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RELATIONS BETWEEN THE ELECTRICAL POTENTIAL, pH GRADIENT, PROTON FLUX AND PHOSPHORYLATION IN THE PHOTOSYNTHETIC MEMBRANE

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SUMMARY

The transmembrane electrical potential ($\Delta\varphi$), the proton flux ($\overline{\dot{H}^{+}}$), the rate of electron transport ($\overline{\dot{e}}$), the pH gradient (ΔpH) and the rate of phosphorylation ($\overline{A\dot{T}P}$) were measured in chloroplasts of spinach. Photosynthesis was excited periodically with flashes of variable frequencies and intensities. A new method is described for determining the rate of electron transport and proton flux. Under conditions where the rate of electron transport and proton flux are not pH controlled the following correlations were found in the range 50 mV $\leqslant \Delta\varphi \leqslant$ 125 mV and 1.8 $\leqslant \Delta$pH $\leqslant$ 2.7:

(1) The pH gradient, ΔpH, increases with $\overline{\dot{H}^{+}}$ independently of $\mathrm{pH_{out}}$ between 7–9.

(2) The rate of phosphorylation, $\overline{A\dot{T}P}$, depends exponentially on ΔpH (at constant $\Delta\varphi$) and is independent of $\mathrm{pH_{out}}$ between 7–9.

(3) The rate of phosphorylation, $\overline{A\dot{T}P}$, depends also on $\Delta\varphi$ (at constant ΔpH and at constant proton flux $\overline{\dot{H}^{+}}$).

(4) The proton flux via the ATPase pathway, $\overline{\dot{H}_{p}{}^{+}}$, depends non-linearly on the ratio of the proton concentrations: $\overline{\dot{H}_{p}{}^{+}} \sim (H_{in}{}^{+}/H_{out}{}^{+})^{b}$, (b = 2.3–2.6). The proton flux via the basal pathway, $\overline{\dot{H}_{b}{}^{+}}$, depends linearly on the ratio of the proton concentrations: $\overline{\dot{H}_{b}{}^{+}} \sim (H_{in}/H_{out})$.

(5) The ratio ΔH^{+}/ATP (e/ATP), i.e. the ratio of the total proton flux, $\overline{\dot{H}_{p}{}^{+}} + \overline{\dot{H}_{b}{}^{+}}$, and the rate of ATP formation, $\overline{A\dot{T}P}$, depends strongly on $\Delta\varphi$ and on ΔpH. The ratio is $\Delta H^{+}/\mathrm{ATP} \approx 3$ (e/ATP $\approx$ 1.5) at ΔpH 2.7 and $\Delta\varphi = 125$ mV.

(6) It is supposed that the reason for the dependence of ΔH^{+}/ATP on $\Delta\varphi$ and ΔpH is the different functional dependence of the basal proton flux $\overline{\dot{H}_{b}{}^{+}}$ and the phosphorylating proton flux $\overline{\dot{H}_{p}{}^{+}}$ on ΔpH and $\Delta\varphi$. The calculation of ΔH^{+}/ATP on the basis of this assumption is in fair agreement with the experimental values. Also the "threshold" effects can be explained in this way.

A preliminary report has been published [1].

(7) The ratio of $\Delta H_p^+/ATP$, i.e. the ratio of the phosphorylating proton flux $\overline{\dot{H}_p^+}$ and $\overline{A\dot{T}P}$, is $\Delta H_p^+/ATP \approx 2.4$.

INTRODUCTION

Since the development of the chemiosmotic theory of phosphorylation [2, 3] attempts have been made to correlate the ATP synthesis with the pH gradient and electrical potential difference across the energy-coupling membrane. In respect to the pH gradient the following correlations were demonstrated:

(1) It has been shown in chloroplasts that a pH gradient, ΔpH, which is induced artificially in the dark, by an acid-base transition, generates ATP [4]. (2) The amount of generated ATP depends on the magnitude of ΔpH and the amount of available protons inside the thylakoids (buffer capacity) [4]. (3) The proton flux is accelerated under phosphorylating conditions [7, 8] and the light-induced pH gradient, ΔpH, is decreased [5, 6, 9]. (4) Four H^+ per generated ATP were calculated from the ΔpH value and the phosphate potential in thermodynamic equilibrium [10].

On the other hand it has been shown that an electrical potential difference, $\Delta\varphi$, measured by electrochromic absorption changes [11–14] is correlated with ATP synthesis as follows: (1) The decay of $\Delta\varphi$ is accelerated during phosphorylation [15, 16, 54]. In chromatophores the acceleration disappears with the removal of the ATPase and appears in full with its recondensation [54]. (2) The rate of $\Delta\varphi$ decay due to proton efflux is proportional to the rate of ATP formation [17, 18]. (3) The extent of $\Delta\varphi$ is proportional to the amount of generated ATP [17]. This work shows that this is the case at $\Delta pH \geqslant 2.7$. (4) The functional unit of the electrical events corresponds to the unit of phosphorylation [17, 55]. (5) The ΔpH induced ATP formation can be increased by a superimposed artificial diffusion potential across the thylakoid membrane [19, 20].

These results favour the chemiosmotic theory. However, the functional relations between phosphorylation and ΔpH and $\Delta\varphi$ have not been determined because either the magnitude of ΔpH or $\Delta\varphi$ were not known or because $\Delta\varphi$ and ΔpH were assumed to be negligible. Furthermore, in experiments with artificially induced ΔpH or $\Delta\varphi$ these parameters change in an uncontrolled manner during the experiments. Therefore, in this investigation the rate of ATP formation, $\overline{A\dot{T}P}$, the pH gradient, ΔpH, the intrinsic electrical potential difference, $\Delta\varphi$, and the proton flux, $\overline{\dot{H}^+}$, were measured and the correlations between these four parameters evaluated.

The essential features of our experimental conditions are the following: (1) Photosynthesis was excited by periodical flashes. (2) The experiments were restricted to a range where the rate of electron transport can be varied independently of pH_{out}, i.e. where the rate is light-controlled and not pH-controlled.

Periodical flashes were used because of the following reasons: (a) Flash-induced electrical potential differences $\Delta\varphi$ can be determined more accurately than $\Delta\varphi$ induced by continuous light. The reason being that in continuous light, on chloroplasts slow changes in light scattering interfere with the potential indicating absorption changes. (b) In flash light the magnitude of $\Delta\varphi$ can be varied independently of ΔpH and the average proton flux. This is not possible in continuous light. (c) With periodical flash light the averaged rate of electron transport and the averaged proton flux can be adjusted in a simple way (see Methods).

MATERIALS AND METHODS

Chloroplasts. These were freshly isolated from spinach as described elsewhere [21]. Additionally 10 mM ascorbate had been added during grinding. The spinach was grown either in a BBC-phytocell or obtained from the local market. The chloroplasts were stored at 0 °C in the suspension medium and were used within 2 h of preparation. As revealed from the rather long half lifetime (300–500 ms) of the flash-induced electrical potential difference, the thylakoid membranes of these chloroplasts had a very low basal conductivity. The reaction volume was 1 ml and contained $2 \cdot 10^{-2}$ M tricine adjusted to the required $\mathrm{pH_{out}}$ with NaOH, 10^{-4} M benzylviologen, $5 \cdot 10^{-3}$ M $MgCl_2$, 10^{-2} M KCl, $5 \cdot 10^{-3}$ M K_2HPO_4 containing ^{32}P with approximately 10^6 counts/min, 10^{-2} M sucrose, $3 \cdot 10^{-4}$ M ADP and chloroplasts giving a chlorophyll concentration of $2 \cdot 10^{-4}$ M. For the ΔpH determination 9-aminoacridine was added to a final concentration of $6 \cdot 10^{-7}$ M. The optical pathlength was 1.4 mm.

Excitation. Photosynthesis was excited with periodical flash light; frequency: 0.1–100 Hz; duration of the single turnover flashes: 20 µs; wavelength: 610–730 nm; intensity: non-saturating and saturating single turnover flashes; for inducing higher electric potential differences flash groups (2–5 flashes per group) with darktimes of 2 ms between the flashes were used.

Registration. The optical measurements were carried out with a repetitive pulse spectrophotometer with double beams. Depending on the time resolution 20–1800 signals were sampled, thereby, improving the signal to noise ratio [22].

Electrical potential differences. $\Delta\varphi$ values were measured by electrochroism, i.e. by absorbance changes ΔA (at 515 nm) caused by a shift of absorbance bands in a transmembrane electrical field [11–14]. It was shown that the electrical potential difference $\Delta\varphi$ is linearly related to the absorbance change ΔA:

$$\Delta\varphi = 50\,\text{mV}.\ \Delta A/\Delta A_1 \qquad (1)$$

(ΔA_1, initial absorption change in a saturating single turnover flash.) With another method of calibration it was shown recently that the proportionality factor may be bigger than 50 mV [23]. However, the results of this work do not rely on the absolute value but only on the proportionality between $\Delta\varphi$ and ΔA [24, 25]. For the sake of simplicity we use in the following the factor 50 mV. $\Delta A > \Delta A_1$ was induced by flash groups. The optical bandwidth of the measuring light at 515 nm was 10 nm; intensity: $\leqslant$ 10 erg/cm^{2}s; electrical bandwidth: 1.4 kHz.

