibrates across the membrane according to the Nernst equation and taking the internal osmotic volume stated above. The potential developed by the addition of calibrating pulses of SCN^- and the change in potential resulting from its uptake upon illumination were measured against a double-junction Orion reference electrode (model 90-02) using the same pH meter and recorder set-up described for the gas-sensing ammonia electrode. The reaction conditions were also the same with both electrodes. The measurements were carried out in a 25 ml beaker containing, in a final volume of 6 ml: 0.2 mM sodium succinate, 2.66 mM sodium phosphate, 5 mM magnesium acetate, 20 mM sucrose and 0.2 mM ADP, where indicated, buffer at pH 9.0, as indicated in the text and chromatophores at concentrations of 0.08–0.16 mg bacteriochlorophyll/ml. The reaction mixtures were stirred with a magnetic bar and illuminated with a saturating light intensity of $1 \cdot 10^6$ erg $\cdot$ cm^{-2} $\cdot$ s^{-1} (see Fig. 1). All experiments were performed at 25°C and in all of them the external pH remained constant during the whole experiment.

Measurements with fluorescent probes. Both ΔpH and $\Delta\psi$ were also determined from the light-induced changes in fluorescence of 9-aminoacridine and ANS, respectively. The calibrations and calculations were as described earlier [6]. The measurements were carried out in cuvettes containing a 3 ml reaction mixture which was identical to that described above for the selective electrode measurements, except that the chromatophore concentration varied between 0.005 and 0.01 mg bacteriochlorophyll/ml.

Determinations of the phosphorylation potential. These measurements were performed in the presence of [^{32}P]phosphate under conditions of illumination and reactant concentrations identical to those used for measurements of ΔpH and $\Delta\psi$ with either the selective electrodes or the fluorescent probes. The samples were illuminated for 20 min. A shorter period was previously found [6] to be sufficient for reaching a steady-state extent of phosphorylation under fluorescent probe conditions and the 20 min period was confirmed as sufficient for the selective electrode conditions. The reactions were stopped by addition of HCl to a final concentration of 0.05 M and after centrifugation ATP, ADP and P_i were assayed as outlined by Leiser and Gromet-Elhanan [6]. ΔG_p was calculated employing a value of 9.17 kcal/mol for the standard

free energy of ATP hydrolysis at pH 9.0, 10 mM Mg^{2+}, 0.1 ionic strength and 25°C [19].

Results

Effect of light intensity

The effect of light intensity on proton uptake and on ΔpH has been studied in detail in chloroplasts, where ΔpH was measured either by the distribution of $^{14}CH_3NH_2$ [20] or by the changes in fluorescence of 9-aminoacridine [15]. The extent of proton uptake showed a strict dependence on the light intensity over an external pH range between 6.0 and 9.0. ΔpH, when measured by both methods, responded to increasing light intensities similarly to the proton uptake at an external pH of 8.0 or 9.0, but when the external pH was below 7.0 it reached saturation much earlier than the proton uptake activity [15,20]. The saturation of ΔpH at lower light intensities when assayed at the low pH values was explained by the existence of a natural buffering group operating at low pH [15]. Thus, at pH values below 7.0, the first few protons transported into the osmotic volume would markedly shift the internal pH, while most of the other protons would undergo buffering. But at an external pH of 8.0 or above, the buffering region would be reached only after most of the protons have been pumped in. $\Delta\psi$ should behave at all external pH values as ΔpH does at the low external pH since, irrespective of the external pH, entrance of the first few ions will be enough to build up the maximal $\Delta\psi$. Therefore, when assayed at an external pH of 8.0 or 9.0, $\Delta\psi$ would be saturated at much lower light intensities than either the proton uptake or ΔpH.

Higher chloroplast or chromatophore concentrations need a higher light intensity in order to reach saturating illumination conditions. Since measurements with selective electrodes require much higher chromatophore concentrations than those using fluorescent probes, about 0.1 mg bacteriochlorophyll/ml [8] as compared to less than 0.01 mg bacteriochlorophyll/ml [6,16], respectively, it is important to use a light intensity which will saturate even the highest bacteriochlorophyll concentrations used.

