Protolytic Reactions in Photosystem II:
a New Model for the Release of Protons Accompanying the Photooxidation of Water

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Photosystem II, Watersplitting, Model of H+-Production

Using pH indicator dye techniques we have investigated the pH changes in dark-adapted chloroplasts following excitation by short flashes. Two types of pH indicator, cresol red and neutral red, were used, to follow the pH changes either inside or outside the thylakoids, or the net change when the membrane was made permeable to protons by uncoupling agents.

(1) With cresol red which showed the net pH changes inside and outside the thylakoids, an oscillation of the flash yield of H+ occurred with a periodicity of 4 (minima on the first and fifth flashes, the yield on the third being not significantly different from the yields on the second and fourth flashes). The pH changes did not occur in synchrony with O_2-evolution.

(2) The net flash yields without addition of electron acceptor were similar to those with benzylviologen. The results were comparable with those obtained with the glass electrode technique by Fowler and Kok (C. F. Fowler and B. Kok, Biochim. Biophys. Acta **357**, 299—307 [1974]).

(3) The net flash yields with ferricyanide as electron acceptor of photosystem I were higher than those in the absence of acceptor, or with benzylviologen. On the first and fifth flashes a net acidification was always observed.

(4) In the presence of 3-(3,4-dichlorphenyl)-1,1-dimethylurea (DCMU) a rapid acidification also occured on the first flash, while the pH changes induced by subsequent flashes were inhibited.

(5) The uncoupler methylamine did not inhibit the proton uptake outside the thylakoids.

(6) With neutral red as indicator for the net pH change inside and outside the thylakoids, the same oscillation of the flash yield occured as with cresol red.

(7) With neutral red in the precense of an external buffer, as a pH indicator for the internal aqueous phase alone, an oscillation of the flash yield with a periodicity of 4 also occured. The first and second flash yields were higher compared with the third than the equivalent yields of oxygen.

(8) We discuss the results with respect to a model for the release of protons in the watersplitting enzyme reactions, in which protons are not released in synchrony with O_2, but in the transitions of all the states of the watersplitting enzyme with the exception of $S_1 \rightarrow S_2$.

Our results are consistent with this model when account is taken of the release of protons inside the thylakoids with a periodicity of 2, associated with electron transfer from reduced plastoquinone.

Introduction

According to the hypothesis of Kok *et al.*[1] for the evolution of oxygen in photosynthesis, an accumulation of 4 oxidising equivalents on the so-called S-states is required to oxidize one molecule of oxygen. This mechanism seems to be generally accepted. However, little direct evidence exists as to the mechanism of the watersplitting reactions at the molecular level. Several models have been proposed in the attempt to describe it[2, 3]. Since the release of one molecule of oxygen from water is accompanied by the appearance of 4 protons, one way of approaching the problem is to follow the change of pH due to this reaction during a sequence of short flashes. The first attempt to use this approach was that of Fowler and Kok[4] using a fast and sensitive

glass electrode technique. By measuring the net pH change after each of a train of short flashes on dark-adapted chloroplasts in the presence of uncoupler, they found that H+-release occurred with a periodicity of 4, with minima on the 1st and 5th flashes. The addition of uncoupler was necessary to allow free movements of protons across the chloroplast membrane since it had been previously shown that the watersplitting enzyme system is located so as to release protons inside the thylakoids[5]. Fowler and Kok[4] tentatively concluded that the release of protons accompanying the photooxidation of water followed the same pattern as the evolution of molecular oxygen, *i. e.* 4 H+ are released in a concerted final reaction together with one molecule of oxygen. However the release of protons with the periodicity of 4 could be demonstrated clearly only in the presence of a high concentration of methylamine. Under these conditions the uptake of protons associated with reactions on the acceptor side of photosystem II

appeared to be inhibited, and Fowler and Kok[4] assumed that the contribution from these reactions and from the reactions involving protons on the acceptor side of photosystem I were negligible. Very recently however (while this report was in preparation) Fowler[6] has shown that pH changes occur with a periodicity of 2 accompanying the accumulation of reducing equivalents on the acceptor side of photosystem II, before transfer to photosystem I. From an energetic consideration it would be rather surprising if all 4 protons were released inside the thylakoids at once (see discussion). We therefore proposed to reconsider the results obtained by Fowler and Kok[4], and their conclusions as to the mechanism.

In this paper we show some results using pH indicator dye techniques to observe pH changes in dark-adapted chloroplasts in the presence of different types of electron acceptors and uncouplers. We found consistent results from experiments with two types of pH indicator, cresol red[5] and neutral red[7]. Under appropriate conditions these could be used to follow the pH changes either inside or outside the thylakoids, or the net pH change when the internal protons were allowed to equilibrate across the membrane in the presence of uncoupler.

