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THE PLASTOQUINONE POOL AS POSSIBLE HYDROGEN PUMP IN PHOTOSYNTHESIS

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Summary

The function of the plastoquinone pool as a possible pump for vectorial hydrogen ($H^+ + e^-$) transport across the thylakoid membrane has been investigated in isolated spinach chloroplasts. Measurements of three different optical changes reflecting the redox reactions of the plastoquinone, the external H^+ uptake and the internal H^+ release led to the following conclusions:

(1) A stoichiometric coupling of 1 : 1 : 1 between the external H^+ uptake, the electron translocation through the plastoquinone pool and the internal H^+ release (corrected for H^+ release due to H_2O oxidation) is valid ($pH_{out} = 8$, excitation with repetitive flash groups). (2) The rate of electron release from the plastoquinone pool and the rate of proton release into the inner thylakoid space due to far-red illumination are identical over a range of a more than 10-fold variation.

These results support the assumption that the protons taken up by the reduced plastoquinone pool are translocated together with the electrons through the pool from the outside to the inside of the membrane. Therefore, the plastoquinone pool might act as a pump for a vectorial hydrogen ($H^+ + e^-$) transport. The molecular mechanism is discussed. The differences between this hydrogen pump of chloroplasts and the proton pump of Halobacteria are outlined.

Abbreviations: Chl a_I (*P*-700), reaction center of pigment System I; Chl a_{II} (*P*-680), reaction center of pigment System II.

Introduction

In the primary act of photosynthesis a transmembrane electric field is set up across the thylakoid membrane [1,2]. This field is caused by a charge separation at the reaction centers of System I and II, respectively. The separation takes place through photooxidation of Chl a_I and Chl a_{II}. Each of the two electrons released are shifted from the inside to the outside of the membrane. This occurs within 20 ns [3,4]. The result was first indication for the asymmetric arrangement of the components of the electron transport chain within the membrane (for details see Refs. 5 and 6). In subsequent reactions the protonation at the outer surface and deprotonation at the inner surface of the membrane lead to a net transfer for 1 H^+ from the outside to the inside at each system. This was experimentally established by measuring the H^+ uptake from the outer phase [7] and the H^+ release into the inner thylakoid space under single turnover conditions [8—10] (Fig. 1).

The inward H^+ translocation is followed by an outward H^+ efflux via the ATPase. In toto these movements of protons correspond to a H^+ circulation. However, this circuit is closed only if between System I and II a hydrogen (proton plus electron) is translocated from the outside to the inside (Fig. 1). The electron flow between both systems occurs via a plastoquinone pool, interconnecting at least ten electron transport chains [34], with a capacity of 5—7 electrons/chain [11]. Based on the protolytic properties of redox reactions in quinone systems plastoquinone was inferred to be a candidate for a hydrogen ($H^+ + e^-$) transport through the thylakoid membrane from the outside to the inside [12]. This means that the plastoquinone pool might operate as a pump for hydrogen. If this assumption is correct, then the reduction of the plastoquinone pool via Photosystem II is expected to be accompanied by a proton uptake from the outer aqueous phase with a 1 : 1 stoichiometry of H^+/e. On the

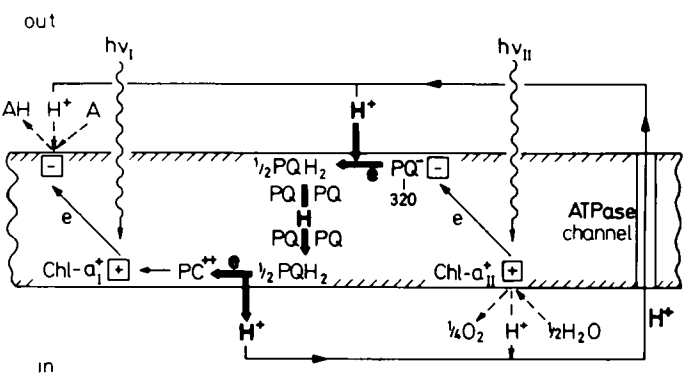

Fig. 1. Zigzag scheme of the vectorial pathways of electrons, protons and hydrogens derived from pulse spectroscopic studies. (1) Excitation of Chl a_I and Chl a_{II}. (2) Photooxidation of Chl a_I and Chl a_{II}. (3) Vectorial transfer of the electrons from the inside to the outside of the membrane. (4) Oxidation of H_2O, reduction and protonation of a terminal proton-binding electron acceptor A and reduction and oxidation of plastoquinone. (5) Proton translocation into the inner phase through protolytic reactions with the charges at the outer (PQ^-) and inner surface (plastocyanine, PC^{2+}) of the membrane. (6) Discharging of the energized membrane through efflux of protons. For details see text.

other hand, the reoxidation of the reduced plastoquinone pool by Photosystem I should give rise to a proton release into the inner phase also with a 1 : 1 stoichiometry of H^+/e.

A first attempt to clarify the role of the plastoquinone pool as a possible hydrogen pump was made by Reinwald et al. [13]. By the application of ms flashes of varying duration it was found that in the range of 1—7 electrons/transport chain there exists a 1 : 1 relationship between the number of electrons transferred into the plastoquinone pool and the number of protons taken up from the outer aqueous phase. These data indicate that the plastoquinone reduction is stoichiometrically coupled with a protonation from the outer side. However, the results do not provide any experimental evidence for the mode of coupling between the plastoquinone pool oxidation and the internal proton release. Hence, a study under comparable experimental conditions was made using the same chloroplast preparation for the proton uptake from the outside and the corresponding proton release into the inner thylakoid space as a function of the number of electrons transferred through the plastoquinone pool.

Preferably all experiments should be done under identical conditions but for principal and practical reasons this is not always possible, e.g. the external phase must be buffered in pH_{in} measurements whereas no external buffer is allowed in pH_{out} measurements. In such cases it has been checked by control experiments or by the application of data from the literature that the modifications of the assay conditions have had no influence on the results (see remarks in Materials and Methods).

All measurements have been carried out at pH_{out} 8. The following results are obtained:

1. There exists a 1 : 1 stoichiometric coupling between the external proton uptake and the electrons taken up by the plastoquinone pool as well as a 1 : 1 coupling between the internal proton release (corrected for the release due to the oxidation of water) and the electron release from the plastoquinone pool. This is valid in the range from zero up to seven redox equivalents/chain.

2. The rate of plastoquinone pool oxidation and the rate of the internal H^+ release due to far-red illumination are identical. This correlation is valid in a range where the rates have been changed more than 10-fold.

3. The results support the assumption that the plastoquinone pool very probably functions as a hydrogen pump. The molecular mechanism is discussed.

4. The principle difference between the mechanism of this hydrogen pump ($H^+ + e^-$) in photosynthesis and that of the proton pump (H^+) of bacteriorhodopsin in Halobacteria is outlined.

Materials and Methods

Spinach chloroplasts were prepared according to the method of Winget et al. [14] except that 10 mM ascorbate was present during the grinding of the leaves. For chloroplast storage in liquid nitrogen 5% Me_2SO was added. The oxygen-evolving capacity of the stored chloroplasts was practically the same as that of freshly prepared chloroplasts.

Excitation conditions. Photosynthesis was excited with repetitive flash groups each containing 1 up to 40 short ($\tau_{1/2} \approx 20$ μs) flashes of saturating intensity. The time between the flashes of a group was 2 ms. The flash light was passed through red filters Schott RG 610. Far-red background illumination, (interference filter IL 722) was applied in order to assure the complete reoxidation of the plastoquinone pool during the time between the flash groups. The effective intensity of the far-red light (and of the actinic light) was independent of the kind of the measured event as the chlorophyll concentrations have been adjusted to give the same absorbance.

