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THE ELECTRIC GENERATOR IN PHOTOSYNTHESIS OF GREEN PLANTS I. VECTORIAL AND PROTOLYTIC PROPERTIES OF THE ELECTRON TRANSPORT CHAIN

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SUMMARY

Light induces the generation of an electrochemical potential difference across the functional membrane of photosynthesis of green plants. Experimental results on the electrochemical phenomena have been largely interpreted in terms of a vectorial alternating electron hydrogen transport system as originally hypothesized by Mitchell.

We asked whether or not the reaction coordinate of the electron transport crosses the membrane, and whether or not the protolytic reactions at either side of the membrane can be understood from the protolytic properties of the redox components involved. For this we studied the flash-light-induced protolytic reactions in the outer and the inner aqueous phase of the chloroplast inner disk membranes. Four sites of protolytic reactions were identified, two at either side of the membrane. One of these sites had to be attributed to the reduction of the terminal electron acceptor at the outer side of the membrane. Evidence is presented for the coupling of the other sites to the oxidation of water at the inner side of the membrane, to the reduction of plastoquinone at the outer side and its oxidation at the inner side, respectively. These results support Mitchell's hypothesis for the generation of an electrochemical potential difference by a vectorial electron transport system.

INTRODUCTION

Prior studies have demonstrated that an electric potential difference is generated across the functional membrane of photosynthesis in green plants [1–4] (for reviews, see refs 5 and 6). Moreover, illumination of chloroplasts causes uptake of protons from the outer phase [7, 8] and release of protons into the inner aqueous phase of the functional vesicles of the primary processes [9–11].

Abbreviations: FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; DCIP, 2,6-dichlorophenolindophenol.

The conversion of radiant energy into an electrochemical potential difference of the proton has been largely interpreted in terms of a vectorial alternating electron hydrogen transport system which goes back to a hypothesis of Mitchell [12, 13] (for an illustration, see Fig. 5). The water splitting system being at the inner side of the membrane, light reaction II activity results in the transport of an electron from water towards an acceptor at the outer side of the membrane. Both light reactions are linked up for in series operation by a hydrogen transfer in the opposite direction. Light reaction I again transfers an electron from a donor at the inner side to an acceptor at the outer side of the membrane.

Earlier studies gave support to this scheme, namely the following results: 1. Both light reactions contribute to the electric potential [3]. 2. Each light reaction promotes the uptake of one proton per electron from the outer phase [3]. 3. The reducing site of light reaction I is located towards the outer side of the membrane [14–16]. 4. There is some indication, that the rate limiting step of the electron transport [10] and plastocyanine, an electron carrier between the two light reactions [17], are located towards the inner side of the membrane.

However, the evidence so far being inconclusive, one has to take into account alternative mechanisms for the electrochemical potential generation, which are not based on a vectorial electron transport chain, e.g. special proton pumps or membrane Bohr effects, as proposed for protolytic reactions in mitochondria and chromatophores [36].

A discrimination between Mitchell's scheme and alternative models will be obtained if the following questions are unequivocally answered: 1. Are reaction sites of the electron transport located on different sides of the membrane? 2. Are the protons bound or released into either aqueous phase stoichiometrically matched by the protolytic properties of the electron carriers in the chain? 3. Do the kinetics of the protolytic reactions correspond to the redox kinetics of the respective electron carriers?

We studied the protolytic reactions at both sides of the functional membrane on excitation with short flashes. Four sites of protolytic reactions were identified, two proton binding sites at the outer side of the membrane, in agreement with prior studies [3], and two sites of proton release into the inner phase. Each of these sites reacts with one proton (terminal acceptor benzylviologene) if one electron is transferred through the chain on excitation with a "single-turnover flash". It is shown that one of these sites is attributable to the reduction of the terminal acceptor at the outer side of the membrane. There is evidence that the other three sites are attributable to the reduction of plastoquinone at the outer side of the membrane, and to the oxidation of water and plastoquinone at the inner side, respectively.

