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PROTON EVOLUTION ASSOCIATED WITH THE PHOTOOXIDATION OF WATER IN PHOTOSYNTHESIS

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SUMMARY

1. We have used a fast and sensitive glass electrode to measure the pH changes associated with single turnovers of photosynthetic electron transport induced by short flashes in spinach chloroplasts.

2. In dark-adapted chloroplast suspensions, containing uncoupling concentrations of gramicidin D or methylamine, the flashyield of proton release oscillated in the same way as the flash yield of O_2; with a periodicity of four, the yield of the third flash being maximal.

3. Based upon the similar behavior of the flash yields of proton and O_2 liberation we conclude that, following the accumulation of 4 oxidizing equivalents, water is decomposed in a final concerted reaction.

4. Because these oscillations can only be seen in the presence of uncouplers we conclude that the O_2 evolving system is located inside the thylakoid membrane.

5. In contrast to the relaxation of System II and O_2 release, which occur in < 1 ms after a flash, the efflux time of the protons released in the decomposition of water was approx. 80 ms. From this we conclude that even in the presence of gramicidin or methylamine, there is a diffusion barrier to the movement of these protons.

6. We also observed the proton influx associated with electron transport from Photosystem II to System I. The half-time of this influx was approx. 30 ms, again slower than the electron transfer time. This half-time was not influenced by gramicidin D.

7. In contrast, methylamine abolished this influx. At the same time, there occurred a net acidification of the external medium during illumination.

8. The proton uptake associated with the reoxidation of the endogenous photoreductant of System I had a half-time of about 0.3 s. The proton uptake associated with the reoxidation of photo-reduced methylviologen showed a half-time of 8 ms.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

INTRODUCTION

The photo-oxidation of water by photosynthetic organisms must result in a release of 4 protons per O_2 molecule evolved. To understand the mechanism of the process, it is important to know when and how these protons are released. It is known that System II requires the sequential accumulation of 4 oxidizing equivalents before O_2 is liberated. One can ask the question whether a single proton is released in each oxidation step, or four protons are released simultaneously with an O_2 molecule. Also mechanisms between these two extremes might be possible.

The observation of the O_2 yields of single flashes given in appropriate time patterns has proved a fruitful approach of the system [1]. Using a glass electrode technique of sufficient sensitivity and time response, we have made a similar analysis of the proton liberation which accompanies O_2 evolution.

MATERIALS AND METHODS

Chloroplasts, isolated from freshly harvested green-house spinach, by methods described previously [2], were stored in suspensions containing 50 mM sodium Tricine buffer, pH 7.3, 50 mM NaCl, 0.4 M sucrose and approx. 2 mg/ml chlorophyll. Observations were made with samples diluted to a chlorophyll concentration of 10–20 μg/ml into a solution containing 0.4 M sucrose and 50 mM NaCl. This dilution resulted in a low buffer concentration (0.5–1 mM) which enhanced the sensitivity of the pH measurements. The final pH of the suspension was between 6.8 and 7.0. Except when stated, no electron acceptors were added in the experiments reported in this paper.

pH measurements were performed as described by Schwartz [3]. To obtain sufficiently rapid response time (< 5 ms) we had to construct our own glass electrodes [4]. The basic configuration of the electrode is that of a cup ($\varphi = 10$ mm, height 20 mm). The bottom of the cup is a very thin (and fragile!) sheet of Corning 015 glass, attached with epoxy or silicon rubber cement. The inside of the cup contains the suspension to be measured and the capillary tip (φ approx. 1 mm) of a Ag/AgCl electrode. This electrode consisted of a disposable pipet filled with a KCl-containing agar gel, in which a silver wire was inserted. Illumination of the silver by the flash was avoided. In one procedure the chloroplasts were allowed to settle during a few minutes on the bottom of pH glass before measurements were made. In another, more rapid procedure for preparing the sample, the pH cup was first filled with buffer solution containing 0.2 M sucrose. Then between 1 and 3 λ of undiluted stock chloroplast suspension was carefully added. Due to the high sucrose content (approx. 0.4 M sucrose) this drop sank and spread evenly as a thin layer over the electrode surface. The cup was placed inside a small beaker containing the reference buffer solution and another silver electrode identical to the one described above. The output from the pH amplifier was connected to a Model 1052 Fabritek signal averager and transferred to a strip chart recorder for a permanent record. Each electrode was calibrated prior to use by the measurement of two standard buffers (pH 7.0, 9.0) having a pH differential of 2 pH units. The minimum electrode response time was determined by the use of a phenazine methosulfate–cytochrome *c* dye system. The response time depended upon electrode thickness, but in most