Fig. 1 illustrates the dependence of proton uptake on the light intensity at two different chromatophore

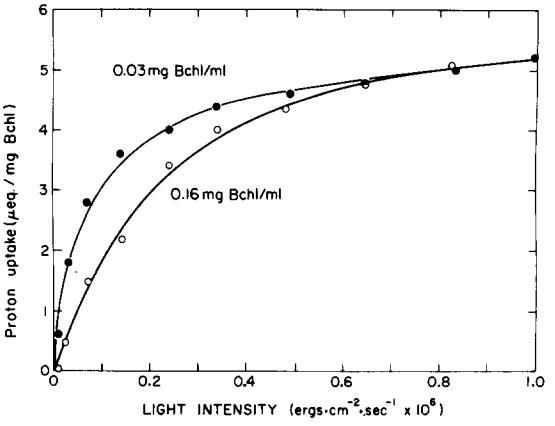

Fig. 1. Proton uptake by *R. rubrum* chromatophores as a function of light intensity. The measurements were performed as described in Materials and Methods with the indicated chromatophore concentrations. Bchl, bacteriochlorophyll.

concentrations. At an intensity of $2.8 \cdot 10^5$ erg · $cm^{-2} \cdot s^{-1}$, which was used by Kell and co-workers [7,8], even the concentration of 0.03 mg bacteriochlorophyll/ml is less than 85% light saturated, and that of 0.16 mg bacteriochlorophyll/ml operates at about 70% saturation. Only above $8 \cdot 10^5$ erg · $cm^{-2} \cdot s^{-1}$ are the recorded values of proton uptake identical with both chromatophore concentrations. So, when tested at an external pH of 8.0 or 9.0, $\Delta\psi$ might be saturated even at $3 \cdot 10^5$ erg · $cm^{-2} \cdot s^{-1}$, whereas ΔpH will require much higher light intensities for its saturation. All the experiments described below were therefore illuminated with an intensity of $1 \cdot 10^6$ erg · $cm^{-2} \cdot s^{-1}$, which is saturating even the highest chromatophore concentration used in these experiments, namely, 0.16 mg bacteriochlorophyll/ml.

Determination of ΔpH with a gas-sensing ammonia electrode

Representative traces of the response of the gas-sensing ammonia electrode to calibrating pulses of NH_4Cl and to illumination are shown in Fig. 2. Although the voltage response to calibrating pulses of ammonia in the absence of chromatophores is logarithmic, with a characteristic increase of 60 mV per decade increase in ammonia concentration, the response observed in the presence of chro-

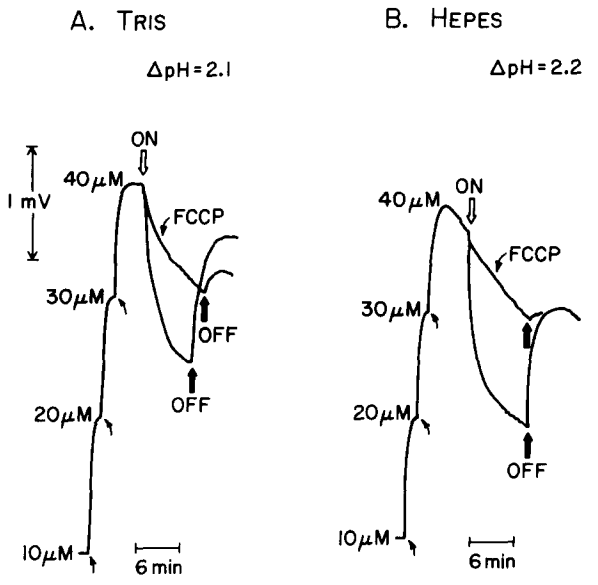

Fig. 2. Light-induced uptake of ammonia by *R. rubrum* chromatophores monitored by a gas-sensing ammonia electrode. The reaction mixture was as described in Materials and Methods with a chromatophore concentration of 0.1 mg bacteriochlorophyll/ml, 10 mM of the indicated buffers at pH 9.0 and without ADP. Calibrating additions of NH_4Cl were made as indicated by the curved arrows, increasing the external concentration in 10 μM increments to the final concentration of 40 μM. The lower trace in each case was run first, then 10 μM FCCP was added and the upper trace was recorded. ΔpH was calculated from the difference between the uninhibited minus the inhibited electrode potentials after 6 min.

