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ELECTRON TRANSPORT CONTROL IN CHLOROPLASTS

EFFECTS OF PHOTOSYNTHETIC CONTROL MONITORED BY THE INTRATHYLAKOID pH

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(1) In isolated chloroplasts (class B) electron flow is controlled mainly by the intrathylakoid pH (pH_{in}). A decrease in pH_{in} due to the light-driven injection of protons inside the thylakoid leads to the retardation of electron flow between two photosystems. This effect can be abolished by uncouplers or under photophosphorylation conditions (addition of Mg^{2+}-ADP with P_i); Mg^{2+}-ATP does not influence the steady-state rate of electron flow, (2) The steady-state pH difference, ΔpH, across the thylakoid membrane was estimated from quantitative analysis of the rate of $P\text{-}700^+$ reduction. In chloroplasts, without adding Mg^{2+}-ADP, ΔpH increases from 1.6 to 3.2 as the external pH rises from 6 to 9.5. Under the photophosphorylation conditions, ΔpH decreases showing a minimum at the external pH 7.5 ($\Delta pH \simeq 0.5–1.0$). (3) The value of photosynthetic control, K, measured as the ratio of the steady-state rates of $P\text{-}700^+$ reduction in the presence of Mg^{2+}-ADP (with P_i) and without adding Mg^{2+}-ADP is dependent on external pH variations, showing a maximum value of $K \simeq 3.5$ at pH_{out} 7.5. This pH dependence coincides with that of the ADP-stimulated ΔpH decrease. (4) Experiments with spin labels provide evidence that the light-induced changes in the thylakoid membrane are sensitive to the addition of uncouplers and are affected only slightly by the addition of Mg^{2+}-ADP and P_i.

Introduction

It is well known [1–14] that in isolated chloroplasts the turnover of plastoquinone is the rate-limiting step in electron flow; the rate of plastoquinol oxidation by PS I is monitored mainly by intrathylakoid pH_{in} [2–5]. Diffusion of plastoquinol across the thylakoid membrane does not limit the rate of plastoquinone turnover (for details see Refs. 13 and 14). The proteolytic action of plastoquinol oxidation by PS I at the internal side of the thylakoid membrane is the rate-limiting step of electron flow between two photosystems [1]. Therefore, one of the reasons for electron-flow retardation may be the back pressure of protons released into the thylakoid in the courses of plastoquinol oxidation by PS I and splitting of water by PS II. According to Ref. 3 the decrease in electron-flow rate by a factor 2–3 demands in uncoupled chloroplasts ($pH_{in} \simeq pH_{out}$) a decrease in the pH value by 2–3 units. Light-induced energization of the thylakoid membrane without adding substrates of

Abbreviations: PS I and PS II, Photosystems I and II; *P*-700 and *P*-680, reaction centres of PS I and PS II; X320, bound plastoquinone, primary electron acceptor of PS II; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, dicyclohexylcarbodiimide; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CAT_{12}, 4-dodecyldimethylammonium 1-oxyl-2,2,6,6-tetramethylpiperidine bromide; $I_{1,14}$, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl.

phosphorylation is known [2–12] to lead to the retardation of electron flow, which can be abolished by the addition of ADP and P_i [9–12]. Recently, Kobayashi et al. [8] suggested that in intact chloroplasts the phosphorylation potential is not involved in the regulation of electron transport. In a previous paper [9] we have put forward the notion that the stimulation of electron flow by the initiation of photophosphorylation, i.e., the effect of photosynthetic control, might be accounted for by the decrease in the electron-flow retardation due to the decrease in transmembrane pH difference under photophosphorylation conditions.

Rumberg and Siggel [3] and Pick et al. [15] have shown that addition of ADP and P_i to coupled chloroplasts leads to a rather small decrease in ΔpH (from 3.0 to 2.6 under external pH 8.0 [3]). In principle this might be considered as evidence in support of the idea of Mitchell [47] that the proton electrochemical gradient acts as the driving force of photophosphorylation. However, this rather small decrease in ΔpH (by 0.4 pH units) cannot explain the phenomenon of photosynthetic control if we assume that in coupled chloroplasts the only factor of photosynthetic control is the acidification of the intrathylakoid space (as shown for uncoupled chloroplasts [3–5]).

The primary goal of the present paper is the reinvestigation of this problem. In the course of our study we have obtained some new data concerning the role of the transmembrane pH difference in photophosphorylation.

Methods

Preparation of chloroplasts. Chloroplasts were prepared from greenhouse bean leaves (*Vicia faba*) as previously described [16]. Chloroplasts were suspended in a medium containing 0.2 M sucrose, 2 mM $MgCl_2$ and 10 mM Tricine-NaOH buffer (pH between 6.5 and 9.5) or Mes-HCl buffer (pH between 4.5 and 6.5). For kinetic measurements at different pH values we also used a suspending medium which contained 0.2 M sucrose, 2 mM $MgCl_2$ and 10 mM phosphate-citrate buffer. Taking into account that the pH value of the suspending medium may be changed due to chloroplast buffering groups we have specially checked the final pH of chloroplast suspension with a Radiometer glass electrode GK2321C. Chloroplasts were suspended at a final concentration of 2–3 mg chlorophyll/ml. Chlorophyll content was measured according to the method of Arnon [17]. The charged spin-labeled detergent CAT_{12} and spin-labeled stearic acid, $I_{1,14}$, were used for testing of the light-induced changes in the membrane surface. CAT_{12} was synthesized by Dr. R.J. Mehlhorn; $I_{1,14}$ was produced by Syva. The final concentration of spin labels in chloroplast suspensions was 10^{-4} M.

ATP formation was measured by the enzymatic [18] and modified luciferin-luciferase [19] methods in collaboration with Dr. S.P. Kuprin and Dr. A.V. Pichugin. All controls including that of adenylate kinase activity were properly made.

Kinetic measurements. The redox state changes of *P*-700 were measured by means of the EPR signal I intensity [20]. The magnetic field was fixed on the low-field peak of signal I from $P\text{-}700^+$. The EPR measurements were made with a Varian E-4 spectrometer at 4 G modulation amplitude and 10 mW microwave power at room temperature. Assays were carried out using different time constants: 0.1 s for the *P*-700 slow changes and 1 ms for the fast changes. To improve the signal-to-noise ratio for the fast kinetic measurements, 4–16 signals were averaged, as a rule, with a Trackor Nothern NS-570 computer to average transients. The EPR spectra of spin labels were recorded at 0.5 G modulation amplitude, 0.1 s time constant and 10 mW microwave power at room temperature.