Average rate of electron transport. This is determined from the flash frequency and the amount of electrons transferred by one flash. In a single turnover flash one electron is transferred from water to the final electron acceptor and an electrical potential difference of $\Delta\varphi_1 = 50$ mV is generated. Arbitrary values of $\Delta\varphi$ are proportional to the amount of charges translocated across the membrane [24, 25] (provided that the membrane capacity remains constant). Therefore, the amount of electrons e transferred through one electron transport chain (e-chain) per flash at $\Delta\varphi$ is:

$$e = 1 \cdot \frac{\Delta\varphi}{\Delta\varphi_1}\left(\frac{\text{electrons}}{\text{e-chain}}\right) \qquad (2)$$

The averaged rate of electron transport is given at any flash frequency (ν) by:

$$\bar{e} = 1\,\frac{\Delta\varphi}{\Delta\varphi_1}\cdot\nu\left(\frac{\text{electrons}}{\text{e-chain}\cdot\text{s}}\right) \tag{3}$$

The number of chlorophyll molecules per electron chain has been determined from the oxygen yield per flash and for the preparations used this value is 700$\pm$100 Chl/e-chain. Therefore Eqn 3 can be written:

$$\bar{e} = \frac{1}{700}\cdot\frac{\Delta\varphi}{\Delta\varphi_1}\cdot\nu = 1.43\cdot 10^{-3}\,\frac{\Delta\varphi}{\Delta\varphi_1}\,\nu\left(\frac{\text{mol electrons}}{\text{mol Chl}\cdot\text{s}}\right) \tag{4}$$

Proton flux. For the determination of the average proton flux the $\Delta H^+/e$ ratio must be known. (ΔH^+, amount of protons translocated per flash.) It has been shown elsewhere that in a single turnover flash two H^+ per transferred electron are translocated across the membrane [25, 26, 31]. Therefore, the average proton influx is twice the average rate of electron transport and it follows from Eqns 3 and 4:

$$\bar{H}^+ = 2\,\frac{\Delta\varphi}{\Delta\varphi_1}\cdot\nu\left(\frac{H^+}{\text{e-chain}\cdot\text{s}}\right) \tag{5}$$

$$\bar{H}^+ = 2.8\cdot 10^{-3}\,\frac{\Delta\varphi}{\Delta\varphi_1}\cdot\nu\left(\frac{\text{mol } H^+}{\text{mol Chl}\cdot\text{s}}\right) \tag{6}$$

The protons which are translocated inwards by each flash must have been released from the inner phase before the following flash, a necessary steady state condition of periodical excitation. Therefore, Eqn 5 also represents the average proton efflux or the proton flux in general. According to Eqn 4 and Eqn 6 it is:

$$\bar{H}^+ = 2\bar{e} \tag{7}$$

In the following we refer only to the proton flux. With Eqns 4 and 5 it is also possible to determine $\bar{e}$ and $\overline{H^+}$ in vivo, i.e. in algae or in whole leaves where proton fluxes cannot be measured by glass electrodes or indicators.

The pH_{in} *value* in the inner space of the thylakoids and $\Delta pH = pH_{out} - pH_{in}$ were measured by the light-induced fluorescence quenching of 9-aminoacridine. According to the method of Schuldiner et al. [27] it is:

$$\Delta pH = \log\frac{Q}{1-Q} + \log\frac{V_{out}}{V_{in}} \tag{8}$$

$Q = F_Q/F$; F = fluorescence of added 9-aminoacridine; F_Q = quenched fluorescence during illumination (see Fig. 1B); V_{out} = volume outside the thylakoids; V_{in} = volume of the inner space of the thylakoids (10 l/mol Chl was used). The reliability of Eqn 8 has been questioned (Rumberg, B., personal communication and [28, 53]). Therefore, the restriction of this method and the range of its application is discussed in detail (see Discussion). The wavelength for excitation of the aminoacridine fluorescence was 367 nm, $\Delta\lambda \approx 10$ nm; light intensity ≈ 60 erg/cm^2 per s. Fluorescence emission was measured at an angle of 90° between 420–470 nm through a Balzers K 2 interference filter, a Balzers 585 nm out-off filter, a 4 mm GG 385 (Schott) and a 3 mm BG 28 (Schott), shielding the photomultiplier from flash light and from scattered 367 nm light.

It was reported in [29] that H_{in}^+/H_{out}^+ in the dark is about 3. The change of H_{in}^+/H_{out}^+ by the light, exciting aminoacridine fluorescence, is about 7. In the range of experimental relevance, i.e. $\Delta pH \geqslant 1.8$ these effects would require a correction of $\Delta pH \leqslant 0.12$ which was within the limits of error and was therefore omitted.

Phosphorylation was measured by ^{32}P as described previously [30]. The measurement of phosphorylation and $\Delta\varphi$ or ΔpH were carried out simultaneously. After the flash illumination and registration of ΔpH or $\Delta\varphi$ trichloroacetic acid was added; the amount of ATP generated was determined and the average rate of ATP formation, $\overline{ATP}$, calculated. It was noticed that there is a slight decrease of the rate of ATP formation of about 10–20 % due to the addition of aminoacridine.

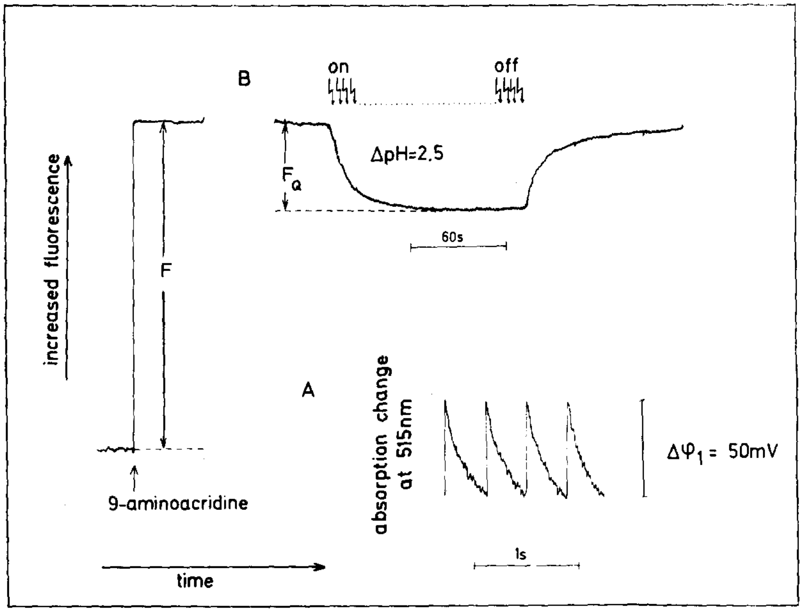

Fig. 1. (A) Absorption change at 515 nm indicating the variation of the electric potential between the flashes in the steady state. (B) Fluorescence change of 9-aminoacridine indicating the change of Δ pH. Periodical excitation with saturating single turnover flashes, flash frequency 2.5 Hz, subject: Spinach chloroplasts.

On the equivalence of $\Delta\varphi$ and ΔpH. Fig. 1B shows a typical trace of a pH_{in} measurement at pH_{out} 8 using for excitation saturating single turnover flashes with a frequency of 2.5 Hz. A stationary fluorescence quenching due to a stationary pH_{in} value is reached within about 20–30 s. In the steady state ΔpH is essentially unmodulated by the periodical excitations (at low frequencies of 0.1 Hz a modulation of ΔpH of at most 4 % is observed). $\Delta\varphi$ in the steady state is depicted in Fig. 1A (note, different time scales). In contrast to ΔpH the electrical potential difference $\Delta\varphi$ is modulated. These differences are due to the different half lifetimes of the flash-induced H_{in}^+ relaxation and $\Delta\varphi$ relaxation ($\tau_{H^+_{in}} \geqslant 10$ s; $\tau_{\Delta\varphi} \leqslant 300$–$500$ ms). Under these conditions $\Delta\varphi/2$ should correspond energetically to 60 mV· ΔpH if (a) the potential decays pseudolinearly between the flashes and if (b) no "underlying" constant electrical potential difference exists. (An "underlying potential" is generated if the next flash is

