

External Proton Uptake, Internal Proton Release and Internal pH Changes in Chromatophores from *Rps. sphaeroides* Following Single Turnover Flashes

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Proton Translocation, Internal pH-Changes, Phosphorylation

The change in internal pH in chromatophores from *Rps. sphaeroides* following short flash excitation has been investigated using the method of the fluorescence quenching of 9-aminoacridine.

(1) A single turnover flash induced a fluorescence quenching of $\approx 0.4\%$. The fluorescence quenching was stimulated by valinomycin, and oligomycin, and inhibited by antimycin A.

(2) The extent of the pH gradient generated by a single turnover flash at the external pH ≈ 8 was estimated to be less than 0.4 unit.

(3) In a multiframe experiment the change in internal pH induced by each flash was found to be not linearly dependent on the number of protons which had been taken up after each flash from the external medium.

We discuss the significance of the results with respect to the mechanism of phosphorylation. The release of H^+ taken up outside the chromatophore into the membrane could not be excluded by the present results.

Introduction

It is well known that continuous light illumination of bacterial chromatophores induces an alkalization of the suspension medium [1]. Later studies with short flash excitation showed rapid H^+ binding on the outside of the chromatophore [2–4]. The extent and the kinetics of this binding have been investigated in more detail recently (for details, see refs 3 and 4). The H^+ -binding was assumed to be correlated with the reduction of an electron carrier on the outside of the chromatophore vesicle and the subsequent release of protons into the intravesicular space [2, 3, 5, 6]. Although some qualitative data exist on the H^+ -release on the inside of the chromatophore [3, 5, 7], little is known on the magnitude of the change in internal pH after short flash excitation.

In this report we wish to present some quantitative data on the change of internal pH under various conditions in chromatophores from *Rps. sphaeroides* bacteria. We used the fluorescence quenching method

of 9-aminoacridine to measure the pH gradient across the chromatophore membrane or the change in internal pH. This method has also been used to measure pH gradient across other types of membrane vesicle systems such as chloroplasts or liposomes [8, 9]. Although some uncertainties exist on the validity of the technique in chloroplasts or liposomes (see ref. 10), the method seems to be suitable for chromatophores [11, 12]. A fluorescence quenching of 9-aminoacridine of about 0.4% was observed after a single turnover flash. The fluorescence quenching was stimulated by valinomycin ($\sim 50\%$ stimulation) and was inhibited by antimycin A (60–70% inhibition under our conditions). The extent of the pH gradient generated by a single turnover flash was estimated to be less than 0.4 pH unit. Furthermore the relation between the number of H^+ taken up at the outside of the chromatophore (measured by the absorbance change of the external pH indicator cresol red) and the change in internal pH (calculated from the fluorescence quenching of 9-aminoacridine) was found to be not a simple linear function. We discussed the relatively low value of ΔpH with respect to its energetic contribution to the electrochemical gradient of protons and its correlation with the amount of H^+ taken up outside the chromatophore. The release of H^+ taken up outside the chromatophore into the membrane could not be excluded by the present results.

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Materials and Methods

Chromatophores from *Rps. sphaeroides* (wild type 2.4.1) were prepared as described in ref. 13. They were stored in liquid nitrogen in a way similar to that described for chloroplasts [14], without losing more than 20% of their phosphorylating activity. After thawing they were used immediately for measurements. The reaction mixture contained 50 mM KCl, 2 mM MgCl₂ and 50 mM N-Tris (hydroxymethyl)-methylglycine (Tricine) at pH 7.9. The bacteriochlorophyll concentration was adjusted to 1.4×10^{-4} M and measured according to the *in vivo* extinction coefficient given by Clayton [15].