As in prior studies [3] the kinetics of the protolytic reactions, as indicated by absorption changes of cresol red, came out considerably slower than the redox-reaction kinetics. The reasons for this being so will be evaluated in a subsequent paper.

EXPERIMENTAL

Chloroplasts

The experiments were carried out with aqueous suspensions of broken chlo-

roplasts. Spinach chloroplasts were prepared as in ref. 18. Typical phosphorylation rates of these chloroplasts after thawing were around 50 nM/ μ M chlorophyll per s. Chloroplasts were suspended in a 20-mm spectrophotometer cell containing the following standard reaction medium: tricine, 1 mM (pH 8); KCl, 20 mM; MgCl₂, 2 mM; benzylviologene, 10 μ M. The average chlorophyll concentration in the cuvette was 10 μ M. Deviations from these conditions are indicated in the legend.

Excitation and record of transient absorption changes

The absorption cell was mounted in a rapid-kinetic spectrophotometer. Photosynthetic electron transport was excited with a saturating flash of light short enough (halftime 15 μ s) to turn over each light reaction only once ("single-turnover flash"). Transient absorption changes were recorded. The signal to noise ratio was increased by averaging over several transients induced by repetitive flashes. This method has been described in detail, elsewhere [19]. At the low intensity of the measuring light (300 erg/cm² per s) the observed absorption changes were independent of the measuring light. The absence of scattering artifacts in experiments at a low time resolution (about 1 s) has been checked by comparison of absorption changes which have been measured with different apertures between the spectrophotometer cell and the photomultiplier cathode.

Electron transport

The number of electrons transferred per flash has been determined from the oxygen evolution under repetitive excitation with "single-turnover flashes" [20, 21]. Because of the repetitive excitation in the experiments on the oxygen evolution as well as in those on absorption changes the influence of the induction phenomena of the water-splitting system [22, 23] was practically zero.

Electric potential generation

The electric potential generated on flash light excitation across the functional membrane of photosynthesis has been measured via the absorption changes around 520 nm. These absorption changes are due to a pseudolinear electrochromic response of chloroplast bulk pigments (here mainly carotenoids) to the strong electric field across the thylakoid membrane. Evidence for this being so has been provided by three independent lines of experiments: kinetic ones [1–3], spectroscopic ones [2, 4], and for chromatophores by the induction of diffusion potentials [2, 4] (for reviews see refs 5 and 6).

Protolytic reactions

As in prior work [3] a pH-indicating dye has been used for measuring pH changes in the external phase of the inner chloroplast membrane system. For three reasons we preferred cresol red for a pH around 8: The peak absorption changes of cresol red at 574 nm are superimposed by very little absorption changes of intrinsic chloroplast pigments (see Fig. 1). Cresol red at a concentration of 30 μ M is a negligible uncoupler and it inhibits photophosphorylation by less than 5%. From the beginning of studies with pH-indicating dyes in submicroscopic membrane systems [25] there has been discussion of whether or not absorbance changes from these dyes indicate pH changes, binding changes or solvatochromic effects [26, 27]. We

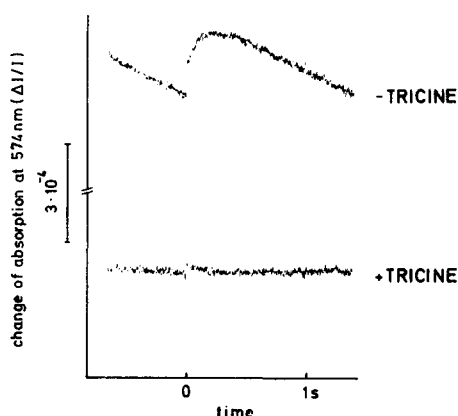


Fig. 1. Absorption changes of cresol red at 574 nm induced by a single-turnover flash of light at $t = 0$. Above: without addition of buffer, pH 8. Below: on addition of tricine-NaOH buffer, 10 mM, pH 8. Standard reaction medium except for buffer. Cresol red $30 \mu\text{M}$. Average over 110 flashes, repetition rate 0.5 cycles/s.