cases was equal to or less than 5 ms. Details concerning the construction and use of the electrode will be presented in another paper. Suspensions were illuminated through the bottom of the cup, by brief saturating (approx. 1 μs) white Xenon flashes (E.G.G. No. FX101B) (approx. 1000 V, 0.5 μF) usually at a rate of 1 per s.

RESULTS AND DISCUSSION

Figs 1A–D illustrate the pH changes induced by a series of flashes (1 per s) given after a 5-min dark period in a suspension of spinach chloroplasts with and without the addition of uncouplers. In the experiment of Fig. 1A no uncoupler was

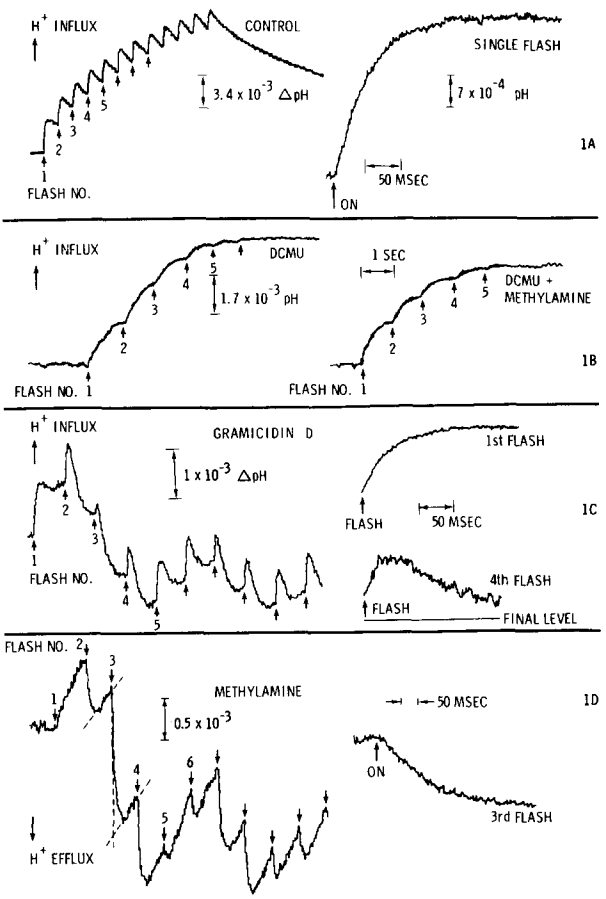


Fig. 1. Changes of pH induced by short saturating flashes in spinach chloroplast suspensions containing no externally added acceptor. Chloroplasts were suspended in a solution containing 0.4 M sucrose and 0.05 M NaCl. Chlorophyll concentrations were between 10 and 20 μg/ml. Flashing rate was 1 per s. Each sample was dark-adapted 5 min prior to measurement. A (left). Changes in pH induced in a control sample by a sequence of flashes. A (right). Changes in pH induced by a single flash, expanded time scale. B (left). Same as A except $1 \cdot 10^{-5}$ M DCMU was added. B (right). Suspension contained $1 \cdot 10^{-5}$ M DCMU and 0.01 M methylamine. C (left). Same as A except for the addition of $2 \cdot 10^{-6}$ M gramicidin D. C (right, upper). First flash of C, left, expanded time scale. C (right, lower). The 4th flash, expanded time scale.