Spectroscopic measurements

Spectroscopic measurements were performed in a 1 ml capillary open quartz cuvette with a 1 mm optical pathway in a similar equipment for repetitive flashes as described elsewhere [13, 16]. Photosynthetic excitation was provided by either a 150 W tungsten lamp filtered through a water bath and Schott filter RG 665, or a 20 μ sec xenon flash. Fluorescence of 9-aminoacridine was excited by a Hanovia Hg/Xe lamp. A monochromator selected wavelengths around 405 nm with an optical bandwidth of 2 nm. The intensity of the fluorescence exciting light was lower than 5×10^2 erg \cdot cm⁻² sec⁻¹. Fluorescence emission was measured at an angle of 90° through a Balzers cut-off filter 585 nm, Schott filters GG 13, GG 15 and bandfilters DAL 458, shielding the photomultiplier from flash light and scattered 405 nm light. Flash-induced fluorescence quenching was usually measured from an average of 256 or 512 flashes at a frequency of 1/18 Hz. The proton uptake on the outside has been monitored by the absorbance change of 50 μ M cresol red at 575 nm under identical conditions to those of fluorescence measurements, *i. e.* in the presence of 100 μ M 9-aminoacridine, but in absence of Tricine buffer, at pH \sim 7.9 and after subtraction of the relatively small background changes in additional presence of 50 mM Tricine-buffer, pH 7.9 and calibrated in a way similar to that described in (ref. 2, 17). Assuming that the buffering capacity of the suspension medium was not affected during the whole experiment (see also ref. 18), the external pH change indicated by the absorbance change of cresol red would then be set proportional to the amount of H⁺ involved. In the presence of 9-aminoacridine

measurements of relatively slow pH change could be altered by the deprotonation of the external amine preceding the diffusion toward the inside of the vesicle [22]. In order to get unambiguous results (*i. e.* in multi-flash experiments) only the rapid H⁺-binding ($\tau_{1/2} < 150$ μ s [2–4]) induced by each flash and not the total change after the flash will be considered.

Calculation of small Δ pH values

Assuming an ideal distribution of the monoamine 9-aminoacridine with the pH-gradient, a complete fluorescence quenching of the amine on the inside of the vesicle and taking into account that the dissociation constant of the amine (pK \sim 10) is negligible compared with the proton concentration inside and outside the vesicle (pH \sim 8), the following equations could be derived [8]:

$$\frac{[A]_{T, in}}{[A]_{T, out}} = \frac{[H^+]_{in}}{[H^+]_{out}} \quad (1)$$

$[A]_{T, in}$, $[A]_{T, out}$ represent the total amine concentration inside and outside the vesicle respectively. The total amine concentration on the inside of the vesicle after illumination is given by:

$$[A]_{T, in} = [A]_{T, out}^0 + ([A]_{T, out}^0 - [A]_{T, out}) \cdot \frac{V_{out}}{V_{in}} \quad (2)$$

where $[A]_{T, out}^0$ is the total amine concentration outside the vesicle before illumination and V_{out} , V_{in} the volumes outside and inside the vesicle respectively. It is assumed that in the dark:

$$[A]_{T, out}^0 = [A]_{T, in}^0 \quad (3)$$

The first term at the right hand side in Eqn (2) gives the internal amine concentration before illumination.

Inserting Eqn (2) into Eqn (1) and assuming that $[A]_{T, out}^0$ and $[A]_{T, out}$ are proportional to F_0 and F respectively (F_0 and F represent the fluorescence of 9-aminoacridine in the dark and during illumination respectively) it results:

$$\frac{[H^+]_{in}}{[H^+]_{out}} = \frac{F_0}{F} + \frac{F_0 - F}{F} \cdot \frac{V_{out}}{V_{in}} \quad (4)$$

Using the symbol used by Schuldiner *et al.* [8], $Q = (F_0 - F)/F_0$, it results

$$\frac{[H^+]_{in}}{[H^+]_{out}} = \frac{1}{1 - Q} + \frac{Q}{1 - Q} \cdot \left(\frac{V_{out}}{V_{in}} \right) \quad (5)$$

Comparison of Eqn (5) with the equation derived by Schuldiner *et al.* [8] shows that both equations differ in the first term on the right hand side which accounts for the relative amount of amine already inside the vesicles in the dark before illumination. This term is, however, negligible with high values of Q , *i. e.* with large changes of pH (note that $V_{out}/V_{in} \gg 1$) [8, 9, 11]. For small values of Q , *e. g.* in the case of flash excitation it is necessary to include this term in the calculation. It results then for $Q = 0$, $[H^+]_{in}/[H^+]_{out} = 1$, *i. e.* $[H^+]_{in} = [H^+]_{out}$. When $[H^+]_{out}$ is kept constant, $[H^+]_{in}$ or its change can be calculated for any value of Q .