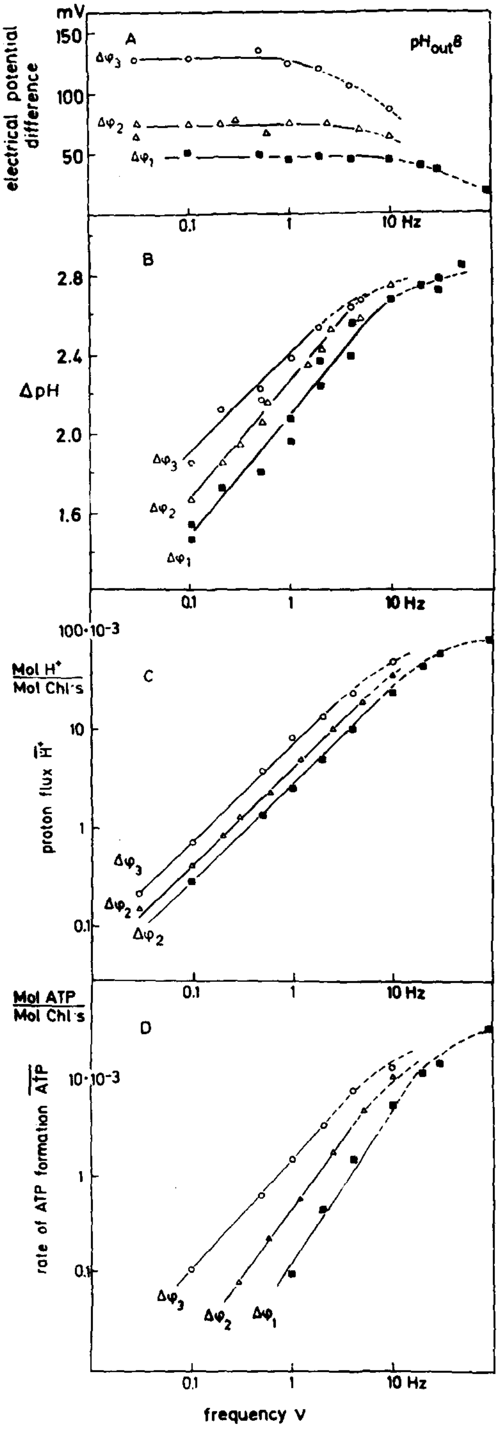

Fig. 2. (A) The electrical potential difference, $\Delta\varphi$, (B) the pH gradient, Δ pH, (C) the proton flux, $\overline{\dot{H}^+}$, and (D) the rate of phosphorylation, $\overline{\dot{A}TP}$, as function of the flash frequency ν. ($\Delta\varphi_1$ initial electrical potential difference induced by one single turnover flash, $\Delta\varphi_2$ induced by a group of two and $\Delta\varphi_3$ induced by a group of five single turnover flashes. Dark time between the flashes within the group is 2 ms.)

fired before $\Delta\varphi$ induced by the first one has declined completely to zero.) With respect to (a) the decay does not correspond to a linear one (see Fig. 1A). With respect to (b) this condition is fulfilled for the maximal used potential $\Delta\varphi_3 = 125$ mV in the entire range up to ΔpH 2.5. For the minimal potential $\Delta\varphi_1 = 50$ mV an "underlying electrical potential" is built up between 0 mV to maximal 20 mV at ΔpH 1.8–2.5 (estimated from the absorption changes at 515 nm). These deviations prevent a simple demonstration of an energetic equivalence of $\Delta\varphi$ and ΔpH in the results below. This has, however, no significant influence on the principle functional relations, outlined below.

RESULTS

The dependency of $\Delta\varphi$, ΔpH, $\overline{H}^+$ and phosphorylation on the flash frequency

Fig. 2 shows the dependence of the different parameters ($\Delta\varphi$, ΔpH, $\overline{H}^+$ and $\overline{\text{ATP}}$) on the flash frequency ν.

In the following, $\Delta\varphi$ is used for describing the initial amplitude as indicated in Fig. 1A. At low frequencies $\Delta\varphi_1$, $\Delta\varphi_2$ and $\Delta\varphi_3$ are independent of ν (solid lines). The decrease of $\Delta\varphi$ at higher frequencies (dashed lines) is due to the fact that the next flash is fired before the rate determining dark reaction of the electron transport (plastoquinone oxidation) is completed [32].

Fig. 2B shows the ΔpH value in the steady state as a function of the flash frequency at different $\Delta\varphi$ values. At lower frequencies ΔpH increases proportional to log ν whereas at higher frequencies this relation does not hold. Fig. 2C shows the rate of the proton flux, $\overline{H}^+$, and Fig. 2D the rate of ATP formation, $\overline{\text{ATP}}$, as a function of the frequency. For the correlations between the described parameters only those values have been used where the magnitude of the electrical potential difference was practically constant (solid lines in Fig. 2).

To prevent confusion between our results and those published earlier by Boeck and Witt [17] who measured the rate of phosphorylation with the pH indicator umbelliferone, their results are plotted in Fig. 3 together with corresponding measurements of this work (data from Fig. 2 and a similar set of measurements in a linear plot). Fig. 3A shows $\Delta\varphi$ and Fig. 3B the ATP yield per flash as a function of the flash frequency. In our work the analysis was restricted to flash frequencies below 10 Hz because under our conditions $\Delta\varphi$ is no longer constant above 10 Hz (Fig. 3A). On the other hand our analysis was extended to very low frequencies (0.1 Hz). In the range between 0.1 and 10 Hz the ΔpH value changes by about 1.2 units which drastically affects the ATP yield (see solid curve Fig. 3B). In the work of Boeck and Witt the analysis was extended to higher frequencies (30 Hz). The constancy of the ATP yield between 1–30 Hz reported in [17] (see dotted line, Fig. 3B) is an oversimplification. According to both measurements there is a slight maximum at 20 Hz. This seems to be due to the fact that with increasing frequency ΔpH increases but $\Delta\varphi$ decreases (see Fig. 3A).

Relation between the proton flux and ΔpH

Fig. 4 shows the dependence of the pH values in the inner space of the thylakoid (pH_{in}) on the flash frequency (phosphorylating conditions). Single turnover flashes were used which set up an electrical potential difference of 50 mV across the thylakoid membrane. The proton flux is depicted on the top of the figure. Due to the large buffer

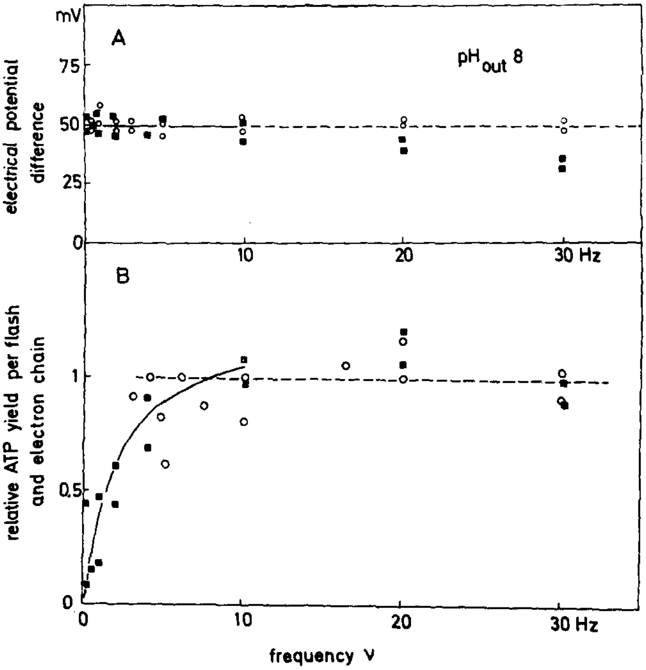

Fig. 3. (A) $\Delta\varphi$ as function of frequency. (B) Relative ATP yield per flash as function of frequency. (○) Data obtained by Boeck and Witt [17], (■) this work (Details see text).

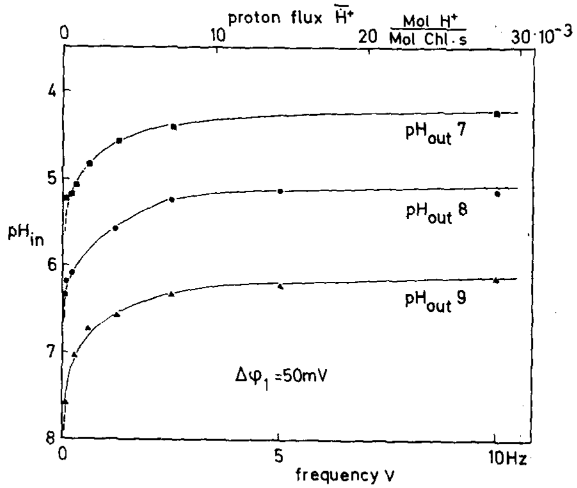

Fig. 4. pH$_{in}$ as a function of the flash frequency and proton flux $\overset{\bullet}{H}{}^{+}$ at pH$_{out}$ 7–9. $\Delta\varphi_1$ = 50 mV.