matophores, at the low range of concentrations of NH_4Cl that was used here as a probe for ΔpH, is nearly linear (Fig. 2). Similar observations were reported by other groups using ion-selective electrodes [8,21]. However, as also shown by these workers, an extensive and rapid uptake, which was reversible in the dark, was observed upon initiation of electron transport by illumination (Fig. 2). In the dark there was a very slow, but continuous drift (see Fig. 2B) and after the light was turned off the trace returned to its expected place along the drifting line. The electrode was somewhat sensitive to the wavelength of light used in this study, as indicated by the potential change observed in the light in the presence of an uncoupler such as FCCP (Fig. 2), or an electron transport inhibitor such as antimycin A, at concentrations which inhibit completely light-induced proton uptake in chromatophores. When the trace obtained in the presence of these compounds was subtracted from that obtained in their absence, the establishment of a steady state was clearly observed (see Fig. 3). ΔpH was therefore calculated on the basis of the light-induced steady-state potential change recorded in the control after subtracting the change recorded in the presence of FCCP or antimycin A.

Light sensitivity seems to be a feature of other ion-selective electrodes, e.g., the Orion Model 94-58 solid-state SCN^- electrode which was reported by Kell et al. [8] to be sensitive to light. Therefore, they recommended the use of the Orion Model 93-07 liquid membrane NO_3^- electrode, which is much less sensitive to light, for determination of the light-dependent uptake of either NO_3^- or SCN^-. In our hands, a slow but persistent potential change was observed upon illumination with the NO_3^- electrode. Therefore, the $\Delta\psi$ calculations, like those of ΔpH, were made only on the basis of the FCCP- and antimycin A-sensitive light-induced SCN^- distribution (see Table II).

As illustrated in Fig. 2, a ΔpH of about 2 pH units is formed across the *R. rubrum* chromatophore membrane upon illumination at an external pH of

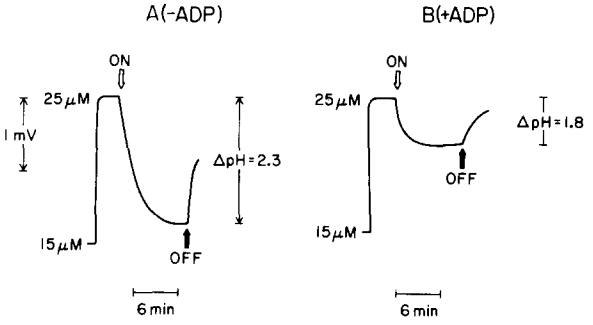

Fig. 3. Decrease of the light-induced ΔpH measured by the gas-sensing ammonia electrode upon initiation of phosphorylation. The reaction mixture was as described in Materials and Methods with a chromatophore concentration of 0.07 mg bacteriochlorophyll/ml and 40 mM glycylglycine buffer at pH 9.0. A, without ADP; B, with 0.2 mM ADP. The calibrating concentrations of NH_4Cl (15 and 25 μM) are shown for each experiment. The traces shown in the figure represent the difference between the light-induced voltage changes obtained from the electrode in the absence and presence of 1 μM antimycin A.

9.0. This ΔpH value is similar to those measured earlier at an external pH of 8.0 [4,6]. The ΔpH measured with the gas-sensing ammonia electrode is independent of the type of buffer used to maintain the external pH at 9.0 (Fig. 2 and Table I). Moreover, under the experimental conditions of pH and chromatophore concentration used here for measurements with the gas-sensing ammonia electrode, the chromatophores responded to the addition of millimolar amounts of the permeant anion, SCN^-, by an increase of ΔpH (Table I) in a manner similar to that reported earlier for ΔpH measured by centrifugation techniques [4] and by fluorescence changes [6,14].