Excitation conditions. Far-red background illumination (interference filter SIF 707, Karl Zeiss Jena; λ_{max} = 707 nm, $\Delta\lambda_{1/2}$ = 5 nm) was applied in order to ensure reoxidation of the electron-transport chain between two photosystems. The intensity of this light, provided by a 150 W incandescent lamp equipped with a water filter and focusing lens, was adjusted to reach the *P*-700 maximal oxidation level. A similar light source without the interference filter was used for effective excitation of both PS I and PS II. Two kinds of flash fired simultaneously with the background far-red light were used. Short flashes ($\tau_{1/2}$ = 7 μs) of saturating intensity were obtained from the pulse lamp described earlier [14]. Long flashes ($\tau_{1/2}$ = 750 μs) were specially used for multiple excitation of *P*-680 by a single flash. The energy released in the discharge circuit was 10 and 100 J, respectively. Continuous white light was cut off by a special shut-

ter made from a commercial mousetrap. The actuation time of the shutter was less than 1 ms. The intensity of light was varied by neutral glass filters.

Results

The kinetics of the light-induced redox changes of P-700

Fig. 1 shows typical patterns of the kinetics of the light-induced redox changes of *P*-700 in a bean leaf (curve a) and isolated chloroplasts (curves b–d). A piece (3 × 20 mm) of bean leaf or isolated bean chloroplasts were illuminated by continuous saturating far-red light (707 nm) after preincubation in the dark for 3 min. The flashes of white light ($\tau_{1/2}$ = 7 or 750 μs) were given simultaneously with far-red light after the maximal yield of *P*-700$^+$ was reached. It follows from Fig. 1 that the level of the reduction of *P*-700$^+$

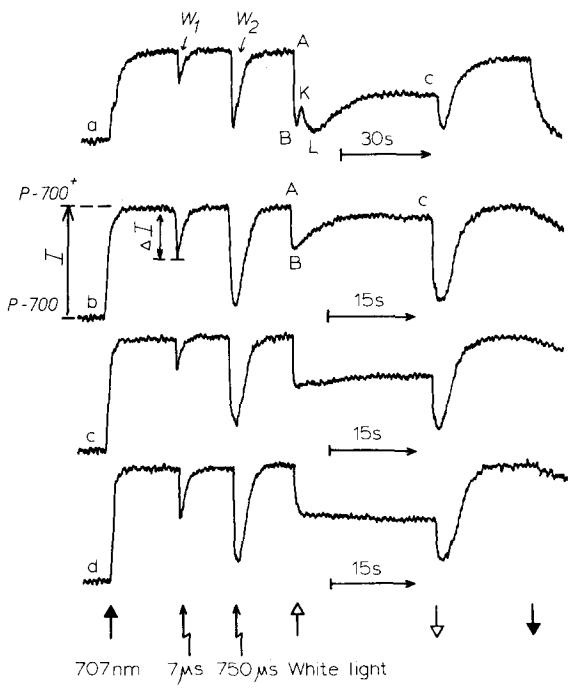

Fig. 1. The time course of light-induced *P*-700$^+$ EPR signal changes in bean leaf (a) and isolated chloroplasts (b, c and d) suspended in Tricine-NaOH buffer (pH 7.5). The straight arrows indicate the moments of switching on (upward) or switching off (downward) continuous light; zig-zag arrows indicate the flashes of various duration. Additions: (b) 20 μM methyl viologen; (c) 20 μM methyl viologen and 10 μM FCCP, (d) 20 μM methyl viologen and 3 μM gramicidin A.

in response to a single flash depends on the pulse duration. It is well known that after one saturating short flash, each reaction centre of PS II donates only a single electron to the electron-transport chain. It is remarkable that in our case only 50% of *P*-700$^+$ is reduced in response to one saturating short flash. This means that in the case of a short flash, only half of the electrons donated from PS II reach the active centre of PS I (*P*-700$^+$). The explanation of this fact can be found in Ref. 14. During the long flash ($\tau_{1/2}$ = 750 μs) more than one electron may be donated to the electron-transport chain from one *P*-680. In this case we observed a complete reduction of *P*-700$^+$. The number (Q) of electron equivalents localized in an electron-transport chain between two photosystems may be evaluated on the basis of the so-called 'work integral' [16,21]. It has been shown before [16] that $Q \approx W \cdot I$, where W represents the area over the kinetic curve of *P*-700 reoxidation by saturating continuous far-red light with intensity I (for details see Refs. 16 and 21). Switching on the continuous white light which effectively excites both PS I and PS II leads to a decrease in the *P*-700$^+$ steady-state concentration due to the electron flow from PS II to *P*-700$^+$. It is well known [16,22–28] that the kinetics of this process may be rather complex. We have found [22–28] that dark-adapted leaves of different species exhibit one or two overshoots. This time-dependent change of *P*-700$^+$ signal intensity in response to a rather strong light exciting both photosystems (Fig. 1, curve a) can be described using the notation introduced in Ref. 24. On illumination of a dark-adapted (or far-red light adapted) leaf by white light, the concentration of *P*-700$^+$ initially formed by far-red light decreases rapidly (phase A-B). This is followed by a relatively slow rise, the rate of which is dependent on the light intensity, to level C showing in many cases a second overshoot, K-L-C. These kinetics have features in common with the slow fluorescence yield changes [29]. Our special study [24–28] had shown that the second wave, K-L-C, appears only after dark or far-red light adaptation of a leaf and depends on the intensity of the actinic light. In an N_2 atmosphere, i.e., without CO_2 and O_2, the overshoot K-L-C is not observed and the steady-state concentration of *P*-700$^+$ is smaller due to the retardation of electron flow in the acceptor side of PS I [28].

In all our experiments described here we have used

methyl viologen (20 μM) as a mediator of electron transfer from PS I to O_2 (a terminal electron acceptor which was present in excess). In this case, the electron transfer to the acceptor side of PS I could not be the rate-limiting step in the chain of pseudocyclic electron transport, and therefore we can be sure that the point of electron-flow control was localized between two photosystems [16]. Under these conditions, the isolated fresh chloroplasts (type B) reveal only one overshoot, A-B-C. Addition of uncouplers (FCCP, gramicidin, NH_4Cl) diminishes the overshoot in isolated chloroplasts (Fig. 1).

Control of electron flow between two photosystems by the intrathylakoid pH_{in}

The second phase, B-C, in the kinetics of the *P*-700 redox changes in isolated chloroplasts may be accounted for by the decrease in the rate of electron flow from PS II to *P*-700$^+$. There is a good correlation between the overshoot A-B-C and the effect of electron-flow retardation [9].