Materials and Methods

Preparation of chloroplasts

Chloroplasts were prepared from spinach, either brought from the market or grown in a greenhouse with supplementary illumination, using a medium containing 0.4 M sucrose, 0.05 M sodium phosphate buffer pH 7.8 and 0.01 M NaCl[8]. Before the final wash the chloroplast pellet from a centrifugation at $1500 \times g$ was resuspended in a buffer-free medium containing 50 mM KCl or sometimes 20 mM KCl and 1 mM $MgCl_2$. After centrifugation for 5 – 10 min at $5000 \times g$ the pellet was resuspended in the buffer free washing medium and adjusted to a concentration of 2 – 4 mg chlorophyll per ml. All experiments were carried out at room temperature using these "broken chloroplasts".

Spectroscopic measurements

Changes of absorption were measured with a single-beam spectrophotometer. The signal to noise ratio was improved by averaging the traces (in general 8 times) using a mini-computer (Digital Equipment Co., PDP 11/10 or LS I-11) and an appropriate programme as previously described in ref. 9. In this programme normalization and subtraction of traces were possible. In order to have a fresh dark-adapted sample for each train of flashes a flow system was introduced using a flow cell with 1 cm optical pathlength. During the flow the measuring beam was closed by a shutter and opened just after the flow was stopped. The time of exposure of the sample to the measuring light before flash excitations was shorter than 5 sec. The behaviour of the absorbance changes after a train of flashes was not modified by preexposure of the chloroplasts to the measuring beam for times varying between 0.5 – 5 sec. The intensity of the measuring light was kept low by decreasing the slit width of the monochromator (less than $100 \, \mathrm{erg \cdot cm^{-2} \cdot sec^{-1}}$). Each experiment was carried out using one chloroplast suspension over a time period of 15 to 40 min. Excitation of photosynthesis was provided by a xenon flash lamp (15 μsec half-bandwidth) with light of wavelengths above 620 nm selected by appropriate filters.

The absorbance changes of the pH indicator dye cresol red were measured at 572 nm[5]. The standard reaction mixture contained 10 mM KCl, 10 μg chlorophyll per ml and 50 μM cresol red adjusted with a fresh solution of NaOH to pH 7.2 – 7.4.

Measurements of the absorbance changes of neutral red were performed at 524 nm in a way similar to that described by Auslaender and Junge[7]. Traces of absorbance changes were recorded, and stored in the computer for chloroplasts in the presence of membrane-impermeable buffer (2 mg per ml bovine serum albumin BSA, pH 7.1 – 7.3). Separate traces of absorbance changes in the presence of internal and external buffers (BSA plus imidazole 4 mM) were then stored, and the stored traces were electronically subtracted. The difference was assumed to show the absorbance changes of neutral red which were sensitive to addition of buffering groups with access to the thylakoids interior, and which therefore reflected the changes in pH in the internal aqueous phase. For the measurements of the absorbance changes of neutral red reflecting pH changes both inside and outside the thylakoids, the traces obtained in the presence of internal and external buffers (BSA and imidazole) were subtracted from those obtained in the absence of any added buffer. The reaction mixture usually contained 10 mM KCl, 20 μg chlorophyll per ml, 10 μM neutral red and 3 μM valinomycin, with other additions as indicated.

Results

Absorbance changes of cresol red in the presence of uncouplers and different electron acceptors

The changes in proton concentration in the suspension medium can be followed by monitoring the colour changes of the pH indicator cresol red[5].

In chloroplasts which have not been dark-adapted, and using the repetitive flash technique, 2 sites of protolytic reactions have been previously identified [5, 10]. One occurs on the electron acceptor side of photosystem II at the plastoquinone level, and the other at the terminal step of photosystem I on reduction of a viologen-type electron acceptor. Proton uptake at the latter site is not observed if ferricyanide is used as terminal electron acceptor [5]. It has also been demonstrated by measuring the proton concentration changes in the presence of uncoupler like carbonylcyanide-p-trifluormethoxyphenylhydrazone (FCCP) or methylamine that the release of protons associated with the watersplitting system is located inside the thylakoids [4, 5]. It is possible to follow the dependence on flash number of the net pH change due to this reaction by using the same technique but with dark-adapted material.

The choice of uncoupler is important since many uncouplers affect the relaxation kinetics of the oxygen evolving enzyme-complex, or other reactions of the electron transport chain. In the present work we tested the suitability of a number of uncouplers, gramicidin, methylamine and the nigericin type ionophore dianemycin as reagents to equilibrate protons. In order to make the membrane freely permeable to protons, gramicidin and methylamine had to be used at high concentrations ($>10^{-6}$ M and $>10^{-2}$ M respectively) [4]. Gramicidin perhaps because of its pore mechanism [11] and the irreversible nature of its action on the membrane [12], did not always give reproducible results. The use of methylamine might also be questioned since many amines affect the oxygen evolving system [13]. However, we found the results obtained with methylamine were similar to those with dianemycin. We preferred to use the latter ionophore in our experiments since its carrier properties [14], reversibility and higher potency (S. Saphon, unpublished) appeared to be most suitable for the purpose.

Fig. 1 shows the net pH changes in a suspension of broken chloroplasts in the presence of dianemycin, observed after a train of flashes at approximately 0.6 sec intervals as indicated by the absorbance changes of cresol red.