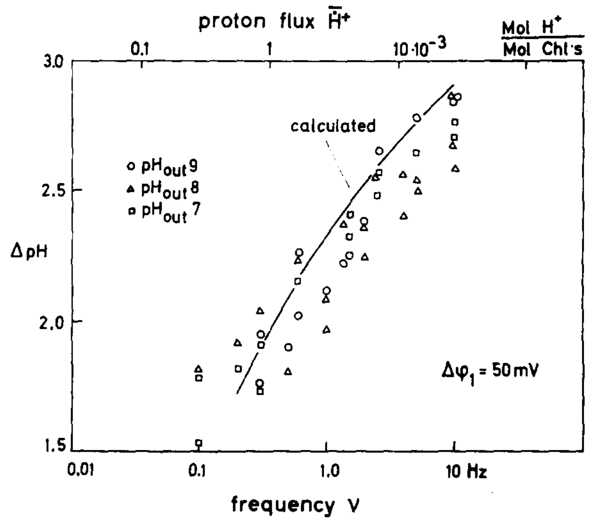

Fig. 5. Δ pH as a function of the flash frequency and proton flux $\bar{H}^+$ (log plot) at pH_{out} 7–9. $\Delta\varphi_1$ = 50 mV (Data from Fig. 4). The curve is calculated according to Eqn 22. Details see Discussion.

capacity of the suspension pH_{out} does not change appreciably during the change of pH_{in}. Because pH_{in} decreases obviously proportional to the decrease of pH_{out}, it is suggested that $pH_{out} - pH_{in} = \Delta pH$ does not depend on pH_{out}.

Fig. 5 shows ΔpH as function of the flash frequency ν (bottom) and proton flux (top) in a log plot at different pH_{out}. ΔpH is within error limits independent of pH_{out} 7–9. This is valid at least for ΔpH values between 1.7 and 2.7. The solid curve is calculated from Eqn 22 (see Discussion).

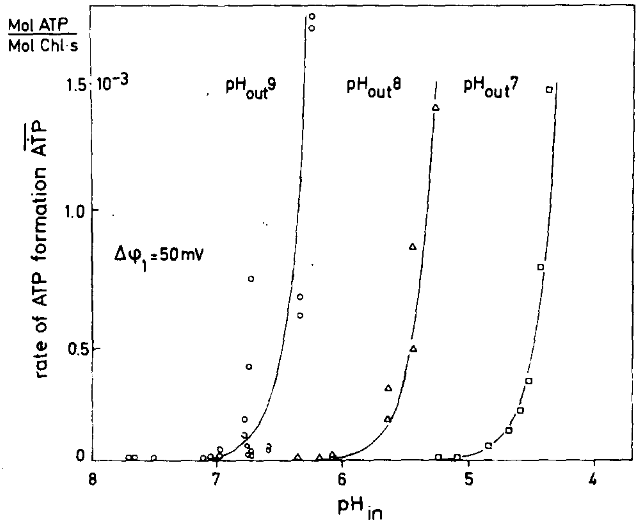

Fig. 6. Rate of ATP formation $\overline{\text{ATP}}$ as a function of pH_{in} at pH_{out} 7–9. $\Delta\varphi_1$ = 50 mV.

Relation between phosphorylation and ΔpH

Fig. 6 shows the rate of ATP formation as a function of the $\mathrm{pH_{in}}$ value at three different $\mathrm{pH_{out}}$ values. The result seems to indicate that there exist "threshold-values" of pH_{in} below which practically no ATP is synthesized. Such "threshold-values" have been reported by several authors [19, 33] and have been interpreted as an indication of a critical ΔpH value necessary for ATP formation (but see Discussion). If we plot log $\overline{\mathrm{A\dot{T}P}}$ versus ΔpH (see Fig. 7A) the functional relation is given by:

$$\log \overline{\mathrm{A\dot{T}P}} = b(\Delta\mathrm{pH}-2.2)+\log \overline{\mathrm{A\dot{T}P}}_{\Delta\mathrm{pH}\,2.2} \tag{9}$$

$b = 2.6$ at 50 mV (see Table I).

The results in Fig. 7A also demonstrate that the rate of ATP formation is independent of $\mathrm{pH_{out}}$ 7–9. Thus, the average rate of ATP formation is not regulated by $\mathrm{pH_{out}}$ nor by $\mathrm{pH_{in}}$ but only by the difference between both, i.e. by the pH gradient ΔpH. This is valid at least in the range of ΔpH 2–2.7. It should be noted that in some experiments the ADP/ATP ratio (about 10 under the conditions described in Methods) has been changed. No difference was observed at ADP/ATP = 1.

The dependence of the phosphorylating proton flux $\overline{H_p{}^+}$ and the basal proton flux $\overline{H_b{}^+}$ on ΔpH

Assuming a constant ratio between $\overline{\mathrm{A\dot{T}P}}$ and the proton flux $\overline{\dot{\mathrm{H}}_p{}^+}$ coupled to phosphorylation, i.e. $\overline{\mathrm{A\dot{T}P}} = \overline{\dot{\mathrm{H}}_p{}^+}/n$, the curve in Fig. 7A shows in respect to the slope

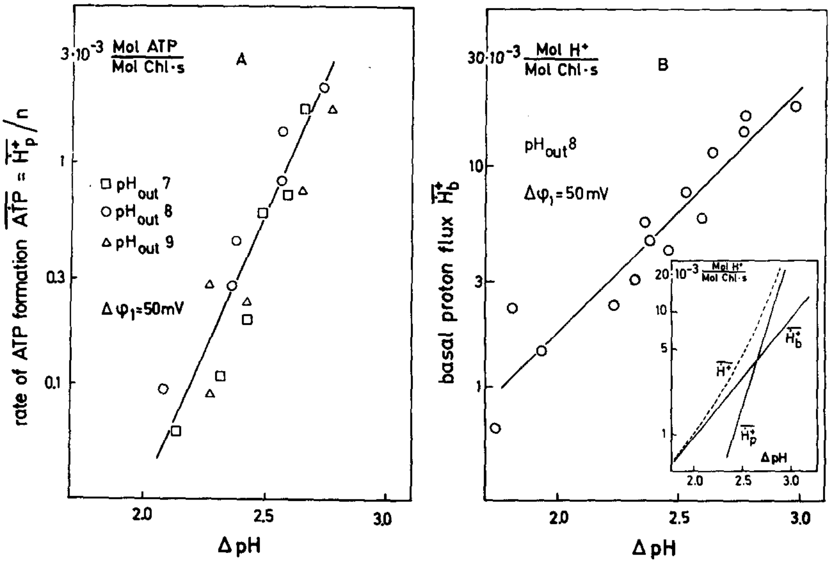

Fig. 7. (A) Rate of ATP formation, $\overline{\mathrm{A\dot{T}P}}$, and phosphorylating proton flux, $\overline{\dot{\mathrm{H}}_p{}^+}$, (log plot) as a function of Δ pH at $\mathrm{pH_{out}}$ 7–9. $\Delta\varphi_1 = 50$ mV (Data from Fig. 6). (B) Basal proton flux as a function of Δ pH at $\mathrm{pH_{out}}$ 8. $\Delta\varphi_1 = 50$ mV. The same reaction medium has been used as under phosphorylating conditions except that ADP was replaced by the same concentration of ATP. Inset: Basal ($\overline{\dot{\mathrm{H}}_b{}^+}$), phosphorylating $\overline{\dot{\mathrm{H}}_p{}^+}$ and total ($\overline{\dot{\mathrm{H}}^+}$) proton flux as a function of Δ pH calculated from Eqns 18, 20 and 22 with the averaged values listed in Table I and $n = 2.4$ (see Discussion).

also the dependence of $\overline{\dot{H}}_p{}^+$ on ΔpH. The absolute value depends on n. (n = number of protons translocated via the ATPase per ATP):

$$\log \overline{\dot{H}}_p^+ = b(\Delta \mathrm{pH} - 2.2) + \log \overline{\dot{\mathrm{ATP}}}_{\Delta \mathrm{pH}\,2.2} + \log n \qquad (9')$$

$b = 2.6$ at 50 mV (see Table I). Fig. 7B shows the ΔpH dependence of the basal proton flux $\overline{\dot{H}}_b{}^+$, i.e. the proton flux which was measured under non-phosphorylating conditions. $\overline{\dot{H}}_b{}^+$ is practically independent of $\mathrm{pH_{out}}$ 7–9 and depends only on ΔpH (unpublished results). The slope is about 1 whereas the slope of $\overline{\dot{H}}_p{}^+$ in Fig. 7A is $b = 2.6$. Thus, the basal proton flux can be described as:

$$\log \overline{\dot{H}}_b^+ = (\Delta \mathrm{pH} - 2.2) + \log \overline{\dot{H}}^+_{b\,\Delta \mathrm{pH}\,2.2} \qquad (10)$$

$\overline{\dot{H}}_p{}^+$, $\overline{\dot{H}}_b{}^+$ and the sum of both, $\overline{\dot{H}}{}^+$, are depicted in the inset in Fig. 7B using $n = 2.4$ for the calculation of $\overline{\dot{H}}_p{}^+$ (Details see Discussion).

Relation between phosphorylation and $\Delta\varphi$

The experiments described above have been carried out at a constant initial electrical potential of 50 mV. Fig. 8 shows the results at three different electrical potentials at $\mathrm{pH_{out}}$ 8. Log $\overline{\dot{\mathrm{ATP}}}$ is proportional to ΔpH as formulated by Eqn 9.