Fig. 2 shows the dependence of the half-time $\tau_{1/2}$ of *P*-700$^+$ reduction, after cutting off the white light, on its duration. Sudden cessation of the white light stops immediately the *P*-700 oxidation, except for the negligible oxidation rate supported by the background far-red light, while electron flow from the reduced plastoquinol pool to *P*-700$^+$ continues until the reservoir between PS II and PS I is exhausted. $\tau_{1/2}$ is the half-time of *P*-700$^+$ reduction during this process. It follows from the top curve in Fig. 2 that during the illumination of the chloroplasts, $\tau_{1/2}$ increases by a factor of 3.5. In the presence of gramicidin (lower curve) this effect does not occur. These results are in accordance with the data obtained by Rumberg and Siggel [3]. The effect of the retardation of electron transport was explained by the inhibitory effect of the light-driven decrease in the internal pH_{in} value on the rate of plastoquinol oxidation by PS I.

The light-induced hindering of electron flow between two photosystems is intimately allied to the photosynthetic control in chloroplasts. It follows from Fig. 3 that in the presence of the phosphoryla-

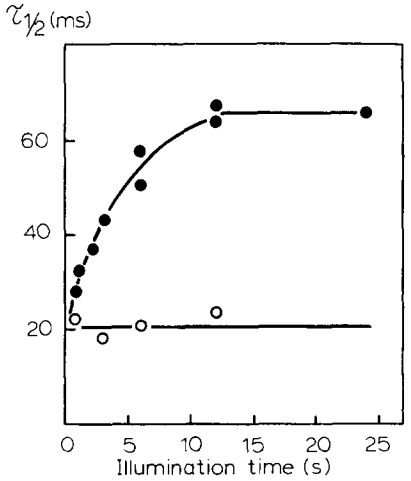

Fig. 2. Half-time ($\tau_{1/2}$) of *P*-700$^+$ reduction in bean chloroplasts, after switching off the white light, as a function of the illumination time without (•) and with (○) gramicidin A (3 μM). Chloroplasts were suspended in Tricine buffer (pH 7.5) in the presence of 20 μM methyl viologen.

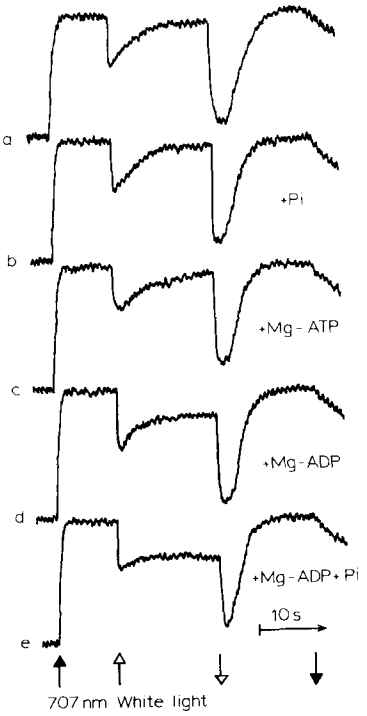

Fig. 3. The time course of *P*-700$^+$ EPR signal changes in bean chloroplasts suspended in Tricine buffer (pH 7.5). Illumination conditions were the same as indicated in Fig. 1. Additions: (a) 20 μM methyl viologen; (b) 20 μM methyl viologen, 10 mM P_i and 10 mM $MgCl_2$; (c) 20 μM methyl viologen, 2 mM ATP and 4 mM $MgCl_2$; (d) 20 μM methyl viologen, 4 mM $MgCl_2$ and 2 mM ADP; (e) 20 μM methyl viologen, 2 mM ADP, 4 mM P_i and 6 mM $MgCl_2$.

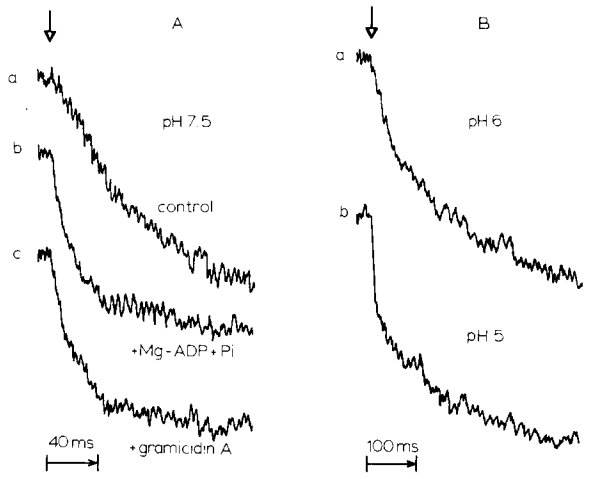

Fig. 4. Decay kinetics of P-700$^+$ EPR signal in bean chloroplasts after switching off the white light; illumination times 12 s. (A) Phosphate buffer, pH 7.5: (a) 20 μM methyl viologen; (b) 20 μM methyl viologen, 2 mM ADP, 4 mM P_i and 6 mM $MgCl_2$; (c) 20 μM methyl viologen and 3 μM gramicidin A. (B) Phosphate buffer, pH 6 (a) and pH 5 (b), in the presence of 20 μM methyl viologen.

tion substrates (Mg^{2+}-ADP and P_i), the overshoot in the kinetic curves of P-700 redox changes does not take place. The effect of Mg^{2+}-ADP and P_i is similar to that of the uncouplers (Fig. 4A). Addition of P_i or Mg^{2+}-ADP separately (Fig. 3, curves b and d) does not influence the kinetics of the light-induced changes of P-700. Mg^{2+}-ADP cannot be replaced by Mg^{2+}-ATP (Fig. 3, curve c). The observed insignificant effect of Mg^{2+}-ADP without added P_i may be accounted for by the presence of a small amount of endogenous P_i. It is remarkable that the effect of Mg^{2+}-ADP and P_i was depressed (not shown) by DCCD, an inhibitor of photophosphorylation. All these data (Figs. 2–4) are some of the clearest pieces of evidence that under phosphorylation conditions the retardation effect caused by the light-induced decrease in pH_{in} is cancelled out not just by the presence of ADP or P_i or ATP but due to initiation of the photophosphorylation process.

Effects of pH on the reactions at the acceptor side of PS II

The kinetic study of flash-induced P-700 redox changes gives some information about the photochemical activity of PS II at different pH values. The functioning of the PS II acceptor side involves a rather peculiar 'two-electron switch' or intermediary electron acceptor R located between the primary acceptor Q and the plastoquinone pool [30–32]. This two-electron switch may reveal itself by the mode of the dark-adapted chloroplasts' response to short-flash illumination [14,30–32].