The intensity of this light was held constant for stoichiometric measurements (850 μW/cm^2) and was varied for kinetic measurements (60—5000 μW/cm^2). The excitation conditions are shown in Fig. 2. Two different types of far-red background illumination were applied: (A) during the flash excitation and the registration of the signals the far-red light was switched off (top scheme), referred to as ($-h\nu_I$)-excitation; (B) the far-red light remained switched on continuously (bottom scheme), designated as ($+h\nu_I$)-excitation.

Registration. The absorption changes were measured with a repetitive flash photometer with double-measuring light beams as described earlier [15]. In order to obtain a maximal signal/noise ratio, the time resolution of the apparatus was limited up to the ms range because the kinetics of the events which have to be analysed in the present study are not faster than a few ms. Furthermore, to improve the signal to noise ratio, 4–1024 signals were averaged, depending on the time resolution and the type of the signal.

Electron uptake and release at the plastoquinone pool

Assay conditions. The electrons taken up by the oxidized plastoquinone pool and the electrons released from the reduced plastoquinone pool have been measured via the absorption increase and decrease at 265 nm as described in Ref. 11. The intensity of the measuring light beam was <20 μW · cm^{-2} (optical bandwidth $\Delta\lambda$ = 5 nm). Optical pathlength 2 cm. The repetition rate of the flash groups was 0.3 Hz for more than one flash/group and 0.04 Hz for the kinetic measurements. The far-red background illumination was switched off during the measurement of the signals (Fig. 2). Measurements with single turnover flashes were performed at a repetition rate of 4 Hz and in the absence of far-red background illumination. In toto, up to 1024 signals were averaged. The reaction medium contained: 10 mM KCl, 5 mM $MgCl_2$, 20 mM Tricine/NaOH

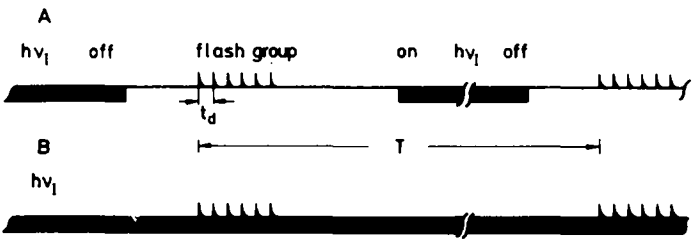

Fig. 2. Scheme of the excitation conditions. The darktime, t_d, between the flashes of a flashgroup was 2 ms and the repetition time, T, of the flashgroup as indicated in Materials and Methods. For details see text.

(pH 8), 1 mM NH_4Cl, 10 μM benzylviologen and chloroplasts (chlorophyll concentration: 12.7 μM).

Numerical evaluation. In order to be able to draw unequivocal conclusions first of all an assignment of the absorption changes is required. Generally, the interpretation of the absorption changes in the ultraviolet region at 265 nm is complex [16] because they reflect at least three plastoquinone components (the primary acceptor plastoquinone molecule X-320, the connector plastoquinone molecule B [17,18] and the plastoquinone pool) and 2 chlorophyll *a* components (Chl a_I and Chl a_{II}). No separation was made for the contribution due to the connector plastoquinone pool molecules and the plastoquinone molecules of the pool because in the present study only the total amount of electrons and protons transferred via these molecules is of interest. Furthermore, the contribution due to Chl a_{II} and X-320 can be neglected because their turnover kinetics are fast in comparison to the ms time resolution of the measuring device and, therefore, remain undetected.

A more complicated pattern arises for the absorption change due to the turnover of Chl a_I because the slow phase (20 ms) of the reduction of Chl a_I^+ corresponds with the kinetics of the plastoquinone pool oxidation.

Therefore, an appropriate correction is required. If n_{PQ}^{total} is the extent of reduction of the plastoquinone pool (number of electrons/transport chain stored in the plastoquinone pool) and $n_{Chl\,a_I^+}$ the number of the Chl a_I^+ molecules/chain which are reduced with the kinetics of the plastoquinone pool oxidation (20 ms), then one obtains

$$\Delta A_{PQ} = \frac{n_{PQ}^{total} \cdot \Delta\epsilon_{PQ}}{n_{PQ}^{total} \cdot \Delta\epsilon_{PQ} + n_{Chl\,a_I^+} \cdot \Delta\epsilon_{Chl\,a_I^+}} \cdot \Delta A_{265} \qquad (1)$$

where $\Delta\epsilon_{PQ}$ and $\Delta\epsilon_{Chl\ a_I^+}$ are differences of the extinction coefficients for the couples PQ/PQ_{red} and Chl a_I^+/Chl a_I, respectively, at 265 nm. Eqn. 1 tacitly implies that maximally one Chl a_I^+/electron transport chain can be formed. Eqn. 1 shows that the largest correction is required for single turnover flashes *, where $n_{PQ}^{total} = 1$ and with $n_{Chl\,a_I^+} = 1$. Taking the $\Delta\epsilon$ values from literature ($\Delta\epsilon_{PQ} = 1.8 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [19] and $\Delta\epsilon_{Chl\,a_I^+} = 6.4 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ [20]), then one obtains $\Delta A_{PQ} = 0.58\ \Delta A_{265}$. However, the absorption change at 265 nm in single turnover flashes has been measured in the absence of far-red background illumination at a repetitive rate of 4 Hz. Under these conditions $n_{Chl\,a_I^+}$ is about 0.3 [21] and according to Eqn. 1 $\Delta A_{PQ} = 0.80\ ^1\Delta A_{265}$. Therefore, the amplitude measured in single turnover flashes is corrected by 20%.

In repetitive groups containing more than three flashes/group and with far-red illumination (Fig. 2) the contribution due to Chl a_I reduction becomes negligible (less than 10%) compared to the accuracy of the measurements. Therefore, the amplitude at 265 nm can be directly used as a measure of plastoquinone reduction, i.e.

$$n_{PQ}^{total} = \frac{\Delta A_{265}}{0.8\ ^1\Delta A_{265}} \qquad (2)$$

* This is also valid for non-saturating short flashes because the saturating curves under conditions, where $n_{Chl\,a_I^+} = 1$ (far-red preillumination) are congruent for both, plastohydroquinone and Chl a_I.

Fig. 3 shows absorption changes at 265 nm induced by a flash group of 40 flashes. In accordance with earlier results maximal seven electrons/chain are taken up by the pool (n_{PQ}^{total}). After the flashing a fast reoxidation is observed (n'_{PQ} = three electrons/chain). This is due to the oxidation by System I donors (Chl a_I, plastocyanine, cytochrome *f*). By far-red illumination which only excites System I (this was checked by oxygen and external proton measurements with and without far-red light) the pool becomes completely oxidized (n_{PQ}) [41].

The uncoupler NH_4Cl was present in the reaction medium in order to eliminate scattering effects, especially at longer flashgroups. But since the reoxidation kinetics are slow in comparison with the reduction kinetics even in the presence of uncouplers [11], the degree of pool reduction attained in a flash group remains practically invariant to the addition of 1 mM NH_4Cl. For the same reason the extent of plastoquinone pool reduction was found to be independent of the electron acceptors used (either benzylviologen or $K_3Fe(CN)_6$ [41]. Therefore, for comparison with the extent of proton uptake or release different acceptors can be chosen. However, as the reoxidation kinetics of the pool is influenced by $K_3Fe(CN)_6$ [41] for the investigation of kinetic effects (e.g. rate of plastoquinone pool reoxidation and internal proton release) benzylviologen has been applied as electron acceptor.

The extent of plastoquinone pool reduction (oxidation) and the oxidation kinetics are practically independent of the internal pH, pH_{in}, between pH_{in} 8 and pH_{in} 6.2 ([35,43], and Siggel, U., personal communication)). Accordingly, the plastoquinone measurements in the presence of NH_4Cl can be compared with the experiments in the absence of an uncoupler.