The other important factor for the estimation of pH gradient is the internal osmotic volume of the chromatophores (see *e. g.* ref. 5). Different values between 40 μl (extrapolated to the osmolarity of our medium) and 140 μl per mg bacteriochlorophyll were given in the literature for chromatophores from *R. rubrum* [19] and *Rps. capsulata* [11, 20]. Recently, values around 100 μl per mg bacteriochlorophyll were determined in *Rps. sphaeroides* chromatophores (Kell, D. and Ferguson, S. private communication). We used the two extreme values of internal volume (40 μl and 140 μl per mg bacteriochlorophyll) to estimate the transmembrane pH difference in order to get some comparative data.

Conditions of measurements and theoretical considerations

The measurement of a transmembrane pH-gradient generated by single turnover flashes using a method which is based on the distribution of an amine is valid if some more conditions are realised than those which already were mentioned in refs 8, 11, 12 for steady-state illumination: (i) the "influx" of the amine should be faster than the efflux of protons. The flash-induced fluorescence quenching was maximal after $\cong 500$ msec (see Fig. 2 A). In this time interval the amount of protons taken up which reappeared in the external medium as indicated by the absorbance change of cresol red [17] was maximally only 15%. The "influx" of the amine represents the diffusion toward the inside of the chromatophore following a concentration gradient according to the mechanism of the amine distribution suggested by Crofts [22]. This diffusion is a dynamic process because the concentration gradient varies with time (and with distance) probably according to the second law of Fick (for details, see

ref. 22 a). One would expect a sigmoidal shape for the distribution curve as a function of time. The degree of sigmoidal depends on the distance from the space with the highest concentration. The nearer it is, the less sigmoidal it will be. In other words the furthest from the space with the highest concentration the longer it takes to reach the equilibrium. If the efflux of H^+ is relatively slow compared with the time needed for the amine to equilibrate between the two phases, the maximum extent after the flash should represent fairly well the equilibrium state. A better evaluation could also be made by extrapolating the relaxation curve of the fluorescence quenching to time zero after the flash. (ii) the amine should not "uncouple" significantly the chromatophore membrane under the conditions used. This has been tested by measuring the decay of the electric potential difference indicated by the carotenoid shift [21]. This decay was not significantly affected by 9-aminoacridine up to 2×10^{-4} M (not shown). (With antimycin-treated chromatophores, however, a slight uncoupling effect was observed (25% acceleration of the electric potential decay at 5×10^{-7} M antimycin and 10^{-4} M 9-aminoacridine. The reason for this uncoupling effect is not clear). (iii) the addition of amine to monitor internal pH-changes should also not alter significantly the intrinsic internal buffering capacity of the chromatophore leading to an underestimation of the pH gradient. If the internal volume of the chromatophore is known (between 40 μl and 140 μl per mg bacteriochlorophyll, see above) as well as the number of H^+ released into the internal space (estimated in the first approximation to be equivalent to the number of H^+ taken up, $\approx 1 H^+$ per 150 bacteriochlorophyll (2–5, see also below), and if we assume a *maximal* change in internal pH by 2 pH-units per flash in the absence of aminoacridine, the *minimal* internal buffering capacity (see Eqn (6)) could be estimated and was found to be in the order $(2-8) \times 10^{-5}$ moles H^+ per liter and pH-unit. If the internal concentration of the amine is known as well as its dissociation constant ($pK \cong 10$), its buffering power could be calculated at the operating pH ($pH \cong 8$). For 10^{-4} M 9-aminoacridine we found a value of 2×10^{-6} moles H^+ per liter and pH-unit, *i. e.* at least 10 times lower than the intrinsic buffering power. Thus, up to this concentration of the amine (10^{-4} M) no significant change in internal buffering capacity should be expected.