The extent of the decrease in P-700$^+$ concentration in response to the short flash (parameter α) corresponds to the relative number of electrons injected into the electron-transport chain by PS II. Fig. 5 shows that PS II most effectively donates electrons to P-700$^+$ in the external pH range 5–7.5. In this pH interval the turnover rate of PS II as electron donor for P-700$^+$ depends only slightly on pH variations. This follows from the fact that the ratio $f = W_2/W_1$ increases slightly with the pH rise from 5 to 8.5. The 'work integrals' W_2 and W_1 were measured from the kinetics of P-700 reoxidation by far-red light after long and short flashes (Fig. 1). The ratio W_2/W_1 gives information about the rate of electron transfer from X320 to the pool of unbound plastoquinone. This reaction is known to be the rate-limiting step ($\tau_{1/2} \simeq$ 0.6 ms) at the acceptor side of PS II [1,2]. The extent of intersystem electron flow may be limited by the supply of electrons to the plastoquinone pool by the PS II secondary acceptor [30]. The effects of

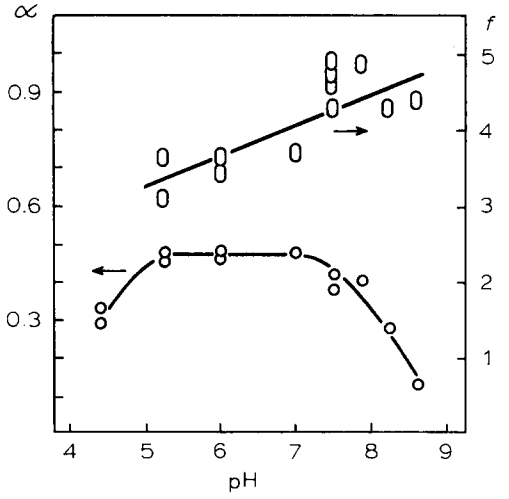

Fig. 5. Effects of the external pH on PS II activity. For details see text and Fig. 1; $\alpha = \Delta I/I, f = W_2/W_1$.

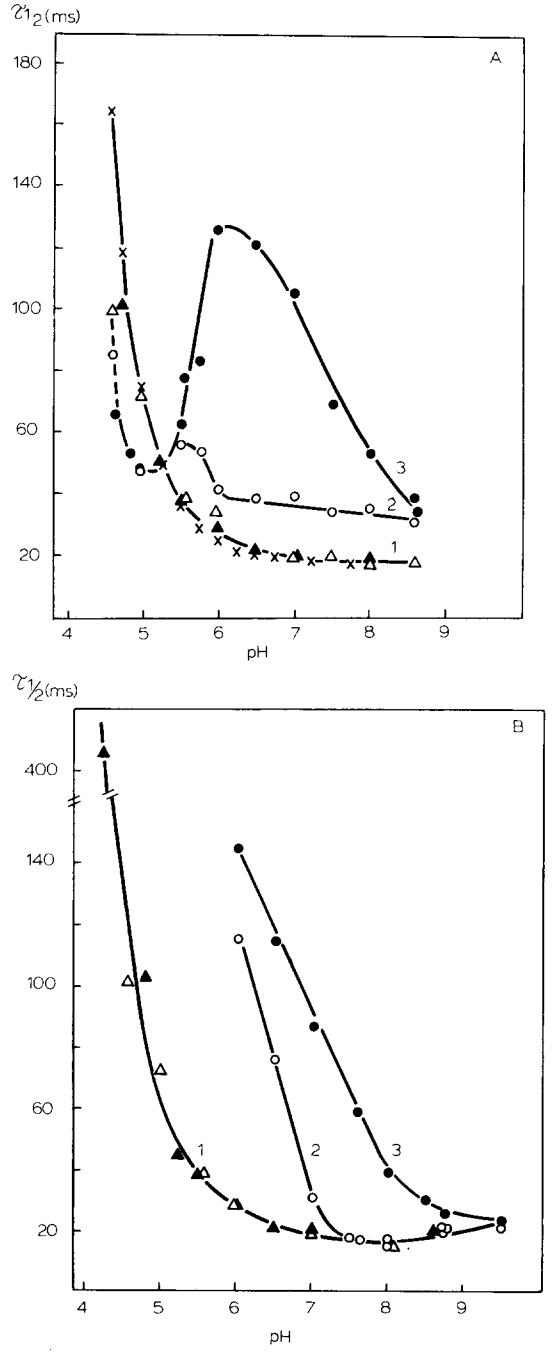

Fig. 6. Effects of the external pH on the half-time ($\tau_{1/2}$) of P-700$^+$ reduction after switching off white light. (A) Chloroplasts suspended in 10 mM Tricine or Mes buffer, (B) chloroplasts suspended in 10 mM phosphate-citrate buffer. Illumination conditions and additions: (A) curve 1: (▲———▲) 20 μM methyl viologen, chloroplasts illuminated by a long flash ($\tau_{1/2}$ = 750 μs); (△———△) 20 μM methyl viologen and 3 μM gramicidin A, illumination 12 s; (X———X) theoretical curve calculated for input parameters pK = 5.5 and $\tau_{1/2}$ = 18 ms at pH 8; curve 2: (○———○) 20 μM methyl viologen, illumination 2 s; curve 3: (●———●) 20 μM methyl viologen, illumination 12 s. (B) Curve 1 as indicated for A; curve 2: (○———○) 20 μM methyl viologen, 2 mM $MgCl_2$ and 2 mM ADP, illumination 12 s; curve 3: 20 μM methyl viologen, illumination 12 s.

the intersystem proton release and electron-flow binary oscillations in flash experiments observed under certain conditions [30–32] were not essential in our experiments (for details see Ref. 14). Furthermore, the presence or absence of two-electron switch effects cannot change the number of electrons accepted by PS II primary acceptors in response to a single flash. It is clear that in this case the number of electrons donated into the electron-transport chain in response to a single long flash, measured from the ratio W_2/W_1, depends on the rate of oxidation of the anion-radical X320$^-$. The fact that parameter f increases rather than decreases with the pH rise is in good agreement with the data of Haehnel [13]. This may be considered as evidence to confirm the conclusion that X320 reduction is not followed by protonation of the anion-radical X320$^-$ [13,33].