External proton uptake, ΔH^+_{out}

Assay conditions. The pH change in the outer aqueous phase was measured by the absorption changes of the pH indicator dye cresol red [10]. The

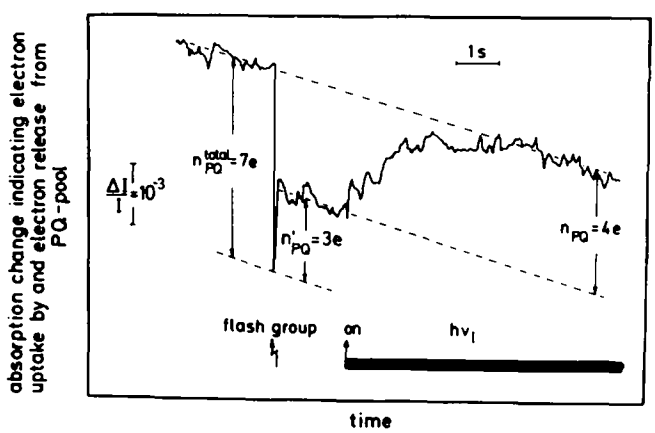

Fig. 3. Absorption change at 265 nm indicating electron uptake by and electron release from plastoquinone pool. Complete reduction of the pool, n_{PQ}^{total} is achieved by a flashgroup of 40 flashes. A fast reoxidation (n'_{PQ} = 3 *e*) is observed due to the System I donors. A further slow reoxidation of the pool, n_{PQ}, is observed by far-red light illumination (intensity 1400 μW/cm^2). Number of averaged flashgroups 32. For details see Materials and Methods.

intensity of the measuring light was $<10\ \mu W \cdot cm^{-2}$ ($\lambda = 571$ nm, $\Delta\lambda = 5$ nm). Optical pathlength 2 cm. In order to eliminate background absorption changes at $\lambda = 571$ nm, which do not indicate pH changes, the signals measured in a strongly buffered solution have been subtracted from the absorption changes obtained in an unbuffered solution.

Repetition rate of the flashgroups was 0.04 Hz. The half-life of the relaxation of the pH change in the outer phase (see Fig. 4, top) was about 3 s (pH 8). Therefore, after 25 s the system has completely decayed in respect of proton uptake and release.

It is known that there exist transient states in chloroplasts. In order to compare plastoquinone signals which are obtained after many excitations ($\approx$200) with signals, reflecting proton uptake or release, obtained by averaging of 4–32 signals the first flashgroups were not sampled. However, this was merely done in order to have comparable conditions. In fact, there is practically no difference between the first and the following flashgroups.

The reaction medium, adjusted to pH 8 by NaOH, contained 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM $K_3Fe(CN)_6$, 12 μM cresol red and chloroplasts giving a chlorophyll concentration of 12.7 μM. Tricine or bovine serum albumin with a concentration of 20 mM or 1.3 mg/ml, respectively, were added for the difference measurements.

Numerical evaluation. As can be seen from Fig. 4 (top) the proton uptake

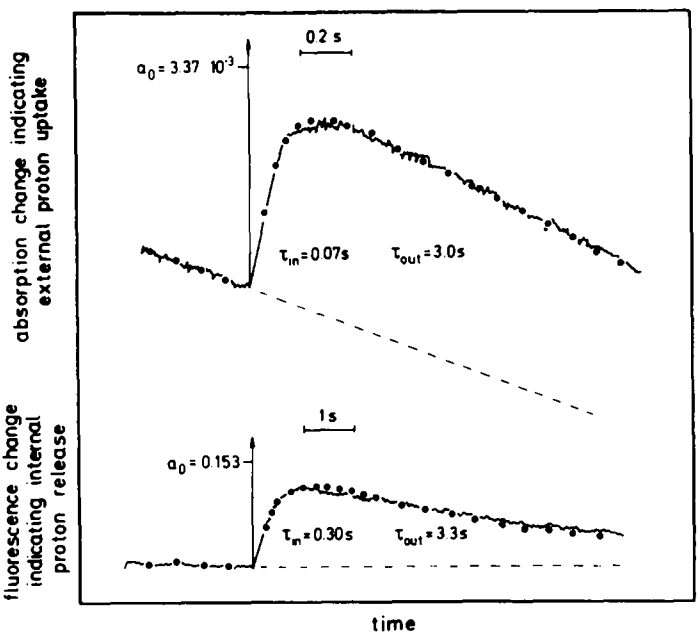

Fig. 4. (Top) Absorption change of cresol red at 571 nm indicating external proton uptake. (Bottom) Fluorescence change of 9-aminoacridine at 454 nm indicating internal proton release. Upward deflection indicates a fluorescence decrease. Excitation condition A as shown in Fig. 2. The circles are calculated according to Eqn. 5 with the indicated half-life times. The initial amplitudes corrected for the proton efflux are indicated by a_0. Excitation by a flashgroup of 40 flashes; number of averaged flashgroups 32. For details see Materials and Methods.

(half-rise time 70 ms) is followed by a slow proton efflux (half-decay time about 3 s). For a quantitative correlation between the degree of plastoquinone reduction and the external proton uptake, the signals obtained with cresol red have to be corrected for the contribution due to proton efflux. For the simplicity we assume the kinetics of proton influx and efflux to be of first order. As the efflux rate of protons is slow in comparison to the influx rate, its rate constant can be calculated directly from the amplitude $a(t_1)$ and $a(t_2)$ measured at times t_1 and t_2, where the influx does no longer interfere with the efflux:

$$\tau_{out} = \frac{(t_1 - t_2) \cdot \ln 2}{\ln\left(\frac{a(t_1)}{a(t_2)}\right)} \tag{3}$$

The half-rise time, τ_{in}, has been calculated from two amplitudes, $a(t_3)$, $a(t_4)$ at short times by applying a Newton-Raphson iteration procedure, i.e. solving the equation

$$F(\tau_{in}/\tau_{out}) = \frac{\exp(-t_3 \cdot \ln 2/\tau_{in}) - \exp(-t_3 \cdot \ln 2/\tau_{out})}{\exp(-t_4 \cdot \ln 2/\tau_{in}) - \exp(-t_4 \cdot \ln 2/\tau_{out})} - \frac{a(t_3)}{a(t_4)} \equiv 0 \tag{4}$$

The curve and the initial amplitude, a_0, have been calculated according to

$$a(t) = a_0 \frac{\tau_{out}}{\tau_{in} - \tau_{out}} [\exp(-t \cdot \ln 2/\tau_{in}) - \exp(-t \cdot \ln 2/\tau_{out})] \tag{5}$$

The correspondence between measured and calculated time courses (see Fig. 4) justifies the applied correction procedure which leads to the initial amplitude, a_0. For the determination of the absolute number of protons taken up, the absorption changes of cresol red have been calibrated by addition of a known amount of HCl.

Fig. 4 (top) shows the time course of the pH-indicating absorption change of cresol red in a flashgroup of 40 flashes. The points are calculated according to Eqn. 5 with a half-rise time τ_{in} = 70 ms and a half-decay time τ_{out} = 3.0 s. The calculation gives the initial amplitude a_0 corrected for the proton efflux. The amplitudes corrected by the method described above have been used for further calculations and conclusions. The initial amplitude a_0 of this signal is independent of far-red background light (checked up to 850 μW/cm^2).

In the presence of $K_3Fe(CN)_6$ as electron acceptor one proton/electron chain is taken up from the outer phase. This proton uptake was shown to be caused by plastohydroquinone formation, probably at the level of the connector plastoquinone molecule B [22]. Accordingly, an oscillatory pattern arises for the proton uptake [22] as well as for the internal proton release. However, under the repetitive flash excitation applied in the present study the detection of these oscillatory effects is prevented by the averaging procedure.