The changes observed under three different sets of experimental conditions will be considered: (1) in the absence of exogeneous acceptors, (2) in the presence of benzylviologen and (3) in the presence of ferricyanide (0.1 − 0.5 mM). In the presence of
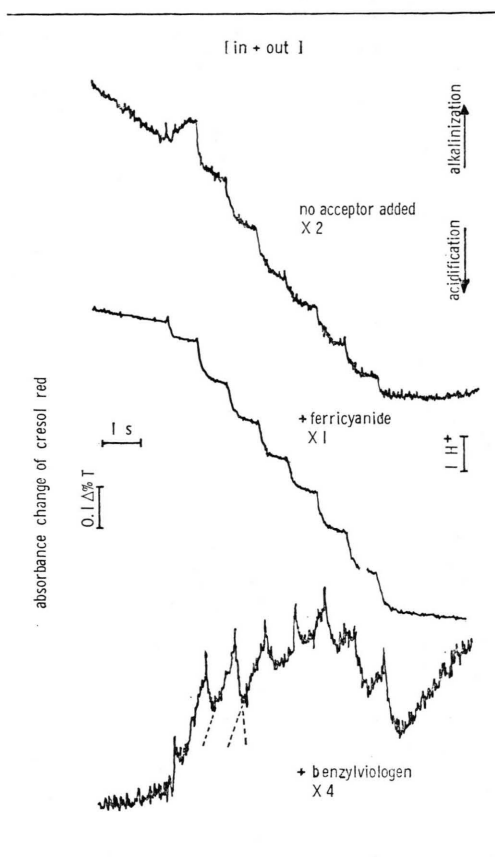

Fig. 1. Absorbance changes of cresol red in the presence of dianemycin and different electron acceptors. All traces were obtained from an average of 8 flashgroups. 8 flashes were fired within the group at $\cong 0.6$ sec intervals. The final concentrations of ferricyanide and benzylviologen were 0.5 mM and 0.1 mM respectively. Calibration of the pH change was performed with chloroplasts in the presence of ferricyanide after a preillumination of approximately 25 flashes, assuming a net release of 1 H$^+$ per flash and electron transport chain. For further details, see Methods.

benzylviologen a rapid proton uptake (<5 msec) occurred after every flash, followed by a more or less strong proton liberation. The rapid alkalinization was probably partly due to the rapid protonation of the reduced benzylviologen [10] since it was not generally observed in the absence of exogeneous electron acceptors or in the presence of ferricyanide. In the absence of exogeneous acceptors the kinetics of the proton release were slower than in the presence of benzylviologen but the net flash yields were not significantly different for the corresponding flash numbers (see also below and Fig. 2). The reason for the slower kinetics probably lies in the slower proton
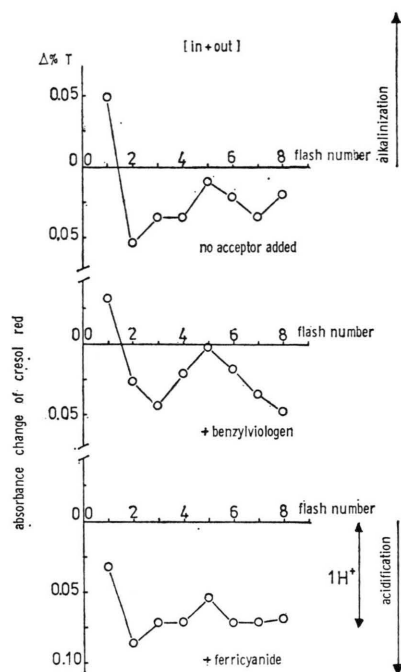
Fig. 2. Net flash-induced pH changes indicated by the absorbance change of cresol red in the presence of dianemycin as function of the flash number. The data were taken from Fig. 1 and represent the net pH change inside and outside the thylakoids.

uptake on reduction of molecular oxygen in a Mehler-type reaction [15]. Both in the presence and absence of benzylviologen a peculiar behaviour was usually seen on the first flash. Accompanying the rapid changes which were complete in less than 0.3 sec, a slower alkalinization was observed (half-time $\cong 0.3 - 0.6$ sec). The rapid changes could be accounted for in terms of the fast H^+-uptake balanced by the H^+-efflux, but the reason for the appearance of the slow H^+-uptake is not yet clear and is under investigation.

In the presence of ferricyanide the net flash-induced changes were much larger than in the first two cases, in accordance with the fact that ferricyanide is not protonated on reduction [5]. No net alkalinization could be detected for any flash.

Clearly, in all 3 cases, the net flash yield of protons depended on the flash number. If we plotted the net flash yield as indicated by the dotted lines in Fig. 1 against the flash number, we obtained a clear oscillation with a periodicity of 4 and minima on 1st and 5th flashes (Fig. 2). The release of protons after the second flash was always high. The yield on

the third flash compared with the second was variable, but the mean changes over a number of experiments were not significantly different. The overall pattern was always similar to that shown. The results obtained here are very similar to those obtained by Fowler and Kok [4] and more recently by Fowler [6] using the glass electrode technique, except for the case of ferricyanide. (We never found a net alkalinization on the first flash in the presence of ferricyanide, see also below.)