Effects of pH on the P-700$^+$ reduction rate

Fig. 6 shows plots of half-times ($\tau_{1/2}$) of the decay kinetics of P-700$^+$ at different external pH values. Parameter $\tau_{1/2}$ was measured from the kinetics of P-700$^+$ reduction after switching off the continuous white light or in response to the long flash given simultaneously with background far-red light. In the presence of the uncoupler, $\tau_{1/2}$ is practically independent of the pH in the range 8.6–6.5. A further decrease in the pH leads to an increase in $\tau_{1/2}$. A pH dependence of the same type was observed in Refs. 3 and 5. Special experiments show that in uncoupled chloroplasts, $\tau_{1/2}$ is practically independent of the duration of illumination (e.g., see Fig. 2). Another type of pH dependence was observed for coupled chloroplasts (without ADP addition) illuminated during 12 s. In this case, the pH decrease from 8.6 to 6 is accompanied by a $\tau_{1/2}$ increase. $\tau_{1/2}$ reaches a maximal value at pH 6 and then drops to a minimum at pH 5 (Fig. 6A). This drop reflects the occurrence of the fast component in the kinetics of the reduction

of P-700$^+$ in coupled chloroplasts at pH values lower than 6 (Fig. 4B). It must be emphasized that the kinetics of P-700$^+$ reduction display only one phase in the pH range from 6 to 9.5. The dip in the pH dependence curve of $\tau_{1/2}$ is not observed with uncoupled chloroplasts. In this case, the P-700$^+$ reduction kinetics can be described by a single exponential law for all pH values between 4 and 9.5.

After pulse illumination, the P-700$^+$ reduction reveals also a single exponential decay. In this case, the pH dependence of $\tau_{1/2}$ is the same as for uncoupled chloroplasts. The simplest possible explanation is that after the single flash illumination there is not significant ΔpH formation. Additional support for this explanation comes from the pH dependence of $\tau_{1/2}$ for coupled chloroplasts illuminated by white light during only 2 s (Fig. 6A) when the ΔpH was rather small.

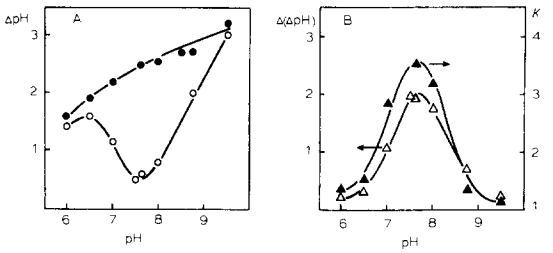

Fig. 7. Effects of the external pH on ΔpH (A), photosynthetic control factor K (B) and ADP-stimulated decrease in ΔpH (B). Chloroplasts were suspended in 10 mM phosphate/citrate buffer and illuminated with white light during 12 s. (A) (●———●) Control chloroplasts (20 μM methyl viologen); (○———○) in the presence of 20 μM methyl viologen, 2 mM ADP and 2 mM $MgCl_2$. (B) (▲———▲) Photosynthetic control factor K measured as the ratio of $\tau_{1/2}$ for chloroplasts with Mg^{2+}-ADP (2 mM) and control chloroplasts (without ADP). (△———△) Δ(ΔpH) The value of ADP-stimulated decrease in ΔpH.

Effects of pH on the value of photosynthetic control and transmembrane pH difference

Fig. 6B shows plots of $\tau_{1/2}$ vs. external pH for chloroplasts suspended in phosphate buffers with different pH values. In order to evaluate the transmembrane steady-state pH difference, ΔpH, generated under phosphorylation conditions, we have measured the pH dependence of $\tau_{1/2}$ in chloroplasts which contained Mg^{2+}-ADP and were illuminated during 12 s with white light. Fig. 6B shows that the addition of Mg^{2+}-ADP accelerates the electron transport from PS II to P-700$^+$. The effect of Mg^{2+}-ADP was most prominent in the pH range 7–8.5.

Assuming that the rate of reduction of P-700$^+$ is controlled solely by the intrathylakoid pH_{in} (this very important assumption will be proved later, see Discussion), we have obtained a calibration curve for pH_{in} values as a function of measured $\tau_{1/2}$ values (Fig. 6, curve 1). This curve corresponds to chloroplasts uncoupled by gramicidin ($pH_{in} = pH_{out}$). A similar curve was also obtained in the case of coupled chloroplasts illuminated by single flashes (in this case ΔpH is negligibly small [2]). The coincidence of these curves (Fig. 6) shows that after gramicidin addition, the steady-state pH_{in} value is practically equal to the external pH.

The data presented in Fig. 7A show that in control chloroplasts (without ADP), the ΔpH formed under steady-state illumination increases from 1.6 to 3.2 with a rise of external pH from 6 to 9.5. After Mg^{2+}-ADP addition the ΔpH value decreases to $\Delta pH \simeq 0.5$, the maximal drop being at the external pH 7.5 (Fig. 7A). The estimated value of ΔpH under phosphorylation conditions at $pH \gtrsim 7.5$ represents the upper limit of ΔpH. Fig. 7B also shows the pH dependence of the value of the photosynthetic control factor K measured as the ratio of half-time ($\tau_{1/2}$) values of P-700$^+$ reduction after switching off the white light for control (without ADP) chloroplasts and chloroplasts containing Mg^{2+}-ADP and P_i. The maximal value of photosynthetic control is observed also at pH 7.5. The pH dependence of the ATP yield with a maximum at pH 7.5–8 was reported also in Ref. 34.

Interaction of spin labels with the chloroplasts

Positively charged spin label CAT_{12} and spin-labeled stearic acid, $I_{1,14}$, with a negatively charged carboxyl group were used as indicators of the light-induced changes in the thylakoid membrane. Each of the EPR signals of both labels, CAT_{12} (Fig. 8) and $I_{1,14}$ (Fig. 9), is registered as a superposition of two signals. (1) An EPR signal from the spin labels dissolved in an aqueous medium (the triplet with the narrow components), and, (2) an EPR signal from the spin labels dissolved in a hydrophobic environment (most probably in the thylakoid interior). Light-induced changes of the EPR spectra of both these

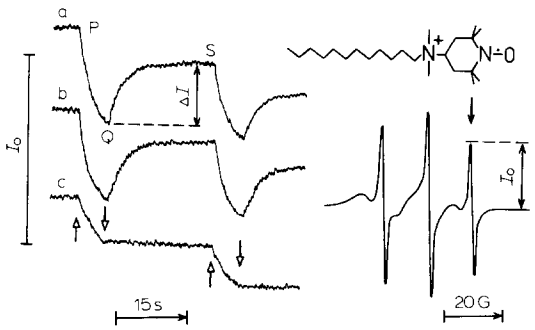

Fig. 8. The EPR signal of CAT_{12} in chloroplasts and light-induced changes in high-field component. Chloroplasts at a chlorophyll concentration of 50 μg/ml were suspended in Tricine buffer (pH 7.5). Switching on (or off) white light is indicated by upward (or downward) arrows. Additions: (a) 20 μM methyl viologen; (b) 20 μM methyl viologen, 2 mM ADP, 4 mM P_i and 6 mM $MgCl_2$; (c) 20 μM methyl viologen and 3 μM gramicidin A.