The proton uptake in the presence of ferricyanide can be correlated directly (a) with the plastoquinone reduction (oxidation) as in the presence of ferricyanide or benzylviologen the degree of plastoquinone pool reduction (oxidation) is the same, and (b) with the internal proton release in the presence of benzylviologen because in accordance with Saphon and Crofts [38] and

Ausländer and Junge [39] we did not find a dependence of the internal proton release on the change of the electron acceptor from benzylviologen to ferricyanide as published by Fowler and Kok [45].

Internal proton release, ΔH^+_{in}

Assay conditions. The proton release into the internal thylakoid space was investigated by two methods: (a) The indicator dye neutral red was applied in the range from 0—6 protons/electron chain. (b) Above this value the fluorescence quenching of 9-aminoacridine was used for indication. The pH_{in}-indicating absorption changes of neutral red were measured as described by Ausländer and Junge [9,39], i.e. the difference signal between bovine serum albumin-buffered and a imidazole-buffered solution was measured. The light intensity was $<10\ \mu W \cdot cm^{-2}$ ($\lambda = 524$, $\Delta\lambda = 10$ nm). Optical pathlength 2 cm. Repetition rate was 0.04 Hz. The fluorescence of 9-aminoacridine was excited at 367 nm, intensity $<20\ \mu W \cdot cm^{-2}$, $\Delta\lambda = 10$ nm, and measured at an angle of 90° at 454 nm through a Balzer K 2-cut-off filter. Repetition rate 0.04 Hz. Optical pathlength was 1.5 mm.

The half-lifes of the relaxation of the 9-aminoacridine signal (see Fig. 4, bottom) and of the neutral red signal (not shown) after one flash group were about 4 s. Therefore, after 25 s the system has completely equilibrated with regard to proton uptake and release. For the same reasons as described for the external proton uptake after five excitations the data storage device was started and up to 32 signals were averaged. However, also in this case no difference between the first and the following flashgroups has been observed. The reaction medium for the neutral red measurement contained 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM benzylviologen, 10 μM neutral red, 0.5 μM valinomycin, 1.3 mg/ml bovine serum albumin (pH 8) and chloroplasts giving a chlorophyll concentration of 10 μM. For the difference measurement 5 mM imidazol (pH 8) was added. The ionophore valinomycin was added in order to eliminate, by accelerated decay, the overlapping absorption change at 524 due to electrochromism. Neither the amplitude nor the kinetics of proton uptake and release in single turnover flashes is influenced by valinomycin.

For the 9-aminoacridine measurement the reaction medium contained: 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM benzylviologen, 4 μM 9-aminoacridine, either 20 mM Tricine or 1.3 mg/ml bovine serum albumin (pH 8) and chloroplasts giving a chlorophyll concentration of 0.13 mM.

Numerical evaluation. The flash-induced pH changes in the internal space up to six H^+/electron chain have been determined by comparison of the empirical and calculated neutral red sensitivity curves (see Ref. 39). The number of protons released at the inside, ΔH^+_{in}, has been calculated in the following way: lowing way:

$$\Delta H^+_{in} = \beta_{in} \cdot \Delta pH_{in} \cdot V_{in} \qquad (6)$$

β_{in}, internal buffer capacity; V_{in}, internal volume, and ΔpH_{in}, change of pH inside. The internal volume of the chloroplast preparation has been determined by the method of Reinwald [40]. It results 50 l/Mol Chl. β_{in} has been determined by adding a known amount of permeating buffer (e.g. imidazol) (as described in Ref. 9). With and without imidazol the same number of pro-

tons are released, therefore the change of ΔpH directly reflects the change of the buffer capacity. As the change of ΔpH_{in} is small under these conditions ($\Delta pH \leqslant 0.2$), the absorption change is proportional to ΔpH, i.e.

$$\Delta H^+_{in} = \beta_{in} \cdot \Delta pH_1 \cdot V_{in} = (\beta_{in} + \beta_{IA})\, \Delta pH_2\, V_{in}$$

$$\beta_{in} = \beta_{IA} \frac{\Delta pH_2}{\Delta pH_1 - \Delta pH_2} = \beta_{IA} \frac{\Delta A_2}{\Delta A_1 - \Delta A_2} \tag{7}$$

Assuming that the imidazol concentration is the same inside and outside, β_{in} was found to be $3 \cdot 10^{-4}$ M and this value was nearly constant in the range up to six H^+ released/chain in accordance with Ausländer [27].

Latest data of Junge et al. [49] led to the conclusion that by the application of imidazol the internal buffer capacity β_{in} becomes underestimated. Therefore, according to these results our absolute values of ΔpH_{in} would be overestimated. However, as the stoichiometry of the H^+/e-ratio in single turnover flashes is independent of the absolute value of ΔpH_{in} and as the internal buffer capacity was confirmed to remain constant in the range of $6.5 \leqslant pH_{in} \leqslant 8$, the stoichiometry of our measurements described in Fig. 6 is not influenced by the above mentioned new β_{in} estimation in Ref. 49.

Above six H^+/electron chain the flash-induced fluorescence quenching of 9-aminoacridine has been used to measure the change of pH_{in} according to Eqn. 8:

$$\Delta pH_{in} = \log \frac{H^+_{in}}{H^+_{out}} = \log\left[\frac{1}{1-Q} + \frac{Q}{(1-Q)} \frac{V_{out}}{V_{in}}\right] \tag{8}$$

Q quenched fluorescence/initial fluorescence, V_{out} volume outside. Comparison of Eqn. 8 with that used by Schuldiner et al. [26] shows that both equations differ in the first term on the right hand side which accounts for the amount of the amine already inside under dark conditions. This term is negligible at high values of Q, i.e. at high $\Delta pH_{in}(V_{out}/V_{in} >> 1)$. For small values of Q it is necessary to take this term into account.

If more than three electrons are injected into the plastoquinone pool the specific reaction pattern of plastoquinone pool oxidation can be used to measure the number of protons released coupled with plastoquinone oxidation by a procedure simultaneously eliminating the buffer capacity and the internal volume. The plastoquinone oxidation is the rate-limiting step of the overall non-cyclic electron transport from water to the terminal acceptor [11]. The capacity of the donor side of System I, which acts as the electron acceptor of the pool (Chl a_1, plastocyanine, the role of cytochrome f is not yet clarified) is three electrons/chain [11]. Accordingly, after switching off the flash group maximally three electrons/chain are removed from the pool during the darktime before the next flashgroup. Therefore, the number of electrons trapped in the pool is $n_{PQ} = n_{PQ}^{total} - 3$ for $n_{PQ}^{total} \geqslant 3$, where n_{PQ}^{total} is the total number of electrons/chain stored in the plastoquinone pool during the flashing.