No conclusions as to the involvement of protons in the S-states can be drawn directly from these results since from Fig. 1 it is clear that the results obtained in the absence of exogeneous electron acceptors or in the presence of viologen represent a superimposition of at least 3 proton involving reactions. Even in the presence of ferricyanide there might be still a complication due to the proton uptake and release on the acceptor side of photosystem II. Another approach to the problem was to use the neutral red technique, which has been shown under appropriate conditions to indicate only the internal pH changes [7] (see later section).

Absorbance changes of cresol red in the presence of DCMU and uncoupler

In an attempt to see if on the first flash a H^+-release occurs inside the thylakoids, experiments were carried out in the presence of DCMU. This agent inhibits the electron transfer from the primary electron acceptor of photosystem II, Q, to the other components of the linear electron transport chain [16]. Charge separation at the photosystem II site can therefore occur only on the 1st flash for a relatively high flash frequency [17]. Fig. 3 (top) shows that the pH changes outside the thylakoids in the train of flashes were inhibited by the addition of DCMU, except for the first flash. After the addition of dianemycin, a fast H^+-release could be detected, followed by a slow H^+-uptake as was observed in the absence of uncoupler. For comparison the changes in the absence of DCMU are also shown in figure 3 bottom (*cf.* Fig. 1). Clearly, a rapid H^+-release on the inside of the thylakoids occurred on the first flash. Also in the presence of relatively high concentration of ferricyanide (3 mM) to oxidize the electron transport components of photosystem I including the plastoquinone pool [18], a similar H^+-release was observed, suggesting that the release is not due to the reoxidation of reduced plastohydroquinone by photosys-
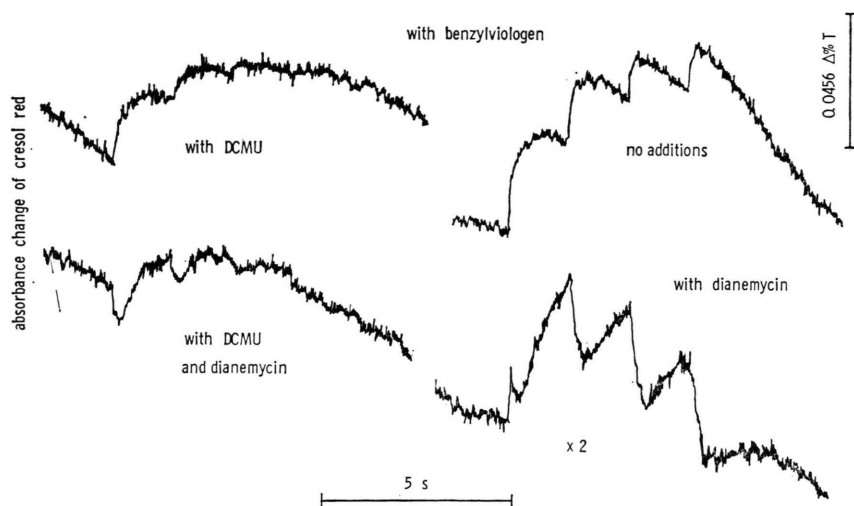
Fig. 3. Effect of DCMU on the flash-induced pH changes indicated by the absorbance changes of cresol red in the presence and absence of dianemycin. DCMU was added after dark-adaptation to a final concentration of 1 μM. The traces were obtained from an average of 4 flashgroups. 4 flashes were fired within the group at $\simeq$ 1.2 sec intervals. For further details, see Methods.

tem I (not shown). The observed H^{+}-liberation on the first flash therefore probably originates from the watersplitting reactions. This observation is in contradiction with that of Fowler and Kok [4] with the glass electrode technique. They did not find any release of H^{+} at all in the presence of DCMU.

Absorbance changes of neutral red in the absence of external and internal buffers

In the absence of any added buffers the colour change of neutral red induced by flash illumination appears to indicate the pH changes of the inner and outer aqueous phases of the thylakoids. In the presence of a buffer with access only to the external aqueous phase, the pH changes on the external phase are suppressed, and the indicator responds to the internal pH changes only.

The technique employed in this work was similar to that described by Auslaender and Junge [7]. Since the absorbance changes due to neutral red occured in the same region as the electrochromic band shift [19], background changes which were not due to pH changes (those changes which remained after addition of external and internal buffers, see Methods) were subtracted from the changes in the absence of any buffer or in the presence of external buffer alone. Despite the fact that the extent of the absorbance change due to neutral red, *e. g.* in the internal aqueous phase, did not correspond to the expected value (see appendix), the technique seemed to give valuable qualitative results.