The data of Fig. 2 and Table I were obtained under nonphosphorylating conditions, i.e., in the absence of ADP. According to the chemiosmotic theory [3], ATP formation should create a drain on the steady-state $\Delta\tilde{\mu}_{H^+}$ generated during light-induced electron transport. A transient decrease in both ΔpH and $\Delta\psi$ measured by changes in the fluorescence of 9-aminoacridine and ANS was previously reported in *R. rubrum* chromatophores suspended at a medium pH of 8.0 [6]. As illustrated in Fig. 3, initiation of phosphorylation by illumination in the presence of ADP decreases by 0.5 pH units the ΔpH measured by the gas-sensing ammonia electrode at a medium pH of 9.0. For phosphorylation of all the ADP added here (1.2 μmol), about 10–12 min were required (see also Ref. 6). Therefore, the transitory nature of the ADP-induced drain is not seen in this case. These results, together with information reported earlier [13], that the rate of photophosphorylation observed in *R. rubrum* chromatophores at pH 9.0 amounts to at least 75% of the maximal phosphorylation rate observed at pH 8.0, indicate that in these chromatophores a medium of pH 9.0 is suitable for measuring ΔpH and the phosphorylation potential.

TABLE I

EFFECTS OF VARIOUS BUFFER SYSTEMS AND OF ADDED $NaSCN^-$ ON THE ΔpH MEASURED BY THE GAS-SENSING AMMONIA ELECTRODE

Reaction conditions were as described for Fig. 2. ΔpH values were calculated from the inhibitor-sensitive light-induced change of the voltage recorded with the ammonia electrode. The values represent averages of three to five determinations for each buffer.

Additions	ΔpH
Tricine-NaOH (10 mM)	2.26 ± 0.15
Tris-maleate (10 mM)	2.19 ± 0.12
Glycylglycine (40 mM)	2.31 ± 0.25
Hepes-maleate (10 mM)	2.26 ± 0.26
Hepes-maleate + NaSCN (1 mM)	2.20
Hepes-maleate + NaSCN (5 mM)	2.65
Hepes-maleate + NaSCN (10 mM)	3.03

Comparison of the measured $\Delta\tilde{\mu}_{H^+}$ and the phosphorylation potential at steady state

In order to enable a comparison of the overall $\Delta\tilde{\mu}_{H^+}$ attained at pH 9.0 with the phosphorylation potential, $\Delta\psi$ must also be measured at the same pH. Table II summarizes the ΔpH and $\Delta\psi$ values as measured, at a medium pH of 9.0, by following the

TABLE II

COMPARISON OF THE STEADY-STATE ELECTROCHEMICAL PROTON GRADIENT DETERMINED BY SELECTIVE ELECTRODES AND FLUORESCENT PROBES UNDER PHOSPHORYLATING CONDITIONS

The reaction mixture was as described in Materials and Methods with 40 mM glycylglycine buffer at pH 9.0 and 0.2 mM ADP. With the selective electrodes the chromatophore concentration was 0.16 mg bacteriochlorophyll/ml and either 40 μM NH_4Cl or 20 μM NaSCN were added for measurements of ΔpH or $\Delta\psi$, respectively. These parameters were calculated from the steady-state FCCP-sensitive light-induced potential changes recorded from the electrodes. With the fluorescent probes the chromatophore concentration was 0.007 mg bacteriochlorophyll/ml, and either 0.66 μM 9-aminoacridine or 7 μM ANS were added for measurements of ΔpH or $\Delta\psi$, respectively. All measurements were made after 20 min of illumination at a light intensity of $1 \cdot 10^6$ erg · cm^{-2} · s^{-1}. The values represent averages of three different determinations.

Method	Assay	Electrochemical proton gradient (mV)		
		$-Z\Delta pH$	$\Delta\psi$	$\Delta\tilde{\mu}_{H^+}$
Selective electrode	Ammonia	118 ± 2		
	Thiocyanate		52 ± 4	
				170 ± 6
Fluorescent probe	9-Aminoacridine	150 ± 6		
	ANS		70 ± 5	
				220 ± 11

TABLE III

COMPARISON OF THE PHOSPHORYLATION POTENTIAL (ΔG_p) AND THE ELECTROCHEMICAL PROTON GRADIENT ($\Delta\tilde{\mu}_{H^+}$) MEASURED IN THE STEADY STATE AT TWO CHROMATOPHORE CONCENTRATIONS