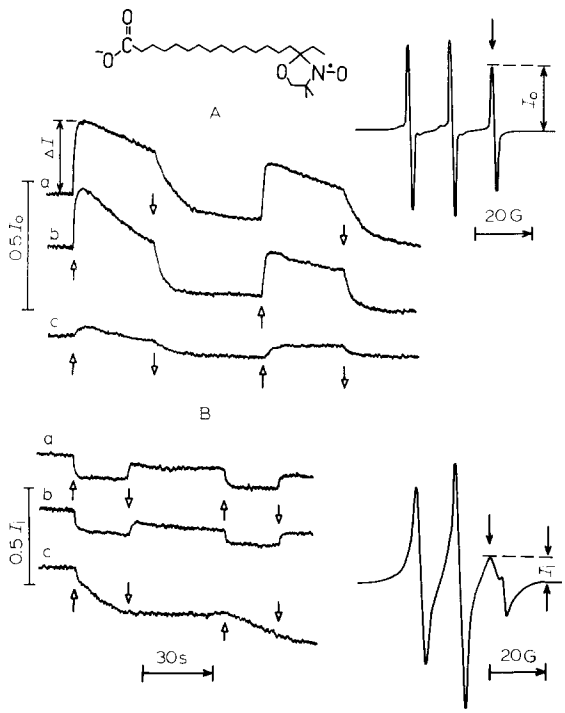

Fig. 9. The EPR signal of $I_{1,14}$ in chloroplasts (Tricine buffer, pH 7.5) and light-induced changes in the amplitude of high-field components. For kinetic measurements magnetic field was fixed as indicated by arrows. (A) Component of the EPR signal from $I_{1,14}$ in aqueous phase; (B) in membrane phase. Additions for a–c were the same as indicated in Fig. 8. Chloroplast concentration: 50 μg/ml (A) and 1 mg/ml (B) chlorophyll.

labels may be a consequence of two effects. (1) A decrease in the EPR signal caused by the reduction of paramagnetic fragments due to interaction with the electron-transport chain, and, (2) reversible changes in the distribution of spin labels between the membrane and aqueous environment in response to membrane surface charge and/or structural changes.

Fig. 8 shows that illumination of coupled chloroplasts which contain CAT_{12} leads to a decrease (phase P-Q) of the narrow high-field component of the EPR signal from the label localized in aqueous medium. A recovery phase, Q-S, i.e., the partial increase in the amplitude of this line, is observed after switching off the light. It is interesting that the kinetic behaviour of CAT_{12} is insensitive to addition of Mg^{2+}-ADP with P_i. The reversible part of the light-induced changes of the EPR spectrum can be accounted for by the redistribution of CAT_{12} between aqueous and membrane phases due to energization of chloroplasts [35]. Light-induced changes in CAT_{12} distribution caused by an increase in CAT_{12} solubility in membranes can be suppressed by the addition of uncouplers (NH_4Cl, gramicidin).

Experiments with the spin label, $I_{1,14}$, which has a negatively charged carboxyl group in the physiological range of pH demonstrate that illumination leads to reversible displacement of this label from the membrane to the aqueous phase. After the light is switched off the solubilization of $I_{1,14}$ in the membrane is increased. These reversible changes are certainly due to the redistribution of spin labels but not to the redox transformations. It was shown by the recording of light-induced changes in the EPR signals of both $I_{1,14}$ fractions: dissolved in an aqueous environment (Fig. 9A) and in a membrane phase (Fig. 9B). The spin label distribution between these fractions depends on the chlorophyll/spin label ratio. In order to enhance the fraction of $I_{1,14}$ in the aqueous (or membrane) phase we have prepared the samples with different chloroplast concentrations. It was possible, therefore, to avoid the misinterpretations which might be due to superposition of the changes of EPR signals from different fractions of $I_{1,14}$. Fig. 9A shows that the amplitude of the narrow line of the EPR spectrum given by $I_{1,14}$ dissolved in an aqueous medium increases initially after switching on the light.

This increase is followed by a rather slow decrease caused by light-induced reduction of the $I_{1,14}$ paramagnetic fragment [36]. After switching off the light a rather fast decay of the narrow line was clearly observed indicating the absorption of $I_{1,14}$ by the membrane. The reversible parts of all these changes are insensitive to addition of Mg^{2+}-ADP with P_i but can be suppressed by uncouplers (NH_4Cl, gramicidin).

The 'hydrophobic' fraction of $I_{1,14}$ is pushed out from the membrane after illumination. Fig. 9B shows that the addition of Mg^{2+}-ADP with P_i does not influence the reversible part of the light-induced changes in the amplitude of the EPR signal given by $I_{1,14}$ dissolved in the membrane phase. This reversible change can be inhibited, however, by uncouplers (NH_4Cl, gramicidin). The opposite signs of the reversible light-induced responses of two $I_{1,14}$ fractions, 'polar' and 'hydrophobic', can be considered as evidence of the spin labels' redistribution effect.

Fig. 10 (curve 2) shows that the retardation of electron flow from PS II to P-700$^+$ can be abolished by the addition of a rather small amount of NH_4Cl (about 1 mM). On the other hand, inhibition of light-induced redistribution of CAT_{12} and $I_{1,14}$ between aqueous and membrane phases needs a 10-fold greater concentration of NH_4Cl (curve 1). These facts mean that the effect of phosphorylation conditions, i.e., the acceleration of electron transport which practically does not influence the distribution of spin labels, can be simulated by the addition of an uncoupler at a rather low concentration. The dependence of chloroplast photophosphorylation activity on the NH_4Cl concentration also is shown in Fig. 10 *. These data clearly demonstrate that ATP synthesis can proceed with only slightly decreased effectivity at the small ΔpH value measured by the electron-flow retardation. It can also be seen that the dependences of ATP-synthetase activity and that of the amplitude of the light-induced spin labels' response on the NH_4Cl concentration can be described by one and the same curve with great precision.