However, the absolute values (i.e. $\overline{\dot{\mathrm{ATP}}}$ at ΔpH 2.2 in Eqn 9) and also the slopes (i.e. b in Eqn 9) are dependent on the electrical potential difference (see Table I). At constant ΔpH, e.g. ΔpH 2.2, an increase from 50 mV to 125 mV gives an approximate 5-fold increase in the rate of ATP formation.

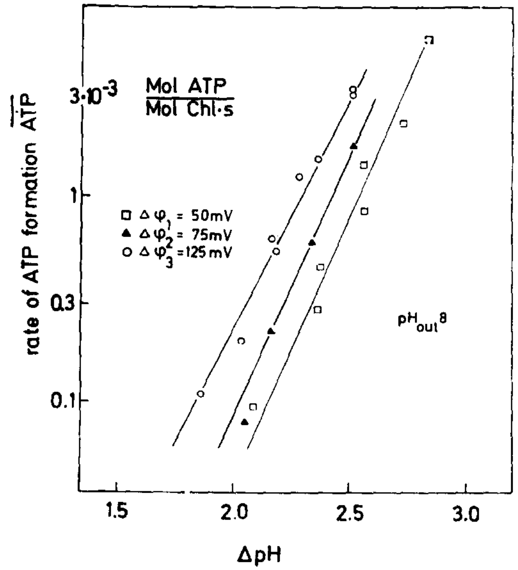

Fig. 8. Rate of ATP formation $\overline{\dot{\mathrm{ATP}}}$ (log plot) as a function of Δ pH at different electrical potential differences. $\mathrm{pH_{out}}$ 8.

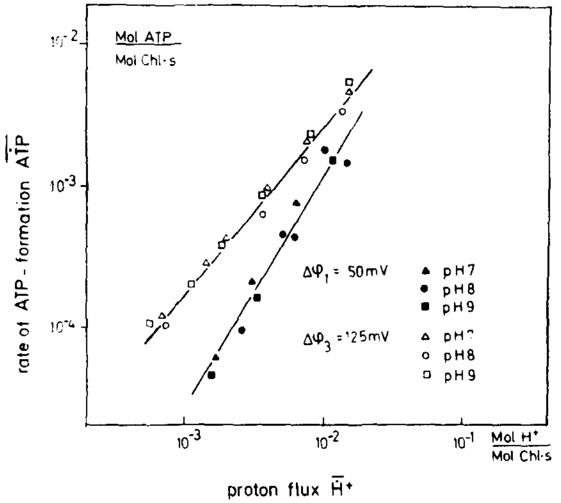

Fig. 9. Rate of ATP formation ($\overline{\dot{\mathrm{ATP}}}$) (log plot) as a function of the proton flux ($\overline{\mathrm{H}^+}$) (log plot) $\mathrm{pH_{out}}$ 7–9 and different electrical potential differences.

In Fig. 9 the rate of ATP formation is plotted versus the proton flux at two electrical potential differences and at $\mathrm{pH_{out}}$ 7–9. The functional relation is given by

$$\log \overline{\dot{\mathrm{ATP}}} = c \cdot \log \frac{\overline{\mathrm{H}^+}}{\overline{\mathrm{H}_1^+}} + \log \overline{\dot{\mathrm{ATP}}_1} \tag{11}$$

($\overline{\mathrm{H}_1^+}$ proton flux at $\nu = 1$ Hz and at $\Delta\varphi_1 = 50$ mV, $\overline{\dot{\mathrm{ATP}}_1}$ rate of ATP formation at the proton flux $\overline{\mathrm{H}_1^+}$). The slope c and the absolute values, $\overline{\dot{\mathrm{ATP}}_1}$, depend on $\Delta\varphi$. They are listed in Table I.

At a constant proton flux, e.g. $\overline{\mathrm{H}_1^+} = 2.8 \cdot 10^{-3}$ (mol H^+)/(mol Chl · s), an increase from 50 mV to 125 mV gives an approximately 4-fold increase in the rate of ATP formation. This stimulation decreases with increasing proton flux.

Relation between $\Delta H^+/ATP$ and ΔpH and $\Delta\varphi$

According to Fig. 9 the rate of ATP formation can be increased by increasing $\Delta\varphi$ at constant proton flux $\overline{\mathrm{H}^+}$. This is only understandable when the yield of ATP formation increases with $\Delta\varphi$. The yield is defined by the ratio of ATP generated per translocated proton: $\mathrm{ATP}/\Delta\mathrm{H}^+$. If the yield is increased due to the energetic properties of $\Delta\varphi$, the yield should be increased also by ΔpH. Fig. 10 shows the dependence of the reciprocal yield, the $\Delta\mathrm{H}^+/\mathrm{ATP}$ ratio, on ΔpH at different $\Delta\varphi$ values. On the right hand side the e/ATP ratio is depicted. Fig. 10 was obtained by dividing the averaged proton flux, $\overline{\mathrm{H}^+}$, with the rate of ATP formation, $\overline{\dot{\mathrm{ATP}}}$, using the data from Fig. 9 and corresponding sets of measurements. At small $\Delta\varphi$ values the stimulation of the ATP yield by ΔpH is large whereas at higher $\Delta\varphi$ values this stimulation is smaller. The curves are calculated from Eqn 23 (for details see Discussion). Fig. 11 shows a plot of $\Delta\mathrm{H}^+/\mathrm{ATP}$ versus $\Delta\varphi$ at different ΔpH. This diagram was obtained from the data of Fig. 10 at constant ΔpH. At low ΔpH the stimulation of the ATP yield by $\Delta\varphi$ is large

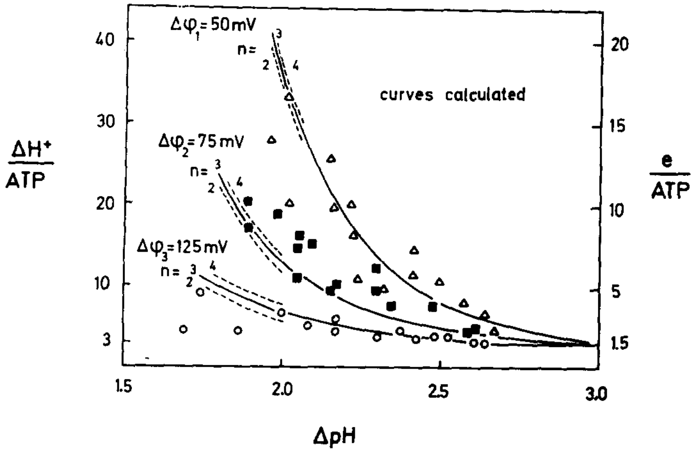

Fig. 10. $\Delta H^+/ATP$ (e/ATP) as a function of Δ pH at different $\Delta\varphi$ values. pH_{out} was varied between 7–9; no principle differences were noticed. The curves are calculated according to Eqn 23.

whereas at high ΔpH the stimulation is smaller. According to the results of Figs 10 and 11 the degree of coupling between proton flux and ATP synthesis, i.e. $\Delta H^+/ATP$ and e/ATP respectively, depends on the pH gradient as well as on the electrical potential difference. It results in a $\Delta H^+/ATP$ ratio ≈ 3 and e/ATP ≈ 1.5, at $\Delta\varphi_3 = 125$ mV and ΔpH 2.7. According to these results the increase of $\overline{ATP}$ with ΔpH at constant $\Delta\varphi$ (Fig. 8) is due to two effects: (a) to an increase of the proton flux (Fig. 5) and (b) to an increase of the ATP yield (Fig. 10). The increase of $\overline{ATP}$ with $\Delta\varphi$ at constant proton flux $\bar{H}^+$ (Fig. 9) is due to an increase of the ATP yield by $\Delta\varphi$.

In Fig. 12 the number of ATP molecules generated per flash and electron chain is plotted as a function of ΔpH at different $\Delta\varphi$ values. The amount of ATP increases with ΔpH. At 50 mV, ATP is measurable only above ΔpH 2.0 suggesting

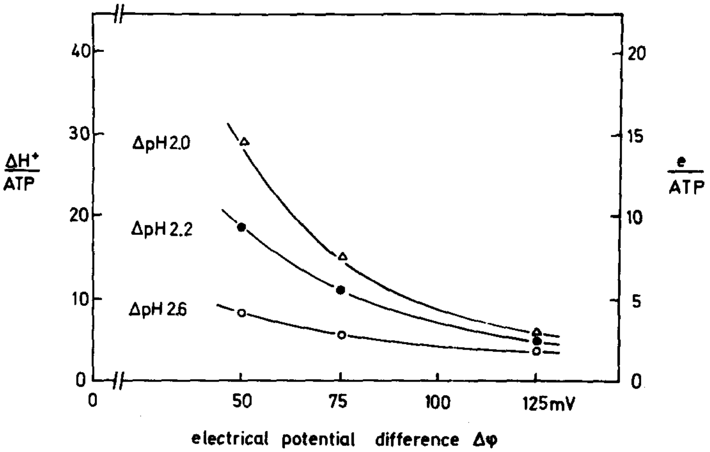

Fig. 11. $\Delta H^+/ATP$ (e/ATP) as a function of $\Delta\varphi$ at different ΔpH values. $pH_{out} = 7–9$.