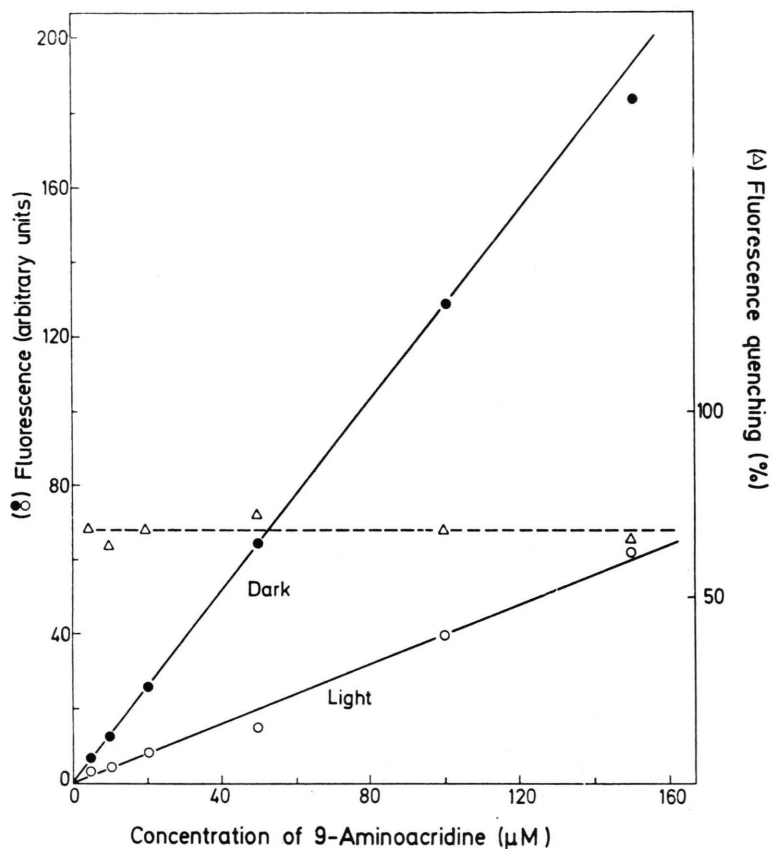


Fig. 1. Fluorescence of 9-aminoacridine in a suspension of chromatophores.

(●) Fluorescence without actinic light.
 (○) Fluorescence under saturating continuous illumination.
 (△) Fluorescence quenching.
 For details see Materials and Methods.

Casadio *et al.* [11] showed that 9-aminoacridine could be used to determine the pH gradient induced by continuous light in *Rps. capsulata* chromatophores. The relative fluorescence quenching was shown to be independent on the concentration of 9-aminoacridine, at least up to 20 μM . We performed similar experiments in *Rps. sphaeroides* chromatophores. As Fig. 1 shows, no change of the fluorescence quenching was observed, at least up to 150 μM . In order to get a good signal to noise ratio, a 9-aminoacridine concentration of 100 μM was used in all flash experiments although with a 5-fold smaller concentration, similar flash-induced signals could have been obtained.

Results and Discussion

Fig. 2 A shows the time course of the fluorescence quenching of 9-aminoacridine induced by a single turnover flash. The rise of the signal was completed after 400–600 msec (half-risetime \cong 200 ms) while the decay was slower ($\tau_{1/2} \cong$ 2–3

sec) and similar to that of the H^+ -efflux measured with cresol red [17]. The rise of the fluorescence quenching reflected the movement of external 9-aminoacridine toward the inside of the chromatophores following the generation of a gradient of H^+ [22] and not the release of protons into the internal space which was found to be faster (see refs 3 and 5). The slower kinetic of the decay of the fluorescence quenching could however reflect the true kinetic of the relaxation of the proton gradient (see above). We estimated the magnitude of the pH gradient induced by one single turnover flash by extrapolating the fluorescence quenching to zero time after the flash as indicated in Fig. 2 A. The "initial" extent was 0.4 to 0.6%, depending on preparations.

Fig. 2 B shows that addition of valinomycin enhanced the fluorescence quenching by approximately 50% in accordance with the known stimulation of the extent of proton binding on the outside and the decrease of the rate of proton efflux by collapsing the transmembrane electric potential difference [17].