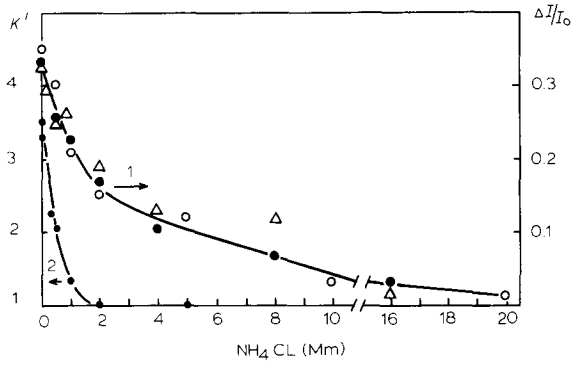

Fig. 10. The reversible light-induced changes, I/I_0 in the amplitude of high-field components of the EPR signals (curve 1) of CAT_{12} (○———○) and $I_{1,14}$ (●———●) and the effect of electron-flow retardation (parameter K', curve 2) as functions of NH_4Cl concentration. ΔI and I_0 were measured as indicated in Figs. 8 and 9. K represents the ratio of $\tau_{1/2}$ (the half-time of P-700$^+$ reduction after switching off the white light, illumination time 12 s) for energized and uncoupled chloroplasts (3 μM gramicidin A or 5 mM NH_4Cl). Experimental conditions for $\tau_{1/2}$ measurements were the same as indicated in Fig. 2 and as indicated in Figs. 8 and 9 for ΔI and I_0 measurements. (△———△) The photophosphorylation activity of chloroplasts (in relative units) at different NH_4Cl concentrations.

The data of this section enable us to conclude that chloroplast energization does not include the formation of a considerable transmembrane pH difference between bulk phases. It is possible to create conditions (e.g., by the addition of a small quantity of NH_4Cl) which considerably reduce ΔpH but only slightly influence the photophosphorylation and light-induced reversible changes in the thylakoid membrane structure tested by charged spin labels. These structural (and/or local charge distribution) changes do not occur in de-energized chloroplasts.

Discussion

Electron-transport control in leaves and isolated chloroplasts

The kinetic behaviour of P-700 in the leaves has a multiphasic character (Fig. 1). The ratio between the amplitudes of two overshoots (A-B-K and K-L-C) depends on the leaf species as well as on various factors: temperature, illumination, intensity during the growth of the plant, the age of the plant and the season [24–28]. Detailed analysis of all these data leads to the conclusion that there are at least two

* The photophosphorylation and spin label experiments were carried out with the same chloroplast samples in each series of our experiments. Special control experiments have shown that spin labels CAT_{12} and $I_{1,14}$ (10^{-4} M) themselves do not inhibit ATP formation.

sites of electron-transport-control in intact chloroplasts (leaves). The acceptor side of PS I may be the rate-limiting step in the chain of non-cyclic electron transport due to the low concentration of $NADP^+$ – the terminal natural acceptor of PS I. Electron flow from the acceptors of PS I may be maintained by activation of the Calvin cycle. It is well known [37–39] that illumination of intact chloroplasts leads to an essential increase in the activity of NADP-glyceraldehyde-3-phosphate dehydrogenase, ribulose-5-phosphate kinase and fructose-1,6-diphosphatase. There are good reasons to believe that these changes are responsible for the *P*-700 kinetic behaviour in the dark- (or far-red)-adapted leaves. The accumulation of $NADP^+$ via the Calvin cycle can lead to the second overshoot K-L-C (Fig. 1), which is most sensitive to the adaptation conditions. We cannot exclude, of course, the light-induced structural changes in photosynthetic apparatus as a possible reason for the complex kinetic behaviour of *P*-700 [23,24].

The second point of electron-transport control is localized between two photosystems. It is common knowledge that the oxidation of plastoquinol by PS I is the rate-limiting step in the chain of non-cyclic (or pseudocyclic) electron transport [1,2]. The reaction rate is diminished by acidification of the inside compartment of the thylakoid [2–5]. The retardation of electron flow from PS II to $P\text{-}700^+$ due to the light-induced increase in proton concentration inside the thylakoid might lead to the first wave, A-B-K, in the kinetic behaviour of *P*-700 in leaves. As mentioned above, the ratio between the two overshoots (A-B-K and K-L-C) was variable for leaves of different species [24–28]. In isolated chloroplasts (class B) in the presence of methyl viologen (acceptor of PS I) and oxygen (terminal acceptor of electron), we have observed only one overshoot, A-B-C, which does not depend on chloroplast adaptation. On the other hand, in leaves the first wave A-B-K is often masked by the second wave K-L-C or does not appear at all. There is a reasonable explanation of all of these facts. It is clear that isolated chloroplasts in the presence of a PS I artificial acceptor (methyl viologen) have only one point of electron-flow control (oxidation of plastoquinol). In dark-adapted leaves the rate-limiting step of electron transfer can be localized in the acceptor side of PS I. On the other hand, the effect of the retardation of electron flow between two photosystems is absent during phosphorylation (Figs. 3, 4 and 6).

There is also another type of electron-transport regulation caused by the depression of photochemical activity of PS II in response to variations of the external and internal pH values. It follows from Fig. 5 that the number of electrons donated by PS II to $P\text{-}700^+$ in response to a single short flash is maximal in the pH range of 5–7.5. The decrease in PS II activity at pH values lower than 5 and higher than 8 may be accounted for by the inactivation of water-splitting reactions. The decrease in photochemical activity of PS II at low pH values might be also caused by stimulation of back reactions in PS II via the effect of pH on the midpoint oxidation-reduction potential of the PS II primary acceptor [40].

Another regulatory effect of pH is connected to a rather sharp pH dependence of the fast component of $P\text{-}700^+$ reduction in energized chloroplasts at pH values less than 6 (Figs. 4 and 6). One of the possible explanations might be connected with the pH-induced structural changes in membranes when the pH inside the thylakoid reaches the threshold value. In this case, the effect of electron-flow retardation may be abolished by a decrease in ΔpH due to the rise of thylakoid membrane permeability. The diminishing of the fast component with a further decrease in external pH (Fig. 6A) can be accounted for by the inhibition of PS II photochemical activity at low pH values (Fig. 5).

The acidification of the intrathylakoid space as the factor of electron-transport control

We have assumed throughout this paper that the rate of $P\text{-}700^+$ reduction by PS II is an unambiguous function of the intrathylakoid pH_{in} value. This very important assumption must now be proved.

Let us consider the pH dependence of $\tau_{1/2}$ (the half-time of $P\text{-}700^+$ reduction after switching off the white light) measured with uncoupled chloroplasts. This pH dependence (Fig. 6, curve 1) can be quantitatively described if we assume that the rate of plastoquinol oxidation is limited by the process of plastosemiquinone deprotonation. In this case, the rate of electron flow from PS II to $P\text{-}700^+$ must be a function of the plastosemiquinone anion radical ($PQ^{\cdot-}$) concentration. Deprotonation of plastosemiquinone ($PQ^{\cdot}H$) formed on the inner side of the thylakoid membrane

depends on the pH_{in} value. As mentioned above, in uncoupled chloroplasts $pH_{in} = pH_{out}$. In this case, the PQ^- concentration, $[PQ^-]$, and, therefore, the rate of electron transfer to $P\text{-}700^+$, i.e., the value $\tau_{1/2}^{-1} \approx [PQ^-]$, can be uniquely determined as a function of the external pH value by the equation $\log [PQ^-]/[PQ^{\cdot}H] = pH - pK$. A good coincidence of theoretical and experimental curves (Fig. 6) was obtained with $pK \simeq 5.5$, corresponding to the pK value of the plastosemiquinone deprotonation reaction [41]. This can be considered as evidence that the rate of electron transport between two photosystems in chloroplasts containing uncoupler is unambiguously determined by plastosemiquinone deprotonation on the inner side of the thylakoid membrane, i.e., by the internal pH_{in} value.