The phosphorylation potential was determined with the chromatophore concentrations corresponding to those used to determine $\Delta\tilde{\mu}_{H^+}$ with the selective electrodes (Expt. 1) or with the fluorescent probes (Expt. 2). All other reaction conditions, namely reactant concentrations and intensity and duration of illumination, were identical, as outlined in Table II. In Expt. 1 four different determinations were made, two with 40 μM NH_4Cl and two with 20 μM NaSCN and the ΔG_p was determined in all four experiments. Similarly, in Expt. 2 two determinations were made with 0.66 μM 9-aminoacridine and two with 7 μM ANS and ΔG_p was determined in all four. BChl, bacteriochlorophyll.

Expt. No.	Chromatophore concentration (mg BChl/ml)	Phosphorylation potential		$\Delta\tilde{\mu}_{H^+}$ (mV)	Apparent H^+/ATP
		ΔG_p (kcal/mol)	ΔG_p (mV)		
1	0.160	13.30 ± 0.53	580 ± 20	170 ± 6	3.41 ± 0.24
2	0.007	12.22 ± 0.41	530 ± 20	220 ± 11	2.41 ± 0.21

distribution of NH_4Cl and SCN^- across the chromatophore membrane with the respective selective electrodes. These values are compared with the ΔpH and $\Delta\psi$ values measured, with the same chromatophore preparation at identical reactant concentrations and medium pH, by following changes in fluorescence of 9-aminoacridine and ANS, respectively. The only difference in the reaction mixtures between the two different methods was in the chromatophore concentration. But the light intensity of $1 \cdot 10^6$ erg · cm^{-2} · s^{-1} was high enough to saturate both chromatophore concentrations (see Fig. 1). The values of ΔpH and $\Delta\psi$ observed here with the fluorescent probes at pH 9.0 are similar to those reported earlier under light-saturating conditions at pH 8.0 [6]. The values obtained with the specific electrodes are lower by about 25% (Table II).

When the phosphorylation potential attained in the steady state was measured under conditions identical to those used for the determination of ΔpH and $\Delta\psi$ by the two different methods, the results summarized in Table III were obtained. As indicated in Tables II and III, only the tested probe was added in each experiment. At the concentrations of the probes used here, and even at 10-fold higher concentrations, neither NH_4Cl, SCN^-, 9-aminoacridine nor ANS had any effect on the measured phosphorylation potential. The apparent H^+/ATP ratio, calculated from the measured steady-state $\Delta\tilde{\mu}_{H^+}$ summarized in Table II and the ΔG_p values summarized in Table III, varies between 2.4 and 3.4. The lower ratio was obtained in the setup used with the fluorescent probes, where in earlier measurements at a medium pH of 8.0, ratios of 2.6–2.7 were obtained [6]. The higher ratio was obtained in the setup used with the selective electrodes. It should be emphasized that even this high H^+/ATP ratio of 3.4 is much lower than the ratio of 5–6 reported by Kell et al. [7] from assays of SCN^- and CH_3NH_2 uptake using flow dialysis.

In all our measurements, performed under a variety of conditions and using different methods, the calculated apparent H^+/ATP ratio was always significantly above 2.0 and seems to be rather close to the ratio of 3.0 observed in chloroplasts [22,23].

Discussion

The chemiosmotic theory envisages that two protons are translocated across the membrane for each ATP molecule synthesized [3]. This stoichiometry requires that the $\Delta\tilde{\mu}_{H^+}$ generated across the membrane in the steady state under continuous illumination should be large enough to account for the ΔG_p measured under identical conditions with an H^+/ATP ratio of 2. A stoichiometry of H^+/ATP of 2 under steady-state conditions has been reported in chromatophores from photosynthetic bacteria only when the $\Delta\psi$ is measured via the carotenoid absorption changes [9,24]. Thus, in *Rhodopseudo-*

monas capsulata [24], the $\Delta\psi$ of 230 mV measured by the carotenoid absorbance changes is about 3–4-fold higher than the $\Delta\psi$ measured here with ANS or SCN^- (Table II). However, the ΔpH that was measured in both cases by the quenching of 9-aminoacridine fluorescence is very similar. We have obtained a ΔpH of 150 mV (Table II), whereas Casadio et al. [24] reported a ΔpH of approx. 190 mV and the latter value was measured in the presence of 50 mM KCl, which they had earlier shown [25] to cause a 40 mV increase of ΔpH.