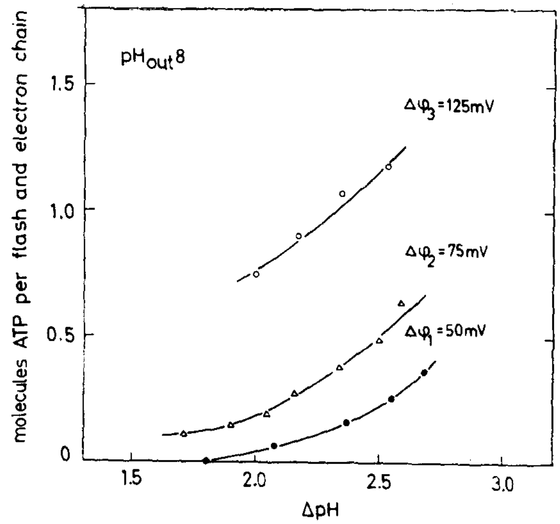

Fig. 12. Molecules ATP generated per flash and electron chain as a function of Δ pH at different $\Delta\varphi$ values. pH_{out} 8.

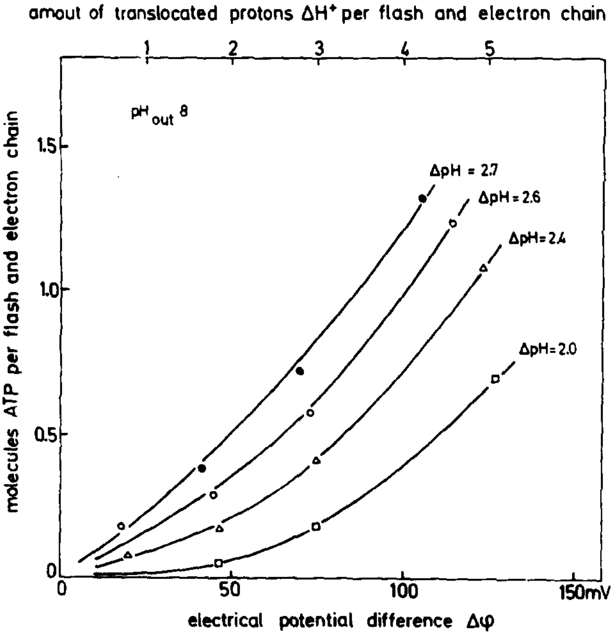

Fig. 13. Molecules ATP generated per flash and electron chain as a function of $\Delta\varphi$ at different ΔpH values. pH_{out} 8. The amount of translocated protons is depicted on the top. The slope at ΔpH = 2.7 and 125 mV corresponds to $\Delta H^+/ATP \approx 3$.

TABLE I

AVERAGED VALUES OF b, k_p, AND $\overline{\dot{\mathrm{A}}\mathrm{TP}}_{\Delta\mathrm{pH}\ 2.2}$ (See Eqns 9 and 19), AND k_b (See Eqns 10 and 21) AND c AND $\overline{\dot{\mathrm{A}}\mathrm{TP}}$ (See Eqn 11)

	$\mathrm{pH_{out}}$ 8			$\mathrm{pH_{out}}$ 7–9	
	b	$\overline{\dot{\mathrm{A}}\mathrm{TP}}_{\Delta\mathrm{pH}\ 2.2}$	k'_p	c	$\overline{\dot{\mathrm{A}}\mathrm{TP}}_1$
50 mV	2.6±0.3	$0.13 \cdot 10^{-3}\ \frac{\mathrm{mol\ ATP}}{\mathrm{mol\ Chl \cdot s}}$	$2.0 \cdot 10^{-10}\ \frac{\mathrm{mol\ ATP}}{\mathrm{mol\ Chl \cdot s}}$	1.6±0.1	$0.15 \cdot 10^{-3}\ \frac{\mathrm{mol\ ATP}}{\mathrm{mol\ Chl \cdot s}}$
75 mV	2.5±0.2	$0.3 \cdot 10^{-3}$	$9.5 \cdot 10^{-10}$	1.4±0.1	$0.3 \cdot 10^{-3}$
125 mV	2.2±0.2	$0.65 \cdot 10^{-3}$	$9.5 \cdot 10^{-9}$	1.2±0.1	$0.6 \cdot 10^{-3}$
				$\overline{\dot{\mathrm{H}}_b^{+}}_{\Delta\mathrm{pH}\ 2.2}$	k_b
				$1.6 \cdot 10^{-3}\ \frac{\mathrm{mol\ H^+}}{\mathrm{mol\ Chl \cdot s}}$	$10^{-5}\ \frac{\mathrm{mol\ H^+}}{\mathrm{mol\ Chl \cdot s}}$

a "threshold" at this ΔpH value. However, with $\Delta\varphi > 50$ mV the "threshold" disappears; with 125 mV the amount of ATP per flash is about 10 times larger at ΔpH 2.2 than that with 50 mV at the same ΔpH value.

Fig. 13 shows a cut at constant ΔpH through the curves plotted in Fig. 12, thus giving the number of ATP molecules per flash and electron chain as a function of $\Delta\varphi$. The corresponding amount of translocated protons ΔH^+ per flash and electron chain is depicted on the top. ΔH^+ is obtained from Eqn 5:

$$\Delta H^+ = \frac{\bar{H}^+}{\nu} = 2 \frac{\Delta\varphi}{\Delta\varphi_1} \frac{H^+}{\text{e-chain}} \tag{12}$$

At ΔpH 2 the amount increases nonlinearly suggesting a "threshold" at 50 mV. At ΔpH 2.7, however, the amount of ATP increases approximately linearly with $\Delta\varphi$, and the "threshold" at 50 mV disappears. The slope of the curve at ΔpH 2.7 and at 125 mV results in a value of $\Delta H^+/\text{ATP} \approx 3$.

DISCUSSION

On the pH_{in} *determination with aminoacridine*

The reliability of a pH_{in} determination from the fluorescence quenching of aminoacridine using Eqn 7 depends on three assumptions [27, 34]:

(1) The uncharged aminoacridine is freely permeable, whereas the charged species is practically impermeable.

(2) Aminoacridine is concentrated in the aequeous inner phase of the thylakoids, there is no significant binding to the membrane.

(3) The fluorescence is quenched completely when the aminoacridine is in the inner space of the thylakoids.

Some evidence has been accumulated showing the validity of these assumptions in chloroplasts [27], liposomes [34] and chromatophores [35]. But on the other hand it was shown in chloroplasts that the fluorescence quenching of aminoacridine depends (a) on the concentration of aminoacridine, (b) on the amount of chloroplasts and (c) on the osmotic volume of the chloroplasts [28, 53]. Such dependencies are not in accordance with the pH_{in} indicating mechanism leading to Eqn 8. It was concluded inter alia that the fluorescence quenching of the acridines is due to an energy depending binding to the chloroplast membranes [28, 36]. (Energy depending fluorescence quenching is, however, not caused by $\Delta\varphi$. This was checked by the addition of valinomycin which accelerates the decay of $\Delta\varphi$ 20-fold. No influence on the magnitude of ΔpH was observed.) Without discussing possible reasons for the differences between the various authors it must be pointed out that as long as the possible binding of aminoacridine is proportional to H_{in}^+/H_{out}^+ the method can be nevertheless used for relative H_{in}^+ measurements. Absolute values can be obtained by calibration with other independent pH_{in} indicating methods.

Rumberg et al. [53] have compared H_{in}^+ concentrations in chloroplasts determined by the aminoacridine method with H_{in}^+ values measured either by the distribution of imidazole [37] or the pH_{in} dependence of the rate of electron transport [5]. Whereas the last two methods give the same results no quantitative agreement between the aminoacridine method and the two other methods has been obtained.

It was, however, shown that at low H_{in}^+/H_{out}^+ ratios $Q/1-Q$ is proportional to H_{in}^+/H_{out}^+. The H_{in}^+/H_{out}^+ range where this proportionality exists depends on the amount of chloroplasts, the concentration of aminoacridine and the osmotic volume of the chloroplasts. The experiments presented in this work are restricted to a ΔpH range where $Q/1-Q$ is proportional to H_{in}/H_{out}. Under our experimental conditions this is true up to about ΔpH 2.7. The range above ΔpH 2.7 where the proportionality is lost was excluded from analysis. In respect to the absolute value it was observed by Rumberg et al. [53] that in the range where the proportionality is valid, the aminoacridine method indicates a H_{in}^+/H_{out}^+ value which is about a factor 2 larger than that measured by the two other methods. Thus, according to these methods the ΔpH values in this work are over-estimated by $\Delta\text{pH} \approx 0.3$.