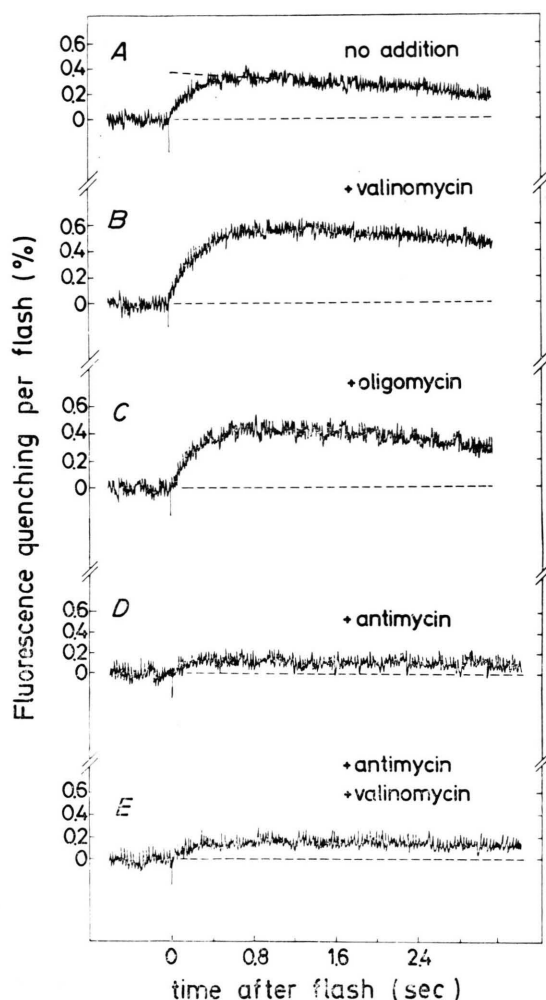


Fig. 2. Fluorescence quenching of 9-aminoacridine following excitation by single turnover flashes. Valinomycin, oligomycin and antimycin A, when added, were adjusted to a final concentration of 10^{-6} M, 10^{-5} M and 5×10^{-7} M, respectively. The traces were obtained from an average of 256 flashes with a frequency of 1/17 Hz. A new sample was replaced after 64 flashes. Further details see Materials and Methods and text.

An enhancement of the fluorescence quenching was also observed after addition of oligomycin which was shown to lower H^+ -efflux [17, 23] (Fig. 2 C). It was suggested that oligomycin blocks the leak of H^+ through "damaged" ATPase systems [17]. In antimycin A-treated chromatophores, the fluorescence quenching of 9-aminoacridine was 60–70% inhibited (Fig. 2 D) and was not significantly relieved by further addition of valinomycin (Fig.

2 E). Antimycin A blocks electron flow in the cytochromes b/c_2 -segment [24], inhibits the millisecond rise phase of the carotenoid band-shift (Phase III) [25], and the second H^+ -binding (H^+_{II}) most clearly seen in the presence of valinomycin under our conditions [2–4]. The results are consistent with the results obtained from different types of experiments based on studies of H^+ -efflux in the presence of protonophores such as the nigericin-type inophore dianemycin [7] or carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) [5]. The effect of the different reagents shown in Fig. 2 corresponded well to the effect for changes due to internal pH.

Fig. 3 A shows the fluorescence quenching of 9-aminoacridine (top) and the absorbance change of the external pH-indicator dye cresol red (bottom) induced by a train of flashes. The dark time of 0.5 s between the flashes was chosen to allow a completed rise of the signal indicating the change in internal pH. In an attempt to evaluate the relationship between the amount of H^+ taken up outside (or released inside) the chromatophore and the change in internal pH, we plotted in Fig. 3 B the flash-induced changes in internal pH as a function of the amount of flash-induced H^+ taken up outside the chromatophore. The changes in internal pH were calculated from the fluorescence quenching extrapolated to zero time after each flash by taking as reference the fluorescence level prior to the flash. The number of H^+ taken up outside the chromatophore was estimated from the absorbance change of cresol red measured *immediately* after each flash, by taking also as reference the absorbance level just before the flash (for explanations and details, see Materials and Methods). We calculated the changes in internal pH with the two extreme values of internal volume of the chromatophore as mentioned in Methods. It resulted that the relationship was not a simple linear function which passes through the origin. (The dashed line in Fig. 3 B was drawn arbitrarily.) Two cases may be considered: (i) all H^+ taken up from the external medium were released into the internal vesicular space. The relationship between the amount of H^+ bound outside, *i. e.* the amount of H^+ injected inside the chromatophore (ΔH^+) and the change in internal pH (ΔpH) represents then directly the buffering capacity β in the internal phase, according to the equation