used cresol red at a concentration of $30 \mu\text{M}$, which exceeds the binding capacity of chloroplasts at $10 \mu\text{M}$ chlorophyll at least by one order of magnitude. By two tests we made certain that the observed absorption changes indicated pH changes: 1. The extent of the absorption changes at 574 nm was reciprocal to the buffer capacity of the suspension. 2. The difference of absorption changes at 574 nm plus/minus buffer (see Fig. 1) was compared with the difference measured at 443 nm. Their ratio was -2.5 . This coincides with the expectation for a shift of cresol red from the protonated (443 nm) to the unprotonated (574 nm) form.

The rise of absorption at 574 nm (Fig. 1) indicates alkalinisation.

Calibration of pH-indicating absorption changes

We calibrated the absorption changes of cresol red into the number of protons bound or released, respectively, by two different methods: 1. By measuring the absorption changes on mixing of a known amount of acid into the suspension. 2. By determining the buffer capacity of the suspension by a glass electrode and taking into account the absorbance over pH curve of the indicator, as in ref. 3. Calibration by these two different methods yielded the same results within a statistical error of 10 %.

The first calibration method had a response time below 1 s, while the second one was rather slow. In the first case a pH difference across the functional membrane of photosynthesis would not have equilibrated in contrast to the second case. The agreement between the calibrations carried out by the two methods reveals that the buffering capacity of the internal phase is small as compared with that of the external phase. This holds for a pH around 8, where our experiments were carried out. This finds support from the fact that the calibrations were independent of the presence of the proton permeability-increasing agent carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP).

It has been reported on the influence of e.g. K^+ -specific ionophores on the ex-

tent of pH-indicating fluorescence changes [28]. This effect was below 5 % at the frozen chloroplast preparations we used in the experiments.

RESULTS

We studied the protolytic reactions on flash excitation of photosynthesis for three different electron acceptor conditions:

1. Benzylviologene. Due to its low midpoint potential (-0.36 V) this acceptor interacts with the reducing site of light reaction I only. Benzylviologene catalyzes the reduction of O_2 [29], which at a pH around 8 leads to the binding of one proton per electron, no matter whether the end product is H_2O_2 or H_2O .

2. Ferricyanide (low concentration). According to its high midpoint potential one might expect ferricyanide to oxidize both photochemically active reaction centers. However, due to kinetic factors at concentrations below 1 mM it accepts electrons efficiently from light reaction I only.

3. Ferricyanide (high concentrations) and ferricyanide plus 2,6-dichlorophenolindophenol (DCIP). At higher concentrations (around 10 mM) ferricyanide competes with light reaction I as an oxidant for plastoquinone [30]. This competition becomes effective even at lower ferricyanide concentrations if the lipophilic cofactor DCIP is added [31]. Recent studies on the action of the electron transport inhibitor dibromothymoquinone have revealed that ferricyanide and ferricyanide plus DCIP can accept electrons from plastoquinone at a site located before plastocyanin [32, 35] (see Discussion). The low pK of the first protonized stage of ferrocyanide ($pK = 4.25$ [33]) makes sure that the reduction of ferricyanide does not lead to the binding of a proton at pH 8.