Fig. 4 shows the absorbance changes of neutral red in the absence of any added buffers with ferri-

cyanide as electron acceptor. Under these conditions the relative contributions to the net changes of the indicator in equilibrium with external and internal aqueous phases would depend on the relative concentration and distribution of endogeneous buffering groups and of the indicator itself [20]. For this reason the changes tended to be somewhat variable. However an inspection of all the traces showed results similar to those obtained using cresol red in the presence of uncoupler (see Fig. 1). The similarity was clearly seen if the net flash yields were plotted against the flash number (Fig. 4 right). Minima occurred on the first and fifth flashes. In the presence of benzylviologen the same pattern could be obtained, although the relative flash yields were different (not shown). The results could however be ambiguous because neutral red may compete with benzylviologen as electron acceptor (their midpoint potentials are similar). We therefore preferred to refer the results to those in the presence of ferricyanide. In the following section the same technique was used in the presence of ferricyanide but with addition of an external buffer, such that the absorbance changes due to the internal pH changes alone could be observed.

Absorbance changes of neutral red in the presence of external buffer only

Fig. 5 shows the absorbance changes of neutral red in the presence of ferricyanide and external buffer, and the net flash yield as a function of the flash number as measured from such a trace. As described in the previous section, the absorbance changes reflected the internal pH changes. A perio-
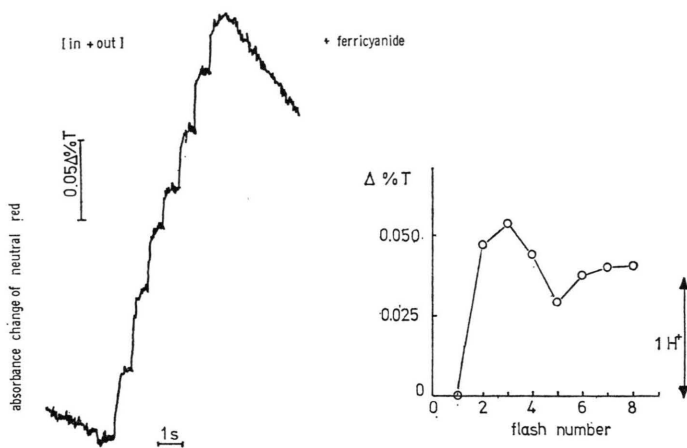
Fig. 4. Absorbance changes of neutral red in the absence of any added buffer (left) and the dependence of the flash yields on the flash number (right). The traces were obtained from an average of 8 flashgroups with 8 flashes within the group as described in Fig. 1. They represent the pH changes inside and outside the thylakoids, for details see text. The final concentration of ferricyanide was 0.1 mM. Calibration of the pH change was performed as described in Fig. 1.
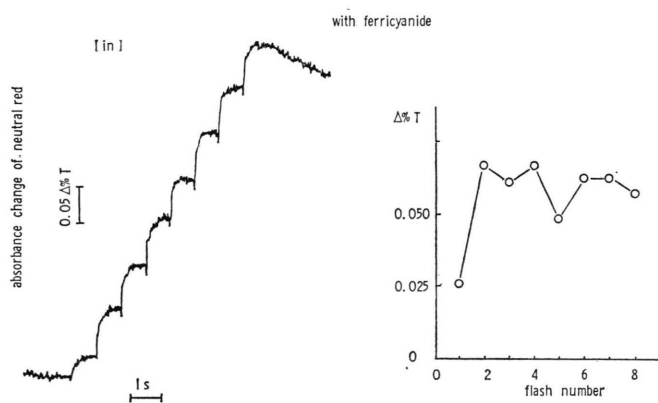

Fig. 5. Absorbance change of neutral red in the presence of external buffer (left) and the dependence of the flash yield on the flash number (right). Conditions otherwise as in Fig. 4. For experimental details, see Methods. The traces represent the internal pH changes alone; for further details, see text.

dicity of 4 was again apparent. The pattern was in no case the same as that for oxygen evolution [1], i. e. the 1st and 2nd flash yields for the internal pH changes were higher compared with the third, than the near zero values for the corresponding yields of oxygen evolution [1]. The proton uptake outside the thylakoids measured with cresol red in the absence of uncoupler was not inhibited under these conditions (not shown). Therefore the observed changes in internal proton concentration were probably due to (i) the proton release accompanying the watersplitting and (ii) the proton release on reoxidation of reduced plastoquinone. This will be discussed in the next section.

Discussion

Evidence from different experimental approaches for the involvement of protons in the reactions of the watersplitting enzyme-complex on transitions of all the states, with the possible exception of $S_1 \rightarrow S_2$ will be presented in a following paper [21].

The direct measurements of pH changes reported here have led to a similar conclusion.