$$\beta = \frac{\Delta H^+}{\Delta pH} \times \frac{1}{V_{in}} \quad (6)$$

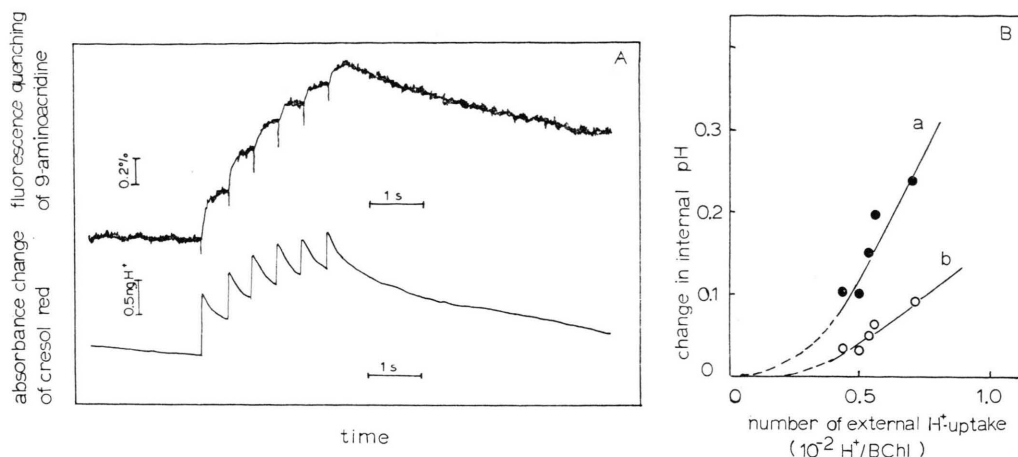


Fig. 3. A. Fluorescence quenching of 9-aminoacridine (top) and absorbance change of cresol red (bottom) induced by a train of flashes. Dark time between the flashes 0.5 s and dark time between the groups of flashes 22 s. For the measurement of the fluorescence quenching 512 traces were averaged using 8 samples and for the measurement of the absorbance change of cresol red 32 traces were averaged using just 1 sample. Further details see Materials and Methods and text. B. Calculated changes of internal pH as function of the number of protons taken up outside the chromatophore. The absolute values of the internal pH changes were calculated from Eqn (5) using internal volumes of 40 $\mu\text{l}/\text{mg}$ BChl (trace a) and 140 $\mu\text{l}/\text{mg}$ BChl (trace b) as mentioned in Methods. The absolute value of the number of proton uptake was determined by addition of known amount of HCl (for first flash, uptake of 1 H^+ per 150 BChl). The calculated internal buffering capacity for a single turnover saturating flash at the external pH of 7.9 were $\approx 6.5 \times 10^{-4}$ mol H^+ per 1 and pH unit.

The internal volume V_{in} is assumed to be constant before and after the flash. The nonlinearity is therefore due to a change in buffering power in the internal phase. Irreversible protonation of buffering groups inside the vesicle (e.g. cytochrome b) [see refs 3, 5] would not be accounted for, since under our conditions the H^+ -efflux measured with an external pH dye was fully accelerated by protonophores such as FCCP or dianemycin [5, 12]. In view of the relatively small change of internal pH in the nonlinear range of the curve in Fig. 3 B, a change of buffering capacity seems therefore unlikely. (ii) The amount of H^+ taken up ($\Delta\text{H}_{\text{out}}^+$) outside the chromatophore is not directly equal to the amount of H^+ released into the inner aqueous phase ($\Delta\text{H}_{\text{in}}^+$) e.g. $\Delta\text{H}_{\text{out}}^+ = \Delta\text{H}_{\text{in}}^+ + \Delta\text{H}_{\text{unknown}}^+$. Assuming a constant inner buffering capacity it results from Fig. 3 B that part of protons taken up from the external medium may be released into a space not detectable by the amine distribution technique, for example, into the interior of the chromatophore membrane. This case is discussed further below.