used. Each flash induced a rapid alkalization of the external medium followed by a slow recovery. Fig. 1A (right) illustrates the flash-induced rise of pH on expanded time and pH scales. Without delay or lag, the pH rises in approximately first order manner with a halftime of 30–40 ms. This time agrees with similar observation of Grünhagen and Witt [5] who used a fluorescent dye to observe rapid pH changes. Evidently our electrode and amplifier have sufficient time resolution and sensitivity to faithfully measure the phenomena. In the experiment of Fig. 1A the recovery is slow, and, except for a more rapid initial phase, approximately first order with a halftime of a few (2–5) seconds. Also this time course has been reported earlier by several workers [2, 5]. In a sequence of flashes the envelope of the pH curve in Fig. 1A (left), the steady-state pH level and flash yield depend upon the rate of flashing and the degree of recovery occurring in each dark time. A plot of the individual flashyields shows a monotonous (first order) decline, without any oscillation which might be correlated with O_2 evolution. Thus, such an oscillation either does not take place or it occurs within a diffusion barrier which prevents it from becoming manifest.

Fig. 1B (left) shows the flash-induced pH changes in a chloroplast suspension containing 10 μm 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The rapid (30 ms) uptake component is completely inhibited, suggesting that it occurs only as a result of equivalents coming from System II. The pH change which does occur also reflects H^+ uptake but is much slower and variable. The rise time may vary between 200 and 600 ms and the amplitude may range from 1/2 to 3/4 of the control. This slow uptake is not seen in the control because the dark recovery occurs within the same time scale. Each succeeding flash yield decreases and approaches zero after 4 or 5 flashes. Recovery of the pH level back to the baseline, if occurring at all, is very slow. The experiment can be repeated after a 2–5-min dark period. Uncoupling concentrations of methylamine (Fig. 1B) and gramicidin D (not shown) do not affect the flash-induced pH change in the presence of DCMU. We conclude from these data that in the presence of DCMU no protons are released inside the thylakoid membrane. The uptake observed in the absence of a System I acceptor is probably correlated with O_2 uptake by the photo reductant of System I resulting in the formation of H_2O_2 (Mehler reaction) [6]. This uptake is known to be quite slow.

Indeed, the addition of methylviologen to this system results in a much faster proton uptake (approx. 8 ms), which probably reflects the autooxidation of photoreduced methylviologen after the flashes. Except for this acceleration viologen does not materially change the flashyield pattern. However, the first flash amplitude becomes consistently near 1/2 of a control which contains only methylviologen. The small pool (about 3 electron equivalents) of System I electron donors seen in Fig. 1B, which become reduced in a few minutes dark might consist of P700, cytochrome *f* and plastocyanin as reported by Marsho and Kok [7]. We assume a complete turnover of Photosystem I on the first flash and arbitrarily set this equal to one equivalent. We cannot however determine the stoichiometry of protons to electrons.

Effects of uncouplers

The experiment shown in Fig. 1C was made in the same way as that of Fig. 1A except that the chloroplast suspension contained $2 \cdot 10^{-6}$ M gramicidin. Inspection of the data reveals the following features:

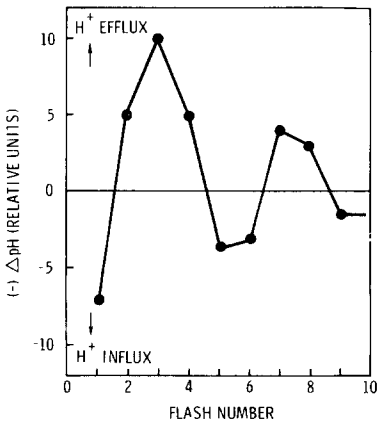
(1) While the initial rapid rise of pH following each flash is hardly affected, the subsequent proton efflux is accelerated by the uncoupler to a halftime of approx. 80 ms (c.f. Fig. 1C, bottom right). Consequently, the flash-induced pH changes are completed within the one second spacing between flashes.

(2) There is very little recovery after the first flash (c.f. the example shown on expanded scales in Fig. 1C, top right). However, after all subsequent flashes, we notice a proton efflux (the size of which is independent of flash number), following each initial pH rise. The extent of the efflux varies with the flash number and can be either smaller or larger than the initial influx.