We therefore propose to discuss our results in terms of a model for the proton release from the watersplitting enzyme complex based on the scheme of Kok et al. [1] for O_2-evolution but which differs from the model proposed by Fowler and Kok [4] for proton release. In our model no protons are involved in the $S_1 \rightarrow S_2$ transition, but a release of H^+ occurs in all other transitions in the sequence from S_2 to S_1 (see diagram). In this diagram the accumulated "charges" are not represented for the sake of simplicity. The total number of protons for the reactions
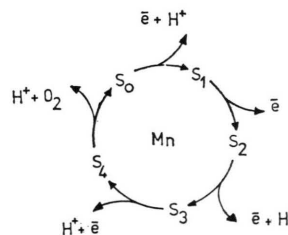
of the S-states on each flash would therefore be 1, 0, 1, 2 for the transitions $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_4 \rightarrow S_0$ respectively.

After $10-15$ min incubation of chloroplasts in the dark, the distribution of the states is 0.25, 0.75, 0, 0 for S_0, S_1, S_2, S_3, respectively [1]. To explain the pertubations which damp the oscillation of the O_2-flash yield in a flash sequence, Forbush et al. [22] introduced a miss factor α and a double hit factor β (for details, see ref. 22). Taking these factors into account, we calculated the expected flash yields of protons due to water decomposition according to our model. The results of such a calculation are shown in Fig. 6 A. The maximum would be expected on the third flash, with minima on the first and fifth flashes, but in comparison with the Fowler-Kok model [4] the flash yields on the first and second flash are higher when compared with that on the third. It should also be noticed that the yield expected on the fifth flash is higher than that on the first flash. For the sake of comparison, if another model is considered, in which protons are released in all the

transitions except for $S_0 \rightarrow S_1$ in a $(0, 1, 1, 2)$ pattern (see above), the yield on the first flash would be almost equal to that of the 4th or 5th flash (not shown). Lastly if a $(1, 1, 1, 1)$ pattern is considered no oscillation would be expected. The predictions of none of these models fit the experimental data of the flash yields of proton release inside the thylakoids obtained by the neutral red technique (Fig. 5). This is because the release of protons on reoxidation of plastohydroquinone also has to be taken into account.

Bouges-Bocquet [23] and Velthuys and Amesz [24] suggested from the results obtained by several types of experiment that a 2-fold accumulation of charge occurs on the acceptor side of photosystem II involving a component called B or R (possibly plastoquinone in a special environment), before electrons are released two at a time to the plastoquinone pool and then to different reaction centers of photosystem I. The reduction on the acceptor side of photosystem II is probably linked to a protonation since proton uptake occurs stoichiometrically with electron
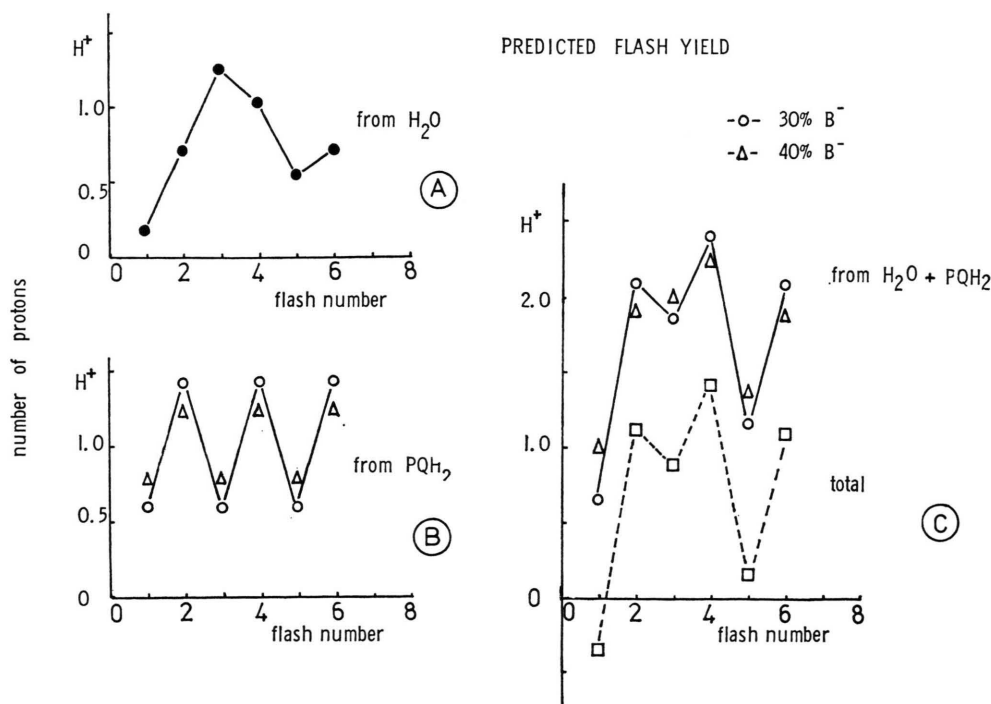

Fig. 6. Predicted flash yields of protons released inside the thylakoids on photooxidation of water (A), on reoxidation of plastohydroquinone (B), on photooxidation of water and plastohydroquinone reoxidation (C), and the predicted total pH changes in the suspension medium (C, dotted line), according to the model presented in this paper. The predicted flash yields of proton release on photooxidation of water were calculated in a way similar to that as described in ref. 22, with a assumption of $\alpha = 0.23$ and $\beta = 0.07$. For the sake of simplicity the lines were drawn only for the case, in which 30% of the reaction centres are in the state $(Q-B)^-$ after dark-adaptation. For details, see text.