This effect is observed under $(-h\nu_1)$-excitation (Fig. 2, top). In order to remove the remaining n_{PQ} electrons from the plastoquinone pool far-red background light $(+h\nu_1)$ was applied (Fig. 2, bottom, and Fig. 3). This light excites only Photosystem I, thereby keeping the acceptors of the pool in the oxidized

state. Hence, the proton release, ΔH^+_{in}, stoichiometrically coupled with the removal of the excess electrons from the pool by far-red light, is given by the difference of proton release observed for $(+h\nu_I)$- and $(-h\nu_I)$-excitation, respectively. One obtains

$$\Delta H^+_{in} = \Delta H^+_{in}(+h\nu_I) - \Delta H^+_{in}(-h\nu_I) = n_{PQ} \cdot (H^+/e)_{PQ} \tag{9}$$

$(H^+/e)_{PQ}$ is the stoichiometric coefficient for the internal proton release due to the oxidation of the plastoquinone pool. It is reasonable to assume that the product $\beta_{in} V_{in}$ is approximately invariant to far-red light illumination because the number of electrons n_{PQ} trapped in the plastoquinone pool is limited to maximal 4, i.e. the change of pH_{in} due to this proton release is small (see Results). Taking into account this assumption, we obtain

$$\Delta H^+_{in} = n_{PQ} \cdot (H^+/e)_{PQ} = (\beta_{in} V_{in})[\Delta pH_{in}(+h\nu_I) - \Delta pH_{in}(-h\nu_I)] \tag{10}$$

where $\Delta pH_{in}(+h\nu_I)$, $\Delta pH_{in}(-h\nu_I)$ are obtained under excitation conditions B and A, respectively (Fig. 2). If one furthermore takes into account the overall stoichiometric coefficient for the proton release due to water oxidation which is 1 (see Results, for subtleties of the proton release pattern see Refs. 22, 28, 38), then the product $(\beta_{in} V_{in})$ is obtained by

$$\Delta H^+_{in}(+h\nu_I) = [1 + (H^+/e)_{PQ}]\, n_e = (\beta_{in} V_{in})\, \Delta pH_{in}(+h\nu_I) \tag{11}$$

where n_e = total number of electrons transferred/flashgroup from water into the plastoquinone pool. The combination of Eqns. 10 and 11 provides the stoichiometric coefficient by elimination of $(\beta_{in} V_{in})$. Finally, the number of protons released into the inner phase due to the abstraction of the excess electrons n_{PQ} from the plastoquinone pool is given by:

$$\Delta H^+_{in} = n_{PQ}(H^+/e)_{PQ} =$$

$$= n_{PQ} : \frac{n_e[\Delta pH_{in}(+h\nu_I) - \Delta pH_{in}(-h\nu_I)]}{n_{PQ}\Delta pH_{in}(+h\nu_I) - n_e[\Delta pH_{in}(+h\nu_I) - \Delta pH_{in}(-h\nu_I)]} \tag{12}$$

The stoichiometric coupling of internal proton release due to the plastoquinone pool oxidation by the acceptor pool (Chl a_I, plastocyanine, cytochrome *f*?) was measured directly for $n_{PQ}^{total} \leqslant 3$ as described below. For $n_{PQ}^{total} < 3$ the stoichiometric coefficient $(H^+/e)_{PQ}$ was found to be 1 (see Results). Therefore, it is reasonable to assume that also for $n_{PQ}^{total} > 3$ the three electrons captured by the oxidized acceptor pool are coupled with the release of three H^+. Then one obtaines the total amount of internal proton release for $n_{PQ}^{total} > 3$:

$$\Delta H^+_{in}(PQ) = \Delta H^+_{in} + 3H^+ \tag{13}$$

The initial amplitudes of the neutral red and the 9-aminoacridine signals were corrected for the proton efflux by the same method as described for the ΔH^+_{out} measurements.

Fig. 4 (bottom) shows the time course of the pH_{in}-indicating fluorescence quenching of 9-aminoacridine in a flashgroup of 40 flashes. (Note that upward deflexion indicates fluorescence decrease.) The points are calculated by the same procedure as described for proton uptake using Eqn. 5 with a half-rise time τ_{in} = 0.30 s and a half-decay time τ_{out} = 3.3 s. The calculation leads to

the initial amplitude, a_0, which is corrected for the proton efflux. These corrected amplitudes have been used for further calculations.

The reliability of the absolute determination of the proton release inside depends on the reliability of the pH_{in} indicators used. Up to now there exists no unequivocal method for pH_{in} measurements. Both methods applied here have some advantages and some disadvantages. Neutral red is enriched at the inside of the membrane and responds very rapidly to pH_{in} changes. However, at prolonged illumination where larger ΔpH_{in} values occur, additional neutral red diffuses to the inside of the thylakoids, thus rising the absorption [39,47]. Therefore, only in short flash groups (low ΔpH_{in}) neutral red has been used as a quantitative indicator [9,27,39,49].

Considerable differences exist in literature concerning the 9-aminoacridine technique [24–26]. Under suitable conditions the method can be used in quantitative way as discussed elsewhere [23,48]. However, at low ΔpH the 9-aminoacridine response shows marked non-linearities which increase with decreasing ΔpH. This has been shown by comparison of the 9-aminoacridine technique with the imidazol technique [31,42]. By comparing the 9-aminoacridine measurements with neutral red measurements in the range $0.2 < \Delta pH < 1.2$ no coincidence of the absolute values is found and a non-linearity of the 9-aminoacridine response is observed. Therefore, at $\Delta pH < 1.0$ the 9-aminoacridine technique should not be used for quantitative measurements. At medium ΔpH both methods give about the same ΔpH. If we apply for example a flashgroup which translocates seven electrons through the e-chain the neutral red measurements gives a ΔpH of 1.4, the 9-aminoacridine measurement a ΔpH of 1.5 (V_{in} = 50 l/Mol Chl). The numerical value of the internal volume can be adjusted so that both methods would give the same ΔpH, since it is not needed for the neutral red measurements. However, this has not been done because, as mentioned above, in this range also the neutral red method has some disadvantages. In the following we have calculated the ΔpH with the internal volume measured directly and we assume that under our experimental conditions 9-aminoacridine indicates the true ΔpH in the range $1.1 \leqslant \Delta pH \leqslant 1.8$.

The results concerning the kinetic correlation between plastoquinone oxidation and proton release inside are independent of the absolute calibration of the 9-aminoacridine signal. At low fluorescence quenching ($Q \ll 1$, $Q(V_{out})/(V_{in}) < 1$) it results from Eqn. 6 and Eqn. 8:

$$\Delta H_{in}^{+} \sim \Delta pH_{in} \approx \log\left(1 + Q\,\frac{V_{out}}{V_{in}}\right) \approx Q\,\frac{V_{out}}{V_{in}} \tag{14}$$

i.e. the kinetics of proton release is proportional to the kinetics of the fluorescence quenching. Only small changes of ΔpH are observed under these conditions and a possible correction factor would be constant [25]. For the interpretation of these measurements it is sufficient to assume that the fluorescence quenching is proportional to the proton release inside.

Oxygen evolution

For the absolute calibration of the number of electrons transferred through the transport chain oxygen measurements were performed with a repetitive flash polarographic device as is described in Ref. 29. In order to avoid any

interference with the deactivation reactions of the watersplitting enzyme system, the repetition rate was 0.4 Hz for flashgroups containing less than five flashes, which is known to be sufficiently high [30].

At flashgroups of more than five flashes the effect of the deactivation becomes negligible. Accordingly the repetition rate was decreased to 0.1 and 0.04 Hz. In a control experiment under this conditions only a slight dependence of the oxygen yield on the addition of NH_4Cl was found. Far-red background illumination of 850 $\mu W/cm^2$ had no influence on the oxygen evolution, thus, indicating that excitation of Photosystem II is negligible. The repetition rates are also sufficiently low to assure a complete reoxidation of the plastoquinone pool between the flashgroups because $K_3Fe(CN)_6$ has been applied as terminal electron acceptor [41]. Reaction medium: chloroplasts (50 μM chlorophyll), 10 mM KCl, 5 mM $MgCl_2$, 300 μM $K_3Fe(CN)_6$, 20 mM Tricine/NaOH, pH 8. Addition in the control experiments: 1 mM NH_4Cl. Optical pathlength, 0.4 cm.

The number of chlorophylls/e-chain was determined to Z = 700 Chl/e-chain for the used chloroplast preparation.