Symons et al. [26] have recently demonstrated that values of the light-induced transmembrane $\Delta\psi$ as calibrated with diffusion potential-induced carotenoid absorbance changes in *Rps. capsulata* are largely overestimated. The reason being that the carotenoid ΔA produced by a diffusion potential consists, besides the change due to the generation of the transmembrane potential itself, also of a change due to an alteration of the negative surface potential. When they corrected the carotenoid ΔA for the surface potential change, the steady-state potentials that they observed in *Rps. capsulata* chromatophores were about 70 mV [26], a value very similar to the $\Delta\psi$ observed here in *R. rubrum* chromatophores (Table II).

The need for a correction of the carotenoid ΔA applies also to the other report of an H^+/ATP ratio of 2 obtained recently in *R. rubrum* chromatophores in which $\Delta\psi$ was measured by the decrease in absorbance of an oxonol dye [9]. But in this case the calibration was even more complicated. It has been reported earlier [17] that the oxonol response cannot be calibrated directly by using potassium gradients in the presence of valinomycin. Therefore, an indirect calibration of the oxonol dye response via the calibrated carotenoid band shift was suggested in *Rps. sphaeroides*, because in this bacterium the logarithm of the oxonol response was shown to be linear with the $\Delta\psi$ estimated from the carotenoid band shift [17]. In *R. rubrum*, however, even the carotenoid band shift cannot be directly calibrated, so Bashford et al. [9] used the carotenoid calibration curve obtained in *Rps. sphaeroides* for estimation of the steady-state $\Delta\psi$ attained in *R. rubrum* as reflected from the oxonol response. The accuracy of this two-step calibration procedure involving two different bacteria, in which the carotenoid absorbance changes are different, is rather questionable and in any case the original carotenoid calibration performed in *Rps. sphaeroides* [17] was not corrected for the nonbulk phase changes as suggested by Symons et al. [26].

Because of the absence of a reliable absolute calibration in methods using optical probes, such as carotenoids or ANS, Kell [11] has promoted the use of ion-distribution methods with hydrophilic ions which do not bind to biological membranes to any significant degree. He assumes that with such ions it is credible that the Nernst potential and bulk phase transmembrane pH gradients are being measured. Kell et al. [7] have recently used a flow dialysis assay of the distribution of the hydrophilic solutes SCN^- and CH_3NH_2 to determine the magnitude of the $\Delta\psi$ and ΔpH generated by the light-driven electron transport in *R. rubrum* chromatophores. They have reported the formation of a $\Delta\psi$ of 100 mV, but could not measure any significant ΔpH unless millimolar concentrations of SCN^- were added. Since they have also determined a phosphorylation potential of 14 kcal $\cdot$ mol^{-1}, the apparent H^+/ATP ratio calculated from their results is 5 or 6 [7]. These data are in contrast with the results obtained above by following the distribution of the hydrophilic solutes SCN^- and NH_4Cl with selective electrodes, especially with regard to the ΔpH values (Tables I and II). Thus, in addition to the formation of $\Delta\psi$, a ΔpH of 2.0–2.3 pH units was recorded here in the absence of SCN^- and increased to 3.0 pH units upon addition of SCN^-. So the overall $\Delta\tilde{\mu}_{H^+}$ recorded here is almost twice as large as that measured by Kell et al. [7].

The large differences in the ΔpH values of more than 2 compared to less than 0.3 as determined, respectively, here and by Kell et al. [7] when using the same general method of distribution of hydrophilic solutes in chromatophores of the same photosynthetic bacterium are surprising, especially since Kell et al. [8] have reported that the selective electrodes and the flow dialysis method gave similar data in submitochondrial particles. A possible reason for the recorded differences in ΔpH seems to be due to the fact that the light intensity of $2.8 \cdot 10^5$ erg $\cdot$ $cm^{-2} \cdot s^{-1}$ that Kell et al. [7] used might not be saturating (see Fig. 1). ΔpH, unlike $\Delta\psi$, has been shown to be strictly dependent on the light intensity,

when measured at an external pH of 8.0 or 9.0 [15,20]. Therefore, ΔpH measured under a non-saturating light intensity will be lower than that measured under a saturating light intensity, whereas $\Delta\psi$ will not be affected by these differences in light intensity.