Later in the sequence, a steady state is reached where the initial proton uptake is balanced by the subsequent efflux, the net changes being zero. In Fig. 2, we plotted as a function of flash number, the difference between the pH level just before each flash and just before each succeeding flash. The plot shows a damped oscillation around zero net change, having a periodicity of 4 and the first maximum at the third flash. This H^+ flash yield pattern clearly resembles the O_2 flash yield pattern obtained under similar conditions, strongly suggesting that the protons are being liberated by the O_2-evolving system [1].

Fig. 1D illustrates the flash-induced pH changes when an uncoupling concentration (10 mM) of methylamine (pK 10.6) is used. In this particular experiment only a quite slow (approx. 500 ms) proton uptake was seen after the first flash (c.f. Fig. 1D top right). This uptake probably is associated with the Mehler reaction as seen in Fig. 1B. Sometimes, after the first flash, one also observes part of the fast proton uptake component (as in the experiments shown in Fig. 1A and 1C) in addition to the slow component. In later flashes the fast uptake component is always absent and we notice an immediate acidification occurring with a half rise time of about 80 ms (Fig. 1D, bottom right). The kinetics are very similar to those of the acidification in the presence of gramicidin which we associated with proton liberation from water by System II. Methylamine thus allows the direct observation of the acid production by each flash, uncomplicated by the simultaneous (rapid and monotonic) uptake as occurs in the presence of gramicidin. The recovery of the acidification in the dark was rather slow (500 ms halftime), probably reflecting the process seen after the first flash. Consequently, a net acid pool is built up with higher rates of flashing or moderately strong continuous light. In Fig. 3, we plotted as a function of flash number the relative yield of protons evolved, computed as illustrated by the dotted lines in Fig. 1D. (Plotting instead the net pH change per flash, in the same way as in Fig. 2, gave a similar result). As with gramicidin D, a flashyield pattern is obtained which oscillates with a period of 4 with the first maximum after the 3rd flash. Similar results can be obtained with NH_4^+ as an uncoupler. However, in all three cases, the second flash results in an anomalously high yield compared to O_2, where characteristically little or no O_2 is evolved on the second flash.

In the second experiment plotted in Fig. 3 (triangles) the flash sequence was given after a pretreatment regimen: 5 min dark, 3 flashes, 5 min dark. It has been shown that such a 3-flash pretreatment increases the relative number of S_0 states in the system, reflected as a low ratio Y_3/Y_4 in the O_2 yield pattern [1]. The 3-flash pretreatment causes also a low Y_3/Y_4 ratio in the proton yield pattern. Similarly (not illustrated), a 1-flash pretreatment causes a very high ratio Y_3/Y_4 in a proton yield sequence, as it does in an O_2 yield sequence.



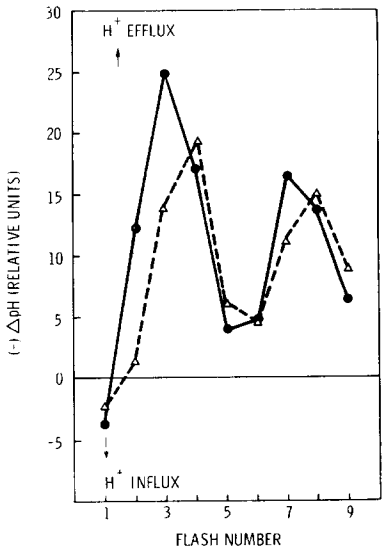


Fig. 2. Changes of pH induced by a sequence of flashes in a chloroplast suspension containing $2 \cdot 10^{-6}$ M gramicidin. Data were taken from Fig. 1C (left). Each data point represents the difference between the pH just before each flash and the pH just before the succeeding flash. Sample was dark adapted 5 min prior to measurement.

Fig. 3. Changes in pH induced by a sequence of flashes in a chloroplast suspension containing $1 \cdot 10^{-2}$ M methylamine. Closed circles: data computed from Fig. 1D left, as shown by dotted lines. Triangles: Flash pattern obtained after a pre-treatment consisting of 5 min dark followed by 3 pre-flashes followed by 5 min dark prior to measurement.