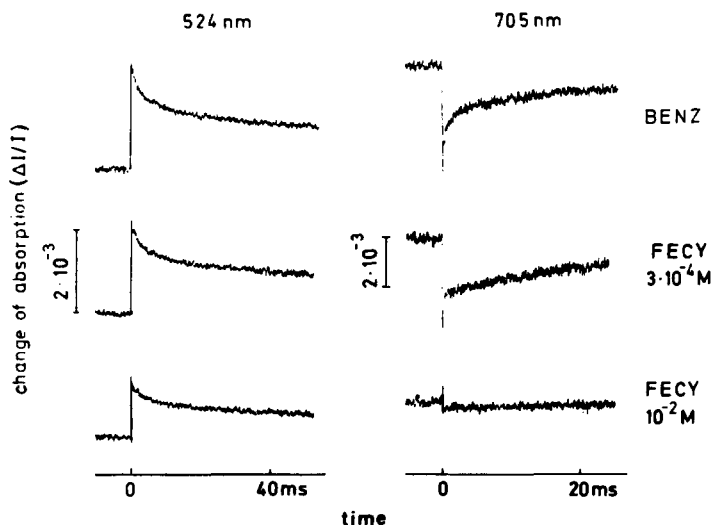


Fig. 2. Absorption changes at 524 nm and at 705 nm under different acceptor conditions induced by a single-turnover flash at $t = 0$. Standard reaction medium, except for electron acceptors. BENZ, benzylviologene; FECY, ferricyanide. Average over 225 (524 nm) and 110 (705 nm) flashes, respectively, repetition rate 0.5 cycles/s.

We have checked whether the above specified acceptor conditions hold true under the conditions of our flash light experiments. To do so we have measured the absorption changes at 705 nm, indicating light reaction I activity [34] and the absorption changes at 524 nm, indicating the electric potential difference with equal contributions from both light reactions [3]. The result is depicted in Fig. 2.

It is known, that in the presence of benzylviologene both light reactions are active [29]. This is reflected by the full extent of the absorption changes at 705 and at 524 nm in the upper row of Fig. 2. In the presence of ferricyanide at low concentrations the extent of both absorption changes remains unaltered. Thus it is obvious that again both light reactions are active. At higher ferricyanide concentrations (Fig. 2, lower row) the absorption change at 705 nm vanishes. However, the appearance of one half of the absorption change at 524 nm demonstrates that light reaction II is still active under these conditions. The same result was obtained with ferricyanide plus DCIP. Light reaction activity and electric potential generation under the three acceptor conditions from Fig. 2 are summarized in Table I.

TABLE I

SUMMARY OF THE EXPERIMENTAL RESULTS FROM FIGS 4-6

$\Delta\varphi$, the electric potential generated (relative units); LR I, light reaction I activity (relative units); LR II, light reaction II activity (relative units); $\Delta H_0^+/\Delta e^-$, protons released into the outer phase per electron; $\Delta H_1^+/\Delta e^-$, protons released into the inner phase per electron.

	$\Delta\varphi$ (524 nm)	LR I (705 nm)	LR II (calculated)	$\Delta H_0^+/\Delta e^-$ (574 nm)	$(\Delta H_0^+ + \Delta H_1^+)/e^-$ (574 nm) (+FCCP)	$\Delta H_1^+/\Delta e^-$ (calculated)
Benzylviologene 1		1	1	-2	0	+2
Ferricyanide ($3 \cdot 10^{-4}$)	1	1	1	-1	+1	+2
Ferricyanide (10^{-2})	$\frac{1}{2}$	0	1	0	+1	+1

We measured the protolytic reactions in the outer phase via the absorption changes of cresol red. Since a pH difference across the membrane relaxes with a half time of several seconds, the absorption changes of cresol red when measured with a 100-ms time resolution indicate pH changes in the outer phase, only.

The absorption changes of cresol red on flash excitation are depicted in the left column of Fig. 3 and Fig. 4, respectively. The calibration procedure of absorption changes into protons per electron ($\Delta H^+/\Delta e^-$) has been specified under Experimental. As obvious from a comparison of the two upper traces (left row) in Figs 3 and 4 about $2 H^+/e^-$ ($1.9 \pm 10\%$ in 10 experiments) were taken up from the outer phase if benzylviologene was the terminal electron acceptor. In the presence of ferricyanide (low concn) only $1 H^+/e^-$ was taken up. These values are inserted into the 4th column of Table I.

As discussed above, both of these acceptors are reduced by light reaction I. However, while reduction of benzylviologene ultimately leads to the binding of $1 H^+/e^-$ the reduction of ferricyanide does not. So we may conclude that the uptake