In Table I, we listed the changes in internal pH (ΔpH) calculated from Eqn (5) using the data given in Fig. 1 and Fig. 2. As mentioned in Materials and Methods the absolute values depend strongly on the internal volume of the chromatophore which lies between 40 and 140 μl per mg bacteriochlorophyll

Conditions	Fluorescence quenching Q (%)	ΔpH	
		40 $\mu\text{l}/\text{mg}$ BChl	140 $\mu\text{l}/\text{mg}$ BChl
saturating continuous light	70	2.6	2.0
single turnover flashes			
no addition	0.4	0.24	0.08
+ valinomycin (10^{-6} M)	0.6	0.32	0.12
+ oligomycin (10^{-5} M)	0.5	0.28	0.10
+ antimycin (5×10^{-7} M)	0.15	0.10	0.03
+ antimycin (5×10^{-7} M)			
+ valinomycin (10^{-6} M)	0.2	0.13	0.04

Table I. Fluorescence quenching of 9-aminoacridine under different conditions: saturating continuous light, single turnover flashes and treatment with valinomycin, oligomycin and antimycin A. The corresponding ΔpH values have been calculated according to Eqn (5) using 2 extreme values of internal volumes of 40 $\mu\text{l}/\text{mg}$ BChl and 140 $\mu\text{l}/\text{mg}$ BChl. Further details see Materials and Methods and text.

(see Methods) and for comparison we calculated ΔpH with both internal volumes which represent the extreme values. Whatever the latter were, the calculated values of the pH gradient across the membrane of *Rps. sphaeroides* chromatophores generated by short flashes, either in the presence or in the absence

of antimycin, were less than 0.4 pH-unit. Similar low value of ΔpH (<0.5 unit) was also found by Packham and Jackson using a different method to determine the flash-induced pH-gradient (Packham, N. K. and Jackson, J. B., private communication). According to these data the energetic contribution of the transmembrane pH differences (<25 mV) to the electrochemical proton gradient is therefore small compared with the electric membrane potential estimated to be of the order of 100 mV in the absence of antimycin [26]. This is especially true in the presence of antimycin. Since phosphorylation of ADP following short flash excitation can take place in both cases, it follows that the major driving force for flash-induced phosphorylation is the electric membrane potential. This conclusion is rather difficult to reconcile with the interpretation of the earlier results of Saphon, Jackson and Witt [17]. They found that ATP-synthesis in single turnover flashes was inhibited either by valinomycin (which collapses the electric membrane potential but does not influence the pH-gradient in presence of antimycin) or by dianemycin (which collapses the pH-gradient but does not influence the electric membrane potential) [12]. Those facts led them to the conclusion that after short flash excitations there was an important energetic contribution of a transmembrane pH difference as well as an electric membrane potential. In view of the data presented in this work, it is necessary to reinterpret these earlier findings. Two possibilities may be considered: (1) Dianemycin collapses, not only the transmembrane pH-gradient but also some "local concentration of H^+ in the membrane" not detected by the amine distribution method, *e. g.* in the region of the ATPase (*cf.* refs 27, 28). The uptake of H^+ increases not only the proton concentration in the inner aqueous phase of the vesicles but also enhances some local proton concentration within the membrane which may be necessary for ATP synthesis. This implies that the oxidation of a hydrogen carrier

occurs within the membrane. Consequently, in the membrane there may be a higher accumulation of protons than it may be expected on the basis of an equilibrium between the inner aqueous phase and the membrane [27, 28]. (2) ATP synthesis needs a pool of H^+ inside the vesicles for field-driven proton flux through the ATPase. In this case, a greater phosphorylation yield at lower pH would be expected. This, however, cannot be easily proved since ATP-synthesis in single turnover flashes is attenuated at lower pH, probably because of the pH-dependent activity of the enzyme [17]. In this context it should be noted that in *R. rubrum* chromatophores, a membrane potential induced by valinomycin/ K^+ alone causes very low ATP formation. Only the presence of a sufficient supply of H^+ (experimentally realized by a coincident acid-base transition and a valinomycin/ K^+ -diffusion potential) a significant amount of ATP is generated [29].

As yet, a clear decision between both mechanisms is not possible. However, (a) the nonlinear correlation between the number of H^+ released into the internal phase (obtained from the change in internal pH assuming a constant inner buffering capacity) and the number of protons bound on the outside of the chromatophore (Fig. 3 B) and (b) the high sensitivity of the flash-induced phosphorylation to dianemycin despite the small transmembrane pH-gradient suggested that there may exist a high local accumulation of H^+ within the membrane as proposed by Williams [27, 28].

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