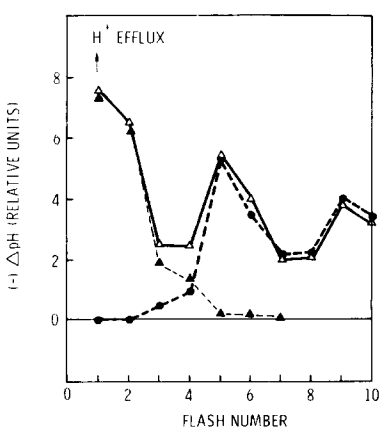


Fig. 4. Comparison yields of protons (open triangles) and O_2 (closed circles), induced by a sequence of flashes in a chloroplast suspension containing $1 \cdot 10^{-2}$ M methylamine plus 50 μM hydroxylamine. Data points were normalized to the average flash yields 7 through 10. Closed triangles: differences between the pH data and the O_2 data.

In another approach to check the correlation between the liberation of O_2 and H^+ we added hydroxylamine to chloroplast suspensions containing 10 mM methylamine. Bouges [8] found that a low concentration of this agent shifts the first maximum in an O_2 yield oscillation pattern from the third flash to the fifth flash. Indeed, as shown in Fig. 4 (triangles) in the presence of 20 μM hydroxylamine the H^+ flashyield is maximal after the 5th and the 9th flash – the oscillation being shifted by two flash numbers. For comparison, the closed circles in Fig. 4 show an O_2 flashyield sequence which was measured in the presence of hydroxylamine. After normalization (for the average of Y_7–Y_{10}) the two yield patterns match quite well for flash numbers greater than 5.

The difference between the respective flashyields of O_2 and H^+, are plotted as closed triangles in Fig. 4. These reveal an additional proton liberation sequence in which the first two flashyields are high and the successive ones decline rapidly. This additional process presumably reflects the oxidation of 2 hydroxylamine molecules which have entered the O_2 evolving centers during the preceding dark period [8]. These are oxidized by the first two flashes prior to the onset of normal O_2 evolution. The question, however, arises why the first two H^+ yields are so high, twice the steady state value (which should reflect 1 proton per reaction center per flash.) As a possible explanation, Dr Cheniae suggested to us that the internally bound hydroxylamine might be in the protonated form NH_3OH^+.

Finally we should report that with high concentrations of hydroxylamine ($>$ 1 mM) the H^+ flashyield becomes constant and independent of flash number. This agrees with earlier reports [9] that under this condition O_2 evolution is abolished and replaced by the photooxidation of hydroxylamine.

DISCUSSION AND CONCLUSIONS

The data presented have lead us to the following conclusions:

(1) The O_2-evolving system is located inside the thylakoid membrane. This conclusion is based primarily on our inability to see H^+ flashyields from Photosystem II except in the presence of uncouplers.

In addition, while it is known that O_2 is released in less than one ms [10] protons appear with a halftime of 80 ms. A decay time of 93 ms was previously reported [5]. This halftime cannot be ascribed to the mechanism of water decomposition itself: in strong light the reaction centers can release an O_2 molecule every 20–40 ms [12]. Therefore, the efflux rate of the protons must increase with their concentration, which implies the existence of a reservoir with an efflux barrier.

(2) Evidently, even in the presence of uncoupling agents, the thylakoid is still posing a diffusion barrier to protons or alternatively there is a delayed release of protons from a secondary storage site. We are unable to distinguish between these two possibilities, however the former is supported by another observation (which will be described in another paper) that protons generated inside the thylakoid membrane at another electron transport site also appears with the same time constant.

(3) Protons are released in synchrony with O_2 during a flash sequence. This leads one to suggest that water may be decomposed in a "concerted" final reaction: Following the accumulation of 4 oxidizing equivalents, presumably as higher oxidation states of manganese [12], there occurs a simultaneous release of 4 protons and one O_2.