This conclusion can be also proved for coupled chloroplasts illuminated by a single flash given simultaneously with a weak background far-red light. In this case, also $pH_{in} \simeq pH_{out}$ because it is well known [2] that in response to a single flash the value of pH_{in} is not essentially changed. Fig. 6 shows that the plot of $\tau_{1/2}$ vs. external pH measured with coupled chloroplasts illuminated by a single flash coincides with that of uncoupled chloroplasts illuminated by continuous white light.

There is a good reason to believe that in coupled chloroplasts illuminated with continuous white light the rate of electron transport between two photosystems is also controlled solely by the pH_{in} value. This statement, certainly, cannot be considered as rigorously proved. We think, however, that indirect evidence makes the alternative mechanisms of electron-flow control for coupled chloroplasts rather improbable. Indeed, experiments with the spin labels (Figs. 8–10) clearly demonstrate that neither surface membrane charge nor structural changes are the factors of photosynthetic control in coupled chloroplasts. These membrane changes might be considered, in principle, as the cause of electron-transport control during the illumination of coupled chloroplasts with continuous white light. In this case, for example, the pK value of the plastosemiquinone deprotonation reaction might be changed due to membrane structural changes or charge redistribution created by chloroplast illumination. However, the data presented in Fig. 10 clearly demonstrate that the retardation of electron flow can be abolished (by the addition of a rather small amount of the uncoupler NH_4Cl) without any significant chloroplast membrane changes which were tested with the spin labels. These facts show that the rate of electron transport between two photosystems in coupled chloroplasts as well as in uncoupled chloroplasts is an unambiguous function of the internal pH_{in} value.

We can now formulate two main conclusions from the data presented in this paper.

(A) The rate of electron flow between two photosystems in uncoupled, coupled and energized chloroplasts and consequently the effect of photosynthetic control are determined solely by the intrathylakoid pH_{in} value in the bulk phase. Effects of photophosphorylation conditions and uncouplers on the rate of electron transport are realized throughout the pH_{in} changes. It is namely the initiation of phosphorylation (and not just the presence of ADP, P_i or ATP) which influences the intrathylakoid pH_{in} value and electron-transport rate by providing an additional channel for proton efflux.

(B) The necessary conditions of ATP synthesis by the photophosphorylation process do not include the formation of a rather large transmembrane pH difference between bulk phases.

Our results are at some variance with the data of Pick et al. [15] who have found that phosphorylation conditions lead only to a relatively small decrease in ΔpH (a maximal effect of about 0.4 units was observed at external pH 9). In Ref. 15, ΔpH was determined using the quenching of the fluorescent probe 9-aminoacridine according to the method of Schuldiner et al. [42]. It is well known (see, e.g., Ref. 2) that the main bulk of protons translocated into the thylakoid are bound to the membrane and dissolved protein buffering groups and only 0.1% of injected protons are able to move freely. It is possible that the rate of plastoquinol oxidation by PS I used in our work as the pH indicator is monitored by the concentration (activity) of unbound protons, i.e., this 'pH-meter' may be insensitive to the number of protons bound to buffering groups. On the other hand, it is not clear whether the light-induced fluorescence quenching of 9-aminoacridine is caused solely by the ΔpH formed. Barber and co-workers [43,44] emphasized the significance of membrane surface charge density in controlling the fluorescence of 9-aminoacridine. From this point of view, interaction of

9-aminoacridine with the chloroplast membrane may be monitored both by changing membrane surface charges and the intrathylakoid pH_{in}. Moreover, Tiemann et al. [45] reported that at low ΔpH, the 9-aminoacridine fluorescence response reveals marked non-linearities. They concluded that at $\Delta pH < 1.0$ this method cannot be used for quantitative ΔpH measurements. The decrease in ΔpH by about 0.3–0.4 units stimulated by phosphorylation was also obtained by Rumberg and Siggel [3] who evaluated this effect from the changes in the rate of ferricyanide reduction. It is possible to explain the divergence with our data by taking into account that the rate of ferricyanide (hydrophilic reagent) reduction can be controlled by its accessibility to the primary acceptor of PS II [46], i.e., by the physical state of the outer surface of the thylakoid membrane. It is clear that the light-induced changes in the membrane surface can be abolished by uncouplers but are only slightly affected by initiation of photophosphorylation. Our experiments with the spin labels (Figs. 8 and 9) show that energization of chloroplasts stimulates the solubilization of the positively charged label CAT but the negatively charged label $I_{1,14}$ is pushed out from the membrane.

From the view of the widely accepted chemiosmotic hypothesis of Mitchell [47] the transmembrane difference of proton electrochemical potentials, $\Delta\mu_{H^+}$ (the sum of ΔpH and electric potential difference, $\Delta\psi$) is the driving force of phosphorylation. Our results show that ΔpH under steady-state conditions at external pH 7.5–8.0 is rather small. These pH values are known [34] to be optimal for the maximal yield of ATP. According to the chemiosmotic concept, it might be proposed that the decrease in the ΔpH steady-state value is compensated by the rise of $\Delta\psi$ due to redistribution of ions across the thylakoid membrane. In this case, the value of the electrochemical proton potential difference $\Delta\mu_{H^+}$ might be supported at a rather high level to provide the photophosphorylation. Critical consideration of this assumption was given in Ref. 48. It is important to note that according to Tillberg et al. [49], the decrease in ΔpH caused by NH_4Cl at low concentrations which practically do not inhibit photophosphorylation is not accompanied by an increase in $\Delta\psi$.

Of course, our results cannot exclude the possibility that energization of chloroplasts leads to the non-equilibrium distribution of protons within the thylakoid membrane creating the local microscopic constraints which are the real driving force of ATP synthesis (see, e.g., Ref. 50). We cannot discuss here these local mechanisms. It should be emphasized, however, that at the molecular (or microscopic) level any local charge and ionic 'constraints' are identical with the mechanical conformational constraints [48].

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