Another factor which is important for the evaluation of the ΔpH and for calculations of the H^+/ATP ratio is the chromatophore concentration used. Higher chlorophyll or bacteriochlorophyll concentration need higher light intensities in order to reach saturating illumination conditions (Fig. 1). This fact is sometimes overlooked. For instance, Elema et al. [27] have determined ΔpH in *Rps. sphaeroides* by using flow dialysis and following either the distribution of $^{14}CH_3NH_2$ or 9-aminoacridine as monitored by its fluorescence quenching. They have used the same setup of illumination for both kinds of measurements, although with CH_3NH_2 a 70–90-fold higher chromatophore concentration was used. They have indeed obtained a much higher ΔpH with 9-aminoacridine than with CH_3NH_2. But they do not discuss at all the possibility that the much lower ΔpH calculated with CH_3NH_2 might be due to the non-saturating light intensity existing at the much higher chromatophore concentrations used.

The measurements of the phosphorylation potential and of $\Delta\psi$ and ΔpH reported by Kell et al. [7] were also performed with different chromatophore concentrations. In the flow dialysis experiments, they used 0.12–0.22 mg bacteriochlorophyll/ml, whereas the phosphorylation potential was measured with only 0.05 mg bacteriochlorophyll/ml. Under the low light intensity used by Kell et al. [7] in all their experiments, the higher bacteriochlorophyll concentrations that they used for flow dialysis measurements of ΔpH were less saturated than the lower concentration that was used for measurements of the phosphorylation potential. This would mean that under their phosphorylation conditions, a larger ΔpH would have been formed than that which they actually measured in their flow dialysis experiments. Since $\Delta\psi$ would be the same, a larger ΔpH will result in a larger $\Delta\tilde{\mu}_{H^+}$ and the calculated apparent H^+/ATP ratio will be reduced.

The above discussion emphasized the importance of using identical conditions for measurements of both the overall $\Delta\tilde{\mu}_{H^+}$ and the phosphorylation potential. This is especially important in light of the recent review by Kell [11] in which he concludes that the bulk transmembrane $\Delta\tilde{\mu}_{H^+}$ is too low to be the only driving force for ATP synthesis. The experimental data leading to this conclusion were derived in part also from results of measurements performed under nonidentical conditions, such as those used by Kell et al. [7] and should therefore be rechecked under identical conditions.

In the experiments described here the reaction mixtures used for measurements of $\Delta\tilde{\mu}_{H^+}$ and the phosphorylation potential were identical. With the selective electrodes the reaction mixture was the same as that used by Kell et al. [7] for the flow dialysis assays, including the concentration of chromatophores (Table I and Fig. 2). But our illumination setup provided more than a 3-fold higher light intensity, which was shown to be saturating (Fig. 1). Under these conditions the generation of a light-induced ΔpH of about 2.0 pH units was observed (Table I and Fig. 2). Together with a $\Delta\psi$ of about 50 mV, an overall bulk transmembrane $\Delta\tilde{\mu}_{H^+}$ of at least 170 mV was measured and the calculated apparent H^+/ATP ratio was 3.4 (Tables II and III). $\Delta\tilde{\mu}_{H^+}$ measured from experiments using fluorescent probes was 220 mV (Table II) and the difference might be due to the possible reflection of some nonbulk changes by these probes. However, here in *R. rubrum* these changes add at the most 30% to the bulk changes recorded by ion distribution, whereas in *Rps. capsulata* the nonbulk changes recorded by the carotenoid ΔA were at least as high as the bulk changes [26]. From the results summarized in Table III, it seems that the transmembrane $\Delta\tilde{\mu}_{H^+}$ generated in *R. rubrum* can serve as the protonmotive force [3] provided that three to four protons are assumed to traverse the membrane for the synthesis of one molecule of ATP. A minimal number of three protons has been observed in chloroplasts under continuous illumination [22,23].

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