Careful inspection of the data reveals complications and a variability which cast doubt on the validity of this conclusion:

One complication is the variability of the size and the kinetics of the pH change induced by the first flash after 5 min dark. The second, more serious complication is an often observed high value of the second flashyield (see for instance Figs 2 and 3). Under similar conditions the second O_2 yield is always close to zero.

A high H^+ yield of the second flash, if associated with O_2 evolution, could imply that a proton is liberated in the step $S_2 \rightarrow S_3$. This suggests a "0, 0, 1, 3" mechanism, one proton being liberated in the third step and the remaining three in the final reaction $S_3 \xrightarrow{h\nu} S_4 \rightarrow S_0 + O_2$. This mechanism would also predict "high" values of Y_6, Y_{10}, etc. in the proton flash pattern compared to the O_2 pattern. Inspecting Figs 2 and 3, we find Y_6 "too high" compared to Y_6 in the O_2 sequence patterns, but too low to fit the high value of Y_2 and a 0,0,1,3 proton liberation mechanism. Actually we have seen both extremes: a few experiments showed a pH oscillation pattern which closely matched the calculated predictions (according to ref. 1) for a 0,0,1,3 mechanism, others precisely matched the O_2 yield pattern. An example is shown in Fig. 5, also the 3 preflash experiment in Fig. 3 shows a negligible Y_2. The latter sequences are compatible only with a "0,0,0,4" mechanisms – in which proton liberation is concerted with O_2 evolution.

To explain this variability we had to assume either (1) proton liberation can occur in two ways (0,0,1,3 or 0,0,0,4) or (2) only the concerted (0,0,0,4) mechanism is involved, but other pH changes interfere to a variable extent.

As will be described in a subsequent paper, we have recently observed an oscillation in the proton efflux associated with electron transport from Photosystem II to System I. This damped oscillation, with a period of 2 ($Y_2 > Y_1$ etc.) can quantitatively account for the occasional high values of Y_2, 4, 6, etc.

Another complication is the depth of the oscillation compared to the amplitude of the uptake in the control. According to a strict interpretation of the linear 4 step model for O_2 evolution [1], two protons should be released on the third flash. If one were to suppose that the single flash yield in the control amounts to between

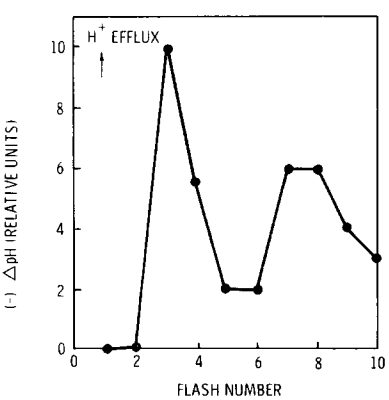


Fig. 5. Changes of pH induced by a sequence of flashes in a suspension layered directly on to the electrode (see Methods for details). Sample contained $1 \cdot 10^{-2}$ M methylamine.

one or two protons, per equivalent, then the amount coming from Photosystem II must be less than required by the model. However, a quantitative assessment cannot be made until the ratio of protons to electrons can be measured in the photosystems.

(4) Proton uptake associated with electron transfer from System II to System I occurs with a halftime of 30 ms. The time, observed with our glass electrode confirms the earlier measurements of Grunhagen and Witt [5] using a fluorescence technique. Again, as in the O_2 system (see No. 1) there is a discrepancy between the electron transfer to quinone ($\leqslant$ 1 ms according to Stiehl and Witt [13]) and the associated proton uptake – suggestive of either a membrane barrier or a mechanistic rate limitation.

(5) Besides accelerating proton flux out of the vesicle, methylamine specifically inhibits the proton uptake normally associated with electron transport between Photosystems I and II. As in the experiment shown in Fig. 1B, this uncoupler does not affect the proton uptake associated with System I. It affects neither the flashyield pattern of O_2 evolution (unpublished results) or the associated proton liberation (compare Figs 1C and 1D). Therefore, the suppression of the rapid initial proton uptake must be a specific effect of the amine, which is also exhibited by NH_4^+. The rapid H^+ uptake step probably reflects the protonation associated with the reduction of plastoquinone. The mechanism of the inhibition of uptake by amine is at present not understood.

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