ΔpH and phosphorylation as a function of pH_{out}

It has been demonstrated in Fig. 5 that up to ΔpH 2.7 the magnitude of the pH gradient is independent of pH_{out} 7–9 (phosphorylating conditions). This is shown at a constant rate of electron transport and constant $\Delta\varphi$. This result implies that in the indicated pH range the same part of the light energy is always conserved as a pH gradient. On the other hand it was reported [38] that in the range above ΔpH 2.7 the ΔpH value depends on pH_{out} with a maximum at about 8.5. This range ($\Delta\text{pH} > 2.7$) is not comparable with the range in our work ($\Delta\text{pH} < 2.7$). Besides this, there is a further difference, we kept $\Delta\varphi$ constant whereas in [38] $\Delta\varphi$ is not known. Furthermore, in [38] the rate of electron transport was not kept constant in contrast to our present experiments. Because the rate of electron transport shows a similar pH_{out} dependence as the ΔpH (maximum at about 8.5), the pH_{out} dependence of electron transport is at least one reason for the observed pH_{out} dependence of ΔpH reported in [38].

It is shown in this work that in the range up to ΔpH 2.7 the rate of phosphorylation is also independent of pH_{out} in the range 7–9. This was demonstrated at constant $\Delta\varphi$ and ΔpH (see Fig. 7a) and at constant proton flux and rate of electron transport (see Fig. 9). The independence of phosphorylation on pH_{out} implies that neither the activity of the enzyme nor the availability of protons consumed chemically in ATP synthesis ("substrate protons") are limited by pH_{out} up to ΔpH 2.7. At low light intensities ($I < 1200$ erg/cm^2 per s) very probably generating $\Delta\text{pH} < 2.7$ it was found by Saha et al. [39] that in continuous light the rate of phosphorylation is also independent of pH_{out} between pH_{out} 7–8 and at pH_{out} 9 is about 20 % higher. This result corresponds within error limits to our observed independence of phosphorylation on pH_{out}. On the other hand it was reported in [40, 41] that the rate of phosphorylation depends strongly on pH_{out} with a maximum at about 8.5. Under these experimental conditions again the rate of electron transport, and therefore ΔpH and the proton flux were not kept constant. The pH dependence of the electron transport is therefore at least one reason for the observed pH dependence of phosphorylation in [40, 41]. Recently, it was reported [42] that even if ΔpH is kept constant the rate of ATP formation depends strongly on pH_{out}. Because our measurements are carried out in the range $1.8 \leqslant \Delta\text{pH} \leqslant 2.7$ and the experiments of Pick et al. [42] in the range $2.7 \leqslant \Delta\text{pH} \leqslant 4$ it has to be concluded that at low ΔpH, i.e. at low rates of ATP formation, phosphorylation is limited only by ΔpH and $\Delta\varphi$ whereas at high rates the pH_{out} dependence of intermediate steps of phosphorylation must be rate determining. However, it must be recalled that for the analysis of the pH dependence, besides the ΔpH, the magnitude of the electrical potential difference must also be kept constant because it is shown in this work that even at constant ΔpH the rate of phosphorylation depends on $\Delta\varphi$. In [42], however, the $\Delta\varphi$ values were disregarded.

The dependence of phosphorylation on $\Delta\varphi$ and ΔpH.

At constant $\Delta\varphi$ the increase of the rate of phosphorylation with increasing ΔpH is due to two effects:

(a) to an increase of the proton flux and (b) to an increase of the ATP yield, i.e. an increase of the coupling between proton flux and ATP synthesis. The yield can also be increased by $\Delta\varphi$ at constant ΔpH and constant $\bar{H}^+$. The lower the ΔpH the higher is the stimulation of the ATP yield by $\Delta\varphi$ and vice versa. Such a stimulation of the ATP yield was also observed qualitatively with an artificial extrinsic diffusion potential as described by Schuldiner et al. [19].

It was reported that there exists a minimal ΔpH ("threshold") below which practically no ATP synthesis takes place [19, 33] (see also Fig. 6). In analogy to a ΔpH "threshold" it was reported by Junge et al. [16] that there also exists a $\Delta\varphi$ "threshold" below which practically no ATP is synthesized. This threshold was interpreted as an electrical triggering level for ATP synthesis [43]. Boeck and Witt [17] found, however, a linear relation between the amount of ATP per flash and the electrical potential difference, i.e. no "threshold". This discrepancy is in principle now explained by the results shown in Fig. 13: at low ΔpH (i.e. ΔpH 2) a "threshold" can be seen at approximately 50 mV, whereas at higher ΔpH this "threshold" at 50 mV vanishes. The "threshold" level depends at constant $\Delta\varphi$ obviously on ΔpH. In analogy the ΔpH "threshold" in Fig. 12 depends at constant ΔpH on $\Delta\varphi$. These observations seem to indicate that there exists a "threshold" energy range. Only above this range ATP synthesis can be observed. It is obviously of no importance whether this energy is provided by $\Delta\varphi$ or ΔpH. An explanation is given below.

Reasonable agreement exists in the literature for the ratio $\Delta H^+/e \approx 2$. No agreement has been obtained in the literature concerning the e/ATP and ΔH^+/ATP ratio. This is inter alia due to different methods of calculating the e/ATP ratio and to different degrees of integrity of the chloroplasts. DelCampo et al. [44] obtained e/ATP $\approx$ 2 (ΔH^+/ATP $\approx$ 4) using the total rate of electron transport, thus, not accounting for a basal flux. Also not accounting for a basal flux, Schwarz [33] obtained ΔH^+/ATP $\approx$ 2. Izawa and Good [45] obtained e/ATP $\approx$ 1 (ΔH^+/ATP $\approx$ 2) proposing a model of independent basal and phosphorylating electron fluxes, i.e. proposing that the basal rate is not changed under phosphorylating conditions. Rumberg and Schröder and Schröder et al. [10, 46, 47] used a model which accounts for a decrease of the basal flux under phosphorylating conditions (due to a pH_{in} decrease) and calculated for the ATPase pathway e/ATP $\approx$ 2 (ΔH_p^+/ATP $\approx$ 4).

Portis and McCarthy [9, 48] obtained from a log $\overline{ATP}$ – pH_{in} plot (pH_{out} 8) a slope of b $\approx$ 3 which was interpreted as the ΔH^+/ATP ratio. However, it is expected that from such a kinetic interpretation only the number of protons involved in the rate determining step can be read out from b which is not necessarily identical with the total number of protons necessary per ATP. Furthermore, they must assume that $\Delta\varphi$ is not changed with increasing light intensity.

In this work we evaluated the parameters which change the $\Delta H^+/ATP$ ratio, i.e. the parameters which determine the degree of coupling between proton flux and the rate of ATP synthesis. It is shown that this ratio depends on ΔpH and $\Delta\varphi$. The value measured in this work is $\Delta H^+/ATP \approx 3$ or $e/ATP \approx 1.5$ (at ΔpH 2.7 and $\Delta\varphi = 125$ mV). At higher ΔpH and $\Delta\varphi$ values $\Delta H^+/ATP$ should be lower than 3 (see also Discussion at the end).

For example in chromatophores in which relative high electrical potentials (200 mV) and high ΔpH (ΔpH 3) are generated a ratio of $\Delta H^+/ATP \approx 2$ was observed [49, 50]. High ΔpH and $\Delta\varphi$ values are also expected in completely intact thylakoids (in algae or highly intact chloroplasts) which have very low permeabilities to ions. In such chloroplasts $e/ATP \approx 1.2$ (or $\Delta H^+/ATP \approx 2.4$) was found by Reeves et al. [51].

On the other hand according to our results at low light intensities (i.e. low ΔpH and low $\Delta\varphi$) the coupling should be weaker. A strong change of ΔpH occurs from 0–10 Hz (see Fig. 4). These values correspond approximately to a change of continuous light from 0–5000 erg/cm^2 s. Thus, above 5000 erg/cm^2 s only a slight change of $\Delta H^+/ATP$ (e/ATP) is expected but below this light intensity an increase of $\Delta H^+/ATP$ should occur. An increase of $\Delta H^+/ATP$ was reported at low intensities [33] and no variation of e/ATP at > 6000 erg/cm^2 s [44, 45]. From the result in [39] at < 1000 erg/cm^2 s also a variation of e/ATP can be concluded. A consequence of these results is that at low light intensities less ATP should be generated in relation to NADPH than at higher intensities. Such an effect has been reported by Heber [52] on chloroplasts with intact outer membrane. However, Heber has interpreted this phenomenon as follows: at high light intensities a linear and a pseudocyclic electron flux (Mehler reaction) is operating whereas with lower light intensities the pseudocyclic electron flux is decreased more than the linear one. Thus, it is assumed that there exists a constant e/ATP ratio but a variable ratio $\overline{\dot{e}}_{linear}/\overline{\dot{e}}_{cyclic}$ whereas according to our results the e/ATP ratio depends on ΔpH and $\Delta\varphi$.