Results

Stoichiometrics of external proton uptake and plastoquinone reduction

For the evaluation of the stoichiometric coupling of electron and proton flux through the plastoquinone pool the following effect has to be taken into consideration. Illumination with flash groups (or continuous light) gives rise to different levels for the extent of plastoquinone reduction, net proton transport and electron transport (O_2 evolution), as is schematically shown in the inset of Fig. 5 (top). The extent of the plastoquinone pool reduction reaches a steady-state level in illumination times of less than 100 ms, whereas the extent of net H^+ transport reaches the steady-state level in a few seconds, depending on the proton permeability. The extent of O_2 molecules evolved proceeds, however, all the time. For the problem to be solved in the present study illumination periods are required sufficiently long to assure a maximal degree of pool reduction. This range is indicated by the hatched area in the inset scheme in Fig. 5 (top) corresponding to flash groups containing 40 flashes with a dark time of 2 ms. The measurements obtained in this time range are shown in Fig. 5 (top, curves A and B). The extent of reduction of the plastoquinone pool, n_{PQ}^{total}, is calculated as described in Materials and Methods. The influence of proton efflux on the extent of external H^+ uptake is rather small (see Fig. 4, top) and has been corrected as described in Materials and Methods. The experimental data for electron transport (O_2 production) and H^+ uptake, ΔH_{out}^+, normalized to the values for single turnover flashes, coincide (Fig. 5, top, curve A), i.e. with $K_3Fe(CN)_6$ as terminal electron acceptor one proton is taken up/electron transferred through the chain. The difference between curves A and B is explained by the following effect. Curve A is due to the electrons which are staying within the plastoquinone pool plus those electrons which have already passed the pool during the flashgroup. Accordingly, for a quantitative discussion of the relations of electron and proton uptake by the plastoquinone pool we have to subtract from curve A the contributions which are due to the electrons which have already passed the pool during the flashgroup. The result is

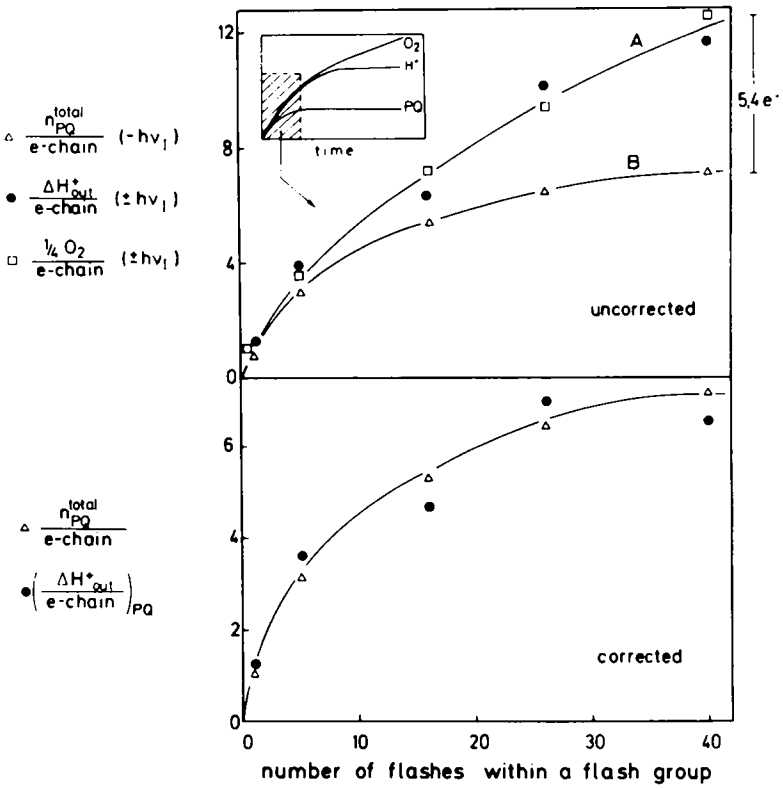

Fig. 5. (Top) Extent of the plastoquinone reduction (n_{PQ}^{total}), external proton uptake (ΔH_{out}^{+}) and oxygen evolution (1/4 O_2)/e-chain as a function of the number of flashes per group. The values are normalized to the corresponding quantities in single turnover flashes. (Absolute values: $\Delta H_{out}^{+} = 1.3 \cdot 10^{-3}$ H^+/Chl; 1/4 $O_2 = 1.4 \cdot 10^{-3}$ e^-/Chl.) For details see Materials and Methods. Inset, schematic time course of oxygen evolution, proton transport and plastoquinone reduction at longer illumination times. (Bottom) Extent of plastoquinone reduction compared with the external proton uptake corrected for those protons which have passed the plastoquinone pool in the period of the flash illumination. The correction procedure is described in the text.

shown in Fig. 5, bottom. The corrected data of curve A (H^+ uptake, ΔH_{out}^{+}) are in good agreement with the data of curve B (plastoquinone pool reduction, n_{PQ}^{total}). The correction has been achieved in the following way:

In the range above 26 flashes/group, the degree of pool reduction is constant and attains the maximal level. Here, the electron flow through the plastoquinone pool is approximately given by the average oxygen yield/flash which amounts to 0.15—0.2 of the yield in single turnover flashes. Accordingly, between the 26th and 40th flash about 2.1—2.8 electrons are transferred through the pool. In the range where the reduction of the pool is below the constant and maximal level of plastoquinone reduction, the number of electrons transferred through the pool is drastically reduced. This is the case because the reoxidation rate of the plastoquinone pool is strongly dependent on its degree of reduction [11]. With data of Stiehl and Witt [11] one obtains the following values. During a group with five flashes (t_d = 2 ms) about 0.4 electrons are transferred through the plastoquinone pool and between the 5th and 16th flash about 1.3 electrons and between the 16th and 26th flash about

1.4 electrons. Summarizing all these values one obtains e.g. after 40 flashes that about 5.4 electrons have passed the pool (Fig. 5, top). Using the 1 : 1 relation between electron transport (measured by oxygen evolution) and proton uptake, the number of protons stored in the plastoquinone pool has been calculated (depicted in Fig. 5, bottom). The data of Fig. 5, bottom, are plotted in a different way in Fig. 6 (left side). It is found that in the whole range from zero up to seven electrons accepted by the plastoquinone pool a corresponding number of protons is taken up in accordance with earlier results [13].

Stoichiometrics of internal proton release and plastoquinone oxidation

In the range up to six H^+/e-chain the internal proton release was measured by the pH_{in}-indicating absorption change of neutral red. The results are shown in Table I. The number of electrons/e-chain, n_e, was determined from the oxygen evolution and the number of H^+/chlorophyll was calculated according to Eqn. 6 using $\beta_{in} = 3 \cdot 10^{-4}$ Mol/l and $V_{in} = 50$ l/Mol Chl. For the evaluation of the number of protons internally released due to plastoquinone pool oxidation by System I, ΔH^+_{in} (PQ), the contribution caused by water oxidation has to be subtracted from the overall extent of internal acidification. It has been inferred that under repetitive flash excitation, assuring steady-state activation of the watersplitting enzyme system, one H^+ is released into the inner phase/electron transferred through System II [22]. Taking into account the value of one H^+_{in} $(H_2O)/e$, then the ΔH^+_{in} (PQ) data depicted in the last column of Table I are obtained.

Above three electrons/chain the 9-aminoacridine technique was used for measuring the internal proton release (see Materials and Methods). The results are shown in Table II.