transfer to the pool [5, 25], and the kinetics of the reoxidation of X-320 [26] are similar to the kinetics of the proton binding when the presence of a proteinaceous diffusion barrier is taken into account [10]. One might therefore expect to observe a release of protons on the inside of the thylakoids with an oscillation of periodicity 2 reflecting the reoxidation of plastoquinone following reduction by the 2 accumulated charges in the $(Q-B)^{2-}$ complex [27] (Q is the primary electron acceptor in photosystem II). The degree of oscillation would however be determined by the existence of charges (protonated or unprotonated) already present before the flash sequence. Several authors came to the conclusion that in dark-adapted chloroplasts $20-40\%$ of the centres are in the state $(Q-B)^-$ in which one charge has been accumulated on the secondary electron acceptor B [28-30]. Taking this finding into account, an approximate prediction of the proton release into the internal phase on reoxidation of plastohydroquinone is shown in Fig. 6 B. (This is necessarily approximate since the mechanism of charge transfer to the quinone pool is not well understood, but undoubtedly complex, see refs. 27 and 31.) The flash yield of proton release expected would be maximal on even numbered and minimal on odd numbered flashes with a periodicity of 2. While this paper was in preparation, Fowler [6] reported a more detailed study of the H^+ changes associated with electron transfer on the acceptor side of photosystem II. His results are similar to ours and may be interpreted as supporting our conclusions, but Fowler has derived a different conclusion which is based on a higher stoichiometry for proton uptake than that expected from our model. However, the measurement of stoichiometries depends upon adequate calibration, and this latter is difficult with the electrode technique. The method of calibration used by Fowler [6] depends on a comparison between the yield of protons from water, either during uncoupled electron flow, or during the steady state, and the yield of the rapid proton uptake in the absence of uncoupler. In the presence of uncoupler, buffering groups both outside and inside the thylakoid are available to the external phase in which the H^+-changes are measured, but the rapid H^+-change in the absence of uncoupler is buffered by the external buffering groups only. Thus, unless precautions are taken to allow for this, the rapid external changes will be over-estimated. These problems will be discussed at greater length in a later paper.

The total amount of H^+ released on the inside the thylakoids would be the sum of the 2 reactions of water decomposition and plastohydroquinone reoxidation (Fig. 6 C). An oscillation of 4 would still be observed, but the difference between the 2nd, 3rd and 4th flashes would not be very significant. It should also be noticed that a superimposition of an oscillation with a periodicity of 2 could be less clearly seen on top of the oscillation with a periodicity of 4 if a charge was stored on B after dark incubation for any fraction of centres greater than 30%. Furthermore, if the changes in proton concentration outside are added to the total changes inside the thylakoids, the net pH change in the suspension medium can be predicted. The changes in pH outside, under our conditions, and as observed by Fowler and Kok [4], are in the first approximation not very significantly different on each flash. Assuming that $1 H^+$ is taken up on each flash in the presence of an electron acceptor such as ferricyanide, the net pH change expected (inside and outside the thylakoids) on each flash is shown in Fig. 6 C.

A comparison of the results obtained from our model (Fig. 6) with the data obtained from experiments using 2 types of pH indicator (Figs 2, 4, and 5) shows very strong similarity. Both the observed pH changes, either inside and outside or inside the chloroplasts thylakoids, fit the predicted changes well. We therefore can conclude that the model presented in this report, i. e. protons released in all transitions of the S-states, except for $S_1 \rightarrow S_2$, describes the watersplitting reactions fairly well. Further experimental evidence will be presented in a following paper.

For the other model for H^+-release accompanying the decomposition of water, e. g. the $(0, 1, 1, 2)$ pattern as mentioned above, the flash yields predicted for the 2nd, 3rd and 4th flashes would not be significantly different when compared with each other, but the yields on the 1st and 5th flashes would be expected to be nearly equal (not shown). This is not in accordance with the experimental data (Figs 4 and 5).

Another argument in favour of our model is provided by the results obtained in the presence of DCMU (Fig. 3). Using our fast indicator technique a release of H^+ inside the thylakoids on the 1st flash was observed. Two explanations are possible: the release is due either (i) to the transition of the S-states of the watersplitting enzyme complex, or

(ii) to the electron transport from the plastoquinone pool to photosystem I. The second case is not likely since with ferricyanide at a concentration high enough to oxidize photosystem I and the plasto-quinone pool [18], a H+-release on the first flash was also observed. One may argue that the release on the first flash in the presence of DCMU is mainly due to the transition $S_1 \rightarrow S_2$ or even to the two transitions $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$. In both cases the predicted results would not fit the observed pH change in the absence of DCMU either inside and outside, or inside the thylakoids only: (i) for H+-release in the transition $S_1 \rightarrow S_2$, the internal pH change on the fifth flash would be expected to be nearly equal to that on the first flash (see above). (ii) for H+-release in the transitions $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ only an overall oscillation with a periodicity of 2 for the internal pH changes due to the plastohydroquinone reoxidation would be expected since no oscillation in H+-release would occur on the photooxidation of water. Furthermore, the H+-release on the first flash in the presence of DCMU is well explained by a H+-release in the transition $S_0 \rightarrow S_1$, assuming that the concentration of S_0 is higher in the presence of DCMU than in its absence (see the results from the DCMU-triggered luminescence experiments of Etienne and Lavorel [29, 30]). This is also confirmed by the finding that the H+-release in the presence of DCMU is higher than in its absence (see Fig. 3).