One reason for this dependence of $\Delta H^+/ATP$ on $\Delta\varphi$ and ΔpH may be the following: The measured $\Delta H^+/ATP$ is assumed to be due to the proton efflux via the phosphorylating pathway ($\overline{\dot{H}_p{}^+}$) and to a proton efflux via a non-phosphorylating pathway, i.e. via a basal pathway ($\overline{\dot{H}_b{}^+}$). This assumption is trivial but it becomes important if one considers that $\overline{\dot{H}_p{}^+}$ and $\overline{\dot{H}_b{}^+}$ depend in a different way on $\Delta\varphi$ and ΔpH.

According to results obtained by Rumberg and Schröder [8, 10] and Schröder et al. [46] at pH_{out} 8 and supposing $\Delta\varphi = 0$ there exists an approximate quadratic dependence for $\overline{\dot{H}_p{}^+}$ on $H_{in}{}^+$:

$$\overline{\dot{H}_p^+} \sim (H_{in}^+)^2 \text{ and a linear one for } \overline{\dot{H}_b{}^+} \text{ on } H_{in}{}^+: \tag{13}$$

$$\overline{\dot{H}_b^+} \sim (H_{in}^+)^1 \tag{14}$$

Because in this case $\overline{\dot{H}_p{}^+}$ increases more rapidly with $H_{in}{}^+$ than $\overline{\dot{H}_b{}^+}$, there must be a change in the distribution of protons between basal and phosphorylating pathways as has been proposed by Schröder [47]. Because the phosphorylating flux is preferred at increasing $H_{in}{}^+$, thereby the degree of coupling is increased. With appropriate proportionality factors at high H_{in}^+ the basal flux is negligible ($\overline{\dot{H}_b^+} \ll \overline{\dot{H}_p^+}$) and the measured $\Delta H^+/ATP$ should be identical with the ratio at the ATPase pathway $\Delta H_p^+/ATP$.

Our results are similar to those of Rumberg and Schröder: Assuming a constant coupling between the proton flux via the phosphorylating pathway $\overline{\dot{H}_p^+}$ and ATP synthesis, i.e. $\Delta H_p^+/ATP \equiv n$ or $\overline{\dot{ATP}} = \overline{\dot{H}_p^+}/n$ (n = number of protons translocated via the ATPase pathway per generated ATP) it results according to Eqn 9′:

$$\overline{\dot{H}_p^+} \sim (H_{in}^+/H_{out}^+)^b \tag{15}$$

($b = 2.2$–2.6). According to Eqn 10 under non-phosphorylating conditions where only the basal flux $\overline{\dot{H}_b^+}$ exists can it results at pH_{out} 7–9.

$$\overline{\dot{H}_b^+} \approx (H_{in}^+/H_{out}^+)^1 \tag{16}$$

In respect to the dependence of $\overline{\dot{H}_p^+}$ on H_{in}^+ Rumberg and Schröder observed an exponent of $b \approx 2$ (using continuous illumination and supposing $\Delta\varphi = 0$). Portis and McCarty observed under similar conditions an exponent of $b \approx 3$ [9].

In respect to the dependence of $\overline{\dot{H}_b^+}$ on H_{in}^+ our result confirms that of Schröder et al. [46, 47]. The difference between the results obtained by these authors and ours is that we change besides H_{in}^+ also H_{out}^+ and observe under the above outlined conditions ($\Delta pH \leqslant 2.7$) that $\overline{\dot{H}_p^+}$ and $\overline{\dot{H}_b^+}$ do not depend on H_{in}^+ but on H_{in}^+/H_{out}^+.

On the basis of Eqn 15 and Eqn 16 the dependence of $\Delta H^+/ATP$ on ΔpH and $\Delta\varphi$ can now be calculated

$$\frac{\Delta H^+}{ATP} = \frac{\overline{\dot{H}^+}}{\overline{\dot{ATP}}} = \frac{\overline{\dot{H}_p^+} + \overline{\dot{H}_b^+}}{\overline{\dot{H}_p^+}/n} \tag{17}$$

According to Eqn 9′,

$$\overline{\dot{H}_p^+} = k_p \exp(2.3b\,\Delta pH) \text{ with} \tag{18}$$

$$k_p = n \cdot \overline{\dot{ATP}}_{\Delta pH\,2.2} \cdot \exp(-2.3 \cdot 2.2b) = n \cdot k'_p \tag{19}$$

According to Eqn 10,

$$\overline{\dot{H}_b^+} = k_b \cdot \exp(2.3\,\Delta pH) \text{ with} \tag{20}$$

$$k_b = \overline{\dot{H}_b^+}_{\Delta pH\,2.2} \exp(-2.3 \cdot 2.2) \tag{21}$$

The total proton flux is:

$$\overline{\dot{H}^+} = \overline{\dot{H}_p^+} + \overline{\dot{H}_b^+} = k_p \exp(2.3b\,\Delta pH) + k_b \exp(2.3\,\Delta pH) \tag{22}$$

With Eqns 17, 18 and 20 it follows that,

$$\frac{\Delta H^+}{ATP} = n + \frac{k_b}{k'_p} \exp\{2.3\,\Delta pH(1-b)\} \tag{23}$$

k'_p and b depend on $\Delta\varphi$ (see definitions and Table I).

It must be reminded that these equations are valid in the range $1.8 \leqslant \Delta pH \leqslant 2.7$ and $50\ mV \leqslant \Delta\varphi \leqslant 125\ mV$. It is especially expected that at high ΔpH, i.e. at high rates of ATP formation the "turnover number" of the ATPase may be rate determining.

In Fig. 7B inset the calculated proton fluxes $\overline{\dot{H}_p^+}$ and $\overline{\dot{H}_b^+}$ and the total proton flux $\overline{\dot{H}^+}$ are depicted in dependence on ΔpH. At lower ΔpH it is $\overline{\dot{H}_p^+} < \overline{\dot{H}_b^+}$ and the rate of ATP formation should be low. At higher ΔpH it is $\bar{\dot{H}}_p^+ > \bar{\dot{H}}_b^+$ and the rate of ATP formation must be high. According to this result the range of inversion ($\overline{\dot{H}_p^+} \approx \overline{\dot{H}_b^+}$) is responsible for the "threshold" phenomena discussed above. The calculated total flux $\overline{\dot{H}^+}$ (Eqn 22) is compared with the experimental values in Fig. 5 (solid line).

The calculated $\Delta H^+/ATP$ (Eqn. 23) for $n = 2$ and 3 and 4 is depicted in Fig. 10. The dotted curves for $n = 2$ and 4 are parallel to the solid one for $n = 3$. There is reasonable agreement between the shape of the calculated curves and the experimental values. Two ways are possible to estimate the ratio valid for the ATPase pathway, i.e. $\Delta H_p^+/ATP \equiv n$.

1. With ΔpH 2.7 and $\Delta\varphi = 125$ mV the correction term in Eqn. 23 is $\Delta H^+/ATP - n = 0.6$ (calculated with data of Table I). Because the measured value at ΔpH 2.7 and $\Delta\varphi = 125$ mV is $\Delta H^+/ATP \approx 3$ (see Fig. 10) it follows

$$\Delta H_p^+/ATP \equiv n \approx 2.4$$

Because n is probably a whole number and regarding the possible margin of errors in the constants of Eqn. 23, it may be expected that the true value is $n = 2$.

2. On the other hand, according to Eqn. 17 or 23 $\Delta H^+/ATP$ should converge to n at high ΔpH and $\Delta\varphi$, i.e. at $\bar{\dot{H}}_p^+ \gg \bar{\dot{H}}_b^+$. Up to this point our investigations were carried out, however, only up to ΔpH 2.7 with rates of maximal $\overline{A\dot{T}P} = 3 \cdot 10^{-3}$ mol ATP/mol chlorophyll s (Fig. 2). The reason is that for the elaborated relationships the knowledge of ΔpH was necessary. Above ΔpH 2.7 the estimation of ΔpH from Eqn. 8 is, however, questionable. In order to measure n directly by $\Delta H^+/ATP$ one is interested, however, only in $\bar{\dot{H}}_p^+ \gg \bar{\dot{H}}_b^+$. This condition might be realized at 10 times higher rates of $\overline{A\dot{T}P} \approx 30 \cdot 10^{-3}$ mol ATP/mol chlorophyll s, adjusted e.g. by single turnover flashes with 100 Hz. This corresponds approximately to saturating continuous light conditions. Under these conditions $(\bar{\dot{H}}^+/\overline{A\dot{T}P})_{sat} = (\Delta H^+/ATP)_{sa} = (2.3), (3.8), (2.1), (2.1), (1.8), (2.4)$ with the mean value

$$(\Delta H^+/ATP)_{sat} \approx 2.4$$

This value is the same as above. This is possible if $(\Delta H^+/ATP)_{sat} \approx \Delta H^+{}_p/ATP \equiv n$, i.e. $(\bar{\dot{H}}_p^+ \gg \bar{\dot{H}}_b^+)_{sat}$.

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