As can be seen from Table II the maximal change of pH_{in} due to far-red illumination is 0.36. We assume that in this range the internal buffer capacity and the internal volume do not change significantly. However, the dependence of the buffer capacity on the different pH_{in} values created in the various flash

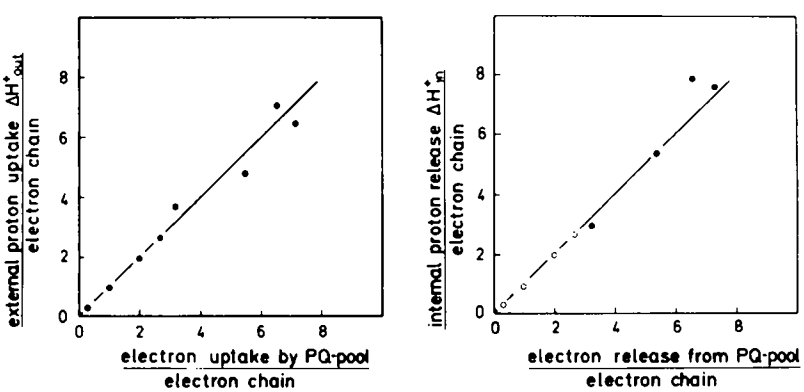

Fig. 6. (Left) External proton uptake, ΔH^+_{out}, as a function of electron uptake by the plastoquinone pool. For details see Materials and Methods. (Right) Internal proton release, ΔH^+_{in}, as a function of electron release from the plastoquinone pool. The data determined with a known internal buffer capacity β_{in} and volume V_{in} are depicted as open circles (see Table I). The range where $\beta_{in}V_{in}$ has been eliminated is indicated by full circles (see Table II). For details see Materials and Methods.

TABLE I

INTERNAL PROTON RELEASE MEASURED BY THE pH-INDICATING ABSORPTION CHANGE OF NEUTRAL RED.

For details see Materials and Methods and text.

n_e (e/e-chain)	Δ pH_{in}	Δ H_{in} (H^+/chlorophyll) · 10^3	Δ H^+_{in} (PQ) (H^+/e-chain)
0.2	0.04	0.6	0.2
1	0.20	3.1	1.1
2	0.38	5.7	2
2.6	0.51	7.7	2.7

groups has no influence on the results. Furthermore, by this method the proton release due to electrons which have already passed the pool is eliminated because we observe with and without far-red light the same number of electrons and protons taken up.

As is shown in Fig. 3, the extent of plastoquinone reduction and oxidation, respectively, can be measured simultaneously by the absorption change at 265 nm. In Fig. 6 (right) the internal proton release summarized in Tables I and II is plotted versus the amount of electrons released from the plastoquinone pool. These data provide for the first time evidence for a 1 : 1 stoichiometric coupling between the number of protons released into the inner phase and the number of electrons released from the plastoquinone pool over its whole capacity from zero up to seven electrons/chain. Furthermore, the external proton uptake due to plastoquinone pool reduction (Fig. 6, left) corresponds to the internal proton release due to plastoquinone pool oxidation. This supports the assumption that each proton taken up from the outer aqueous phase by electron injection into the plastoquinone pool is translocated into the inner phase by electron release from the plastoquinone pool.

Kinetics of plastoquinone reoxidation and internal proton release

An independent approach to obtain the correlation between plastoquinone oxidation and internal proton release provides the investigation of the kinetics of both events. A kinetical coincidence is expected if the H^+ release occurs simultaneously with the electron release from the reduced pool. As shown in Fig. 3 at a reduction degree of the pool exceeding more than three electrons, the reoxidation kinetics in the dark was found to be biphasic: (a) A fast phase, characterized by a half-life time of up to 20 ms[11] and a maximal amplitude corresponding to three electrons/chain, is caused by the electron transfer from the pool to the electron acceptors of the pool which have already been oxidized by the flash light; (b) a slower phase induced by far-red background illumination with a kinetics strongly dependent on the light intensity. The extent of the slow phase corresponds to the excess of electrons, n_{PQ} (Fig. 3) remaining stored within the pool under $-h\nu_I$-excitation. As the internal proton release is found to be coupled with the oxidation of the plastoquinone pool and of water, the kinetics of the H^+ release should be triphasic: two phases corresponding to the above-mentioned kinetical pattern of plastoquinone pool oxidation and one phase due to water oxidation. However, the 9-aminoacridine technique does not allow a time resolution of kinetics in the ms range because

TABLE II

INTERNAL PROTON RELEASE MEASURED BY THE FLUORESCENCE QUENCHING OF 9-AMINOACRIDINE

For details see Materials and Methods and text.

Number of flashes/group	n_e (e/e-chain)	n_{PQ} (e/e-chain)	Q ($+h\nu_1$)	Q ($-h\nu_1$)	ΔpH_{in} ($+h\nu_1$)	Δ pH ($-h\nu_1$)	ΔH^+_{in} (PQ) (H^+/e-chain)	n_{PQ}^{total} (e/e-chain)
5	3.5	0.1	0.071	0.055	1.11	1.01	2.9	3.1
16	7.1	2.4	0.161	0.092	1.50	1.23	5.7	5.4
26	9.5	3.4	0.246	0.121	1.72	1.36	7.8	6.4
40	12.5	4.1	0.268	0.153	1.77	1.47	7.4	7.1

9-aminoacridine responds rather slowly to the transmembrane pH gradient (Fig. 4, bottom). Therefore, the kinetics of the proton release coupled with the fast plastoquinone pool oxidation (20 ms) as well as of water oxidation ($\lesssim$1 ms) is not resolved. Accordingly, the investigation of the kinetic coincidence between the plastoquinone pool oxidation and internal proton release has been restricted to the slow phase which can be separated by a difference measurement in the absence and presence of far-red light, respectively. In Fig. 7 the flash-induced internal pH change for $-h\nu_I$-excitation is represented by curve A, only reflecting the proton release due to the fast phase of the plastoquinone pool oxidation and to the water oxidation. Curve B shows the signal for $+h\nu_I$-excitation reflecting the complete proton release. Therefore, the difference (B — A) represents the proton release due to the slow phase of plastoquinone pool oxidation by far-red light only. The rate of this process is expected to coincide with the rate of the reoxidation of the plastoquinone pool.

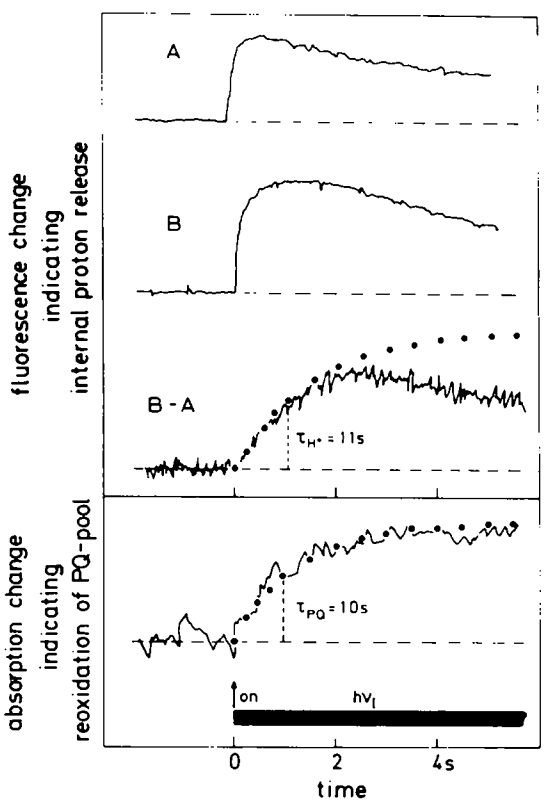

Fig. 7. Fluorescence change of 9-aminoacridine (A, B, B — A) at 454 nm indicating internal proton release. A, excitation conditions A as shown in Fig. 2, top ($-h\nu_I$); B, Excitation conditions B as shown in Fig. 2, bottom ($+h\nu_I$); B — A, difference between B and A indicating internal proton release only due to far-red light. (Bottom) Absorption change at 265 nm indicating reoxidation of the plastoquinone pool due to far-red light. The points are calculated as described in the text using the half-life times indicated in the figure. Exctitation by a flashgroup of 40 flashes, number of averaged flashgroups 32, intensity of far-red light 580 $\mu W/cm^2$. For further details see Materials and Methods.

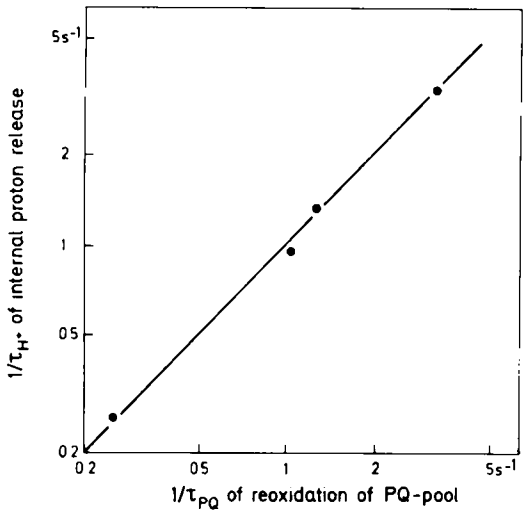

Fig. 8. Reciprocal half-life time $1/\tau_{H^+}$ of internal proton release as a function of $1/\tau_{PQ}$ of the reoxidation of the plastoquinone pool. For details see text.

The time course of the H^+_{in} release (corrected for the H^+ efflux according to Eqns. 4 and 5) and the obtained τ_{H^+} (corresponding to τ_{in}) are indicated by points. In Fig. 7 (bottom) the reoxidation kinetics of the plastoquinone pool at the same intensity of far-red light is shown. The time course was approximated by a first order reaction. The half-life τ_{PQ} of the plastoquinone reoxidation is in good agreement with the half-life, τ_{H^+}, of the proton release. As shown earlier [41] the rate constant of the reoxidation of the plastoquinone pool ($1/\tau_{PQ}$) can be varied by changing the light intensity of far-red light. Fig. 8 shows the coincidence of the rate constants of the reoxidation of the plastoquinone pool ($1/\tau_{PQ}$) and of the internal H^+ release ($1/\tau_{H^+}$). The rate constants vary more than 10-fold changing the far-red light intensity between 60 and 5000 $\mu W/cm^2$.

The applied intensities affected neither the extent of plastoquinone pool reduction (oxidation) nor led to biphasic kinetics. This can be expected since the turnovers of System II excited by $h\nu_1$ illumination are less than 10% of the turnovers of System I [44]. At light intensities above 5000 $\mu W/cm^2$ this coincidence is not longer valid because the 9-aminoacridine signals attain a constant rise kinetics whereas the rate of plastoquinone pool oxidation still further increases. This might be due to a kinetic limitation by the slow 9-aminoacridine transport across the membrane (see Fig. 4, bottom).

The data of Fig. 8 support the suggestion that the reoxidation of the plastoquinone pool is directly coupled to the release of H^+ into the inner space of the thylakoid.

Discussion

We have shown that there exists 1 : 1 stoichiometric coupling (a) between the external H^+ uptake, ΔH^+_{out}, and the electron uptake by the plastoquinone pool (Fig. 6, left) and (b) between the internal H^+ release, ΔH^+_{in} (PQ), and the

electron release from the plastoquinone pool (Fig. 6, right). This stoichiometry holds in the whole range of $n = 0—7$ electrons.

With respect to the kinetics it was additionally shown that the rate of the internal H^+ release is identical with the rate of the electron release from plastoquinone pool (Fig. 7). This relation is valid even if the rate is changed more than 10-fold by corresponding intensities of far-red light (Fig. 8). Recently, it has been discussed that instead of $H^+/e = 1$ with respect to the proton transport through the plastoquinone pool a ratio of $H^+/e = 2$ could arise due to a possible participation of *b*-type cytochromes according to a so-called Q-cycle mechanism proposed by Mitchell [32] or by a double loop of plastoquinone similar to the twin UQ loop in photosynthetic bacteria discussed by Crofts et al. [33]. However, under our experimental conditions a Q-cycle does not operate because the ratio $(H^+/e)_{PQ} = 1$. Furthermore, a Q-cycle should include a relative slow vectorial electron transfer involving cytochromes. This transfer should be 'visible' as a further but slow field-indicating absorption change in addition to the fast phase of $\leqslant 20$ ns. Such a slow phase has also not been observed under our conditions (isolated chloroplasts, pH_{out} 8).

At experimental conditions different from that of this study a deviation from the 1 : 1 : 1 stoichiometry may occur. Under continuous light excitation (i.e. steady-state conditions) a variation of the H^+/e ratio was found depending on the intensity of the exciting light and the proton concentration of the sample [45,46]. Nevertheless, also in such cases the transfer of the additional protons and electrons occurs via the plastoquinone pool, i.e. the plastoquinone pool always acts as a hydrogen pump.

The ability of the plastoquinone pool to interconnect at least ten electron transport chains [34] suggests the existence of clusters or strands of plastoquinone. Accordingly, two possibilities can be considered for the mechanism of the hydrogen pumping through the plastoquinone pool: (a) the diffusion-type mechanism and (b) the hopping-type mechanism. In the diffusion-type mechanism each individual molecule (oxidized or reduced) of the plastoquinone pool is assumed to be able to move statistically between the inside and the outside of the membrane. The hopping mechanism occurs by a statistical hopping of an electron and proton from one plastoquinone to another. If $H(H^+ + e)$ reaches, either via mechanism (a) or via (b), a site next to the oxidized System I donors, the electron from (H) is captured at this place while the remaining proton is released into the inner phase. The random migration ultimately leads to the vectorial transport of $(H^+ + e^-)$ from the outside to the inside because (a) the electron acceptors acting as trap of the electron from the couple $(H^+ + e^-)$ are localized at the membrane inside and (b) the H^+ concomitantly deliberated has a much higher affinity for a hydrophilic phase than for the hydrophobic part of the membrane lipids. It should be mentioned that the internal H^+ release into the inner water phase occurs very likely via protonizable head groups of lipids acting as buffer groups at the inner surface of the membrane [35].

For both models one has to assume that due to the long hydrophobic side chain of plastoquinone, the plastoquinone molecules are soluble within the lipids of the thylakoid membrane. This is supported by the observation that in artificial bilayers of lipids plastoquinones are indeed soluble [36].

The results presented in this work are consistent with the assumption that plastoquinone is a pump for a vectorial hydrogen transport across the thylakoid membrane. No additional molecules are required to explain this mechanism. It is of interest to compare the mechanism of the hydrogen ($H^+ + e^-$) pump via the plastoquinone pool in chloroplasts with that of the light-driven proton pump realized by bacteriorhodopsin in the membrane of Halobacteria (for reviews see Ref. 37). Both pumps differ in their basic functional mechanisms. The plastoquinone pump translocates a neutral atom ($H^+ + e^-$) in the dark, the rhodopsin pump, however, a charged atom (H^+) in the light. The plastoquinone pump acts mechanistically selective because the energy-requiring steps take place in preceding processes at the reaction centers (a) reduction of plastoquinone at the outside is caused by the electrons provided by photooxidation of Chl a_{II} and (b) oxidation of plastohydroquinone at the inside occurs ultimately via photooxidized Chl a_I^+. This means that the overall process of H^+ pumping is subdivided into two sequences: In a vectorial electron transfer from inside to outside at the chlorophyll containing reaction centers and the vectorial H transfer from outside to inside via the plastoquinone pool.

On the other hand, the rhodopsin pump translocates a proton, H^+. Therefore, this translocation must be coupled with the energy requiring act, i.e. with generation of an electric field and ΔpH formation. As a pump of this type has to perform all elementary steps within one highly specialized protein, its reaction cycle is expected to be rather complex. It is, therefore, not surprising that such a pump includes a photoreaction, conformation changes and a sequence of as yet not defined H^+ transfer reaction within the enzyme [37].

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