The discrepancy between our observations and those of Fowler and Kok [4] in the presence of DCMU will be discussed in a following paper.

Lastly, we wish to argue that the release of protons in synchrony with O_2 is improbable on thermodynamics grounds.

Consider the reaction:

$$S_4^{4+} + 2\,H_2O \rightleftharpoons S_0 + 4\,H^+ + O_2 .$$
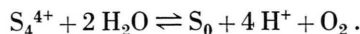
We may make the assumption that S_4^+ and S_0 are distinct chemical species, and that the reaction can be described by an equilibrium constant:

$$K_{eq} = \frac{[S_0] \cdot [H^+]^4 \cdot [O_2]}{[S_4^{4+}] \cdot [H_2O]^2} .$$

We may assume that the concentrations of O_2 and H_2O remain relatively constant under physiological conditions, and rearrange the equilibrium equation with pseudo-constant

$$\frac{[S_4^{4+}]}{[S_0]} = K' \cdot [H^+]^4$$

such that $K' = \dfrac{[O_2]}{K_{eq} \cdot [H_2O]^2} .$

It can be seen that the equilibrium ratio of S_4^{4+} to S_0 would vary with the fourth power of the proton concentration. Since oxygen evolution occurs over a range of pH from $4.5 - 8.5$, we would expect the above ratio to vary by 10^{16} over this pH range. This seems unlikely.

An alternative approach is to include the transition $S_3^{3+} \rightarrow S_4^{4+}$ in our consideration and express the overall change as a redox-half cell.

$$2\,H_2O + S_3^{3+} \rightarrow S_0 + 4\,H^+ + O_2 + \bar{e} .$$
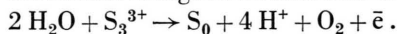
$$E' = E^0 - 4\left(2.3\,\frac{RT}{F}\right)pH + \frac{RT}{F} \ln \frac{[S_0] \cdot [O_2]}{[S_3^{3+}] \cdot [H_2O]^2} .$$

It can be seen that the apparent mid potential of the reaction would vary by about $-240\,mV/pH$ unit, or by $960\,mV$ over the range of pH applicable. All the extra work needed to oxidise water at lower pH would be loaded onto a single electron oxidation. Since the state S_3^{3+} is considered to have a higher redox potential than the lower states, its potential would be expected to be $\geqslant 810\,mV$ at pH 7. Insufficient energy [32] would be avialable from the proton absorbed to oxidise S_3^{3+} at pH values much below 6, assuming a redox potential for Q of $\cong -130\,mV$ [33]. These arguments are valid no matter how many partial reactions intervene between the states S_3^{3+} and S_0. A mechanism involving synchronous release of 4 protons must, in view of the evidence suggesting equal concentrations of the states $S_0 - S_3$ under continuous illumination, and the above argument, be considered unlikely. If further experimental evidence favours such a mechanism then attention will have to be turned to the probability of very strong cooperative interaction between the different oxidation states of the watersplitting complex.

Appendix

We would like to point out that despite the fact that the neutral red technique gave valuable qualitative results (compare Figs 2, 4, and 5), the extent of the observed absorbance change following excitation by short flashes, for the internal aqueous phase in the presence of an external buffer, was much higher than expected from a simple distribution of neutral red molecules between the internal and the external phases.

Let us assume (i) an internal volume of 50 l per mol chlorophyll, (ii) a maximal change in extinction

coefficient of neutral red of $\cong 30\ cm^{-1} \cdot m_M^{-1}$ from pH 8.5 to 5.5 [7, 34] and (iii) an ideal distribution (equal concentration) of neutral red between the outer and inner aqueous phases. With those data a maximal change of transmission per flash of $\cong 5 \times 10^{-6}$ could be estimated. The observed value was of the order of 5×10^{-4}, *i. e.* 100 times higher than the calculated one. Unless it is assumed that there is an accumulation of neutral red molecules on the "inside" of the thylakoids, such that the concentration of the indicator sensing the internal pH increases by 2 orders of magnitude (*e. g.* accumulation on the inside in freely soluble or in aggregated form as well as in the membrane with the polar group facing the inside of the thylakoids, see also ref. 34), this behaviour is not easily understood.

It is however not relevant to our qualitative interpretation since the results were always obtained by a subtraction of traces in the presence and in the absence of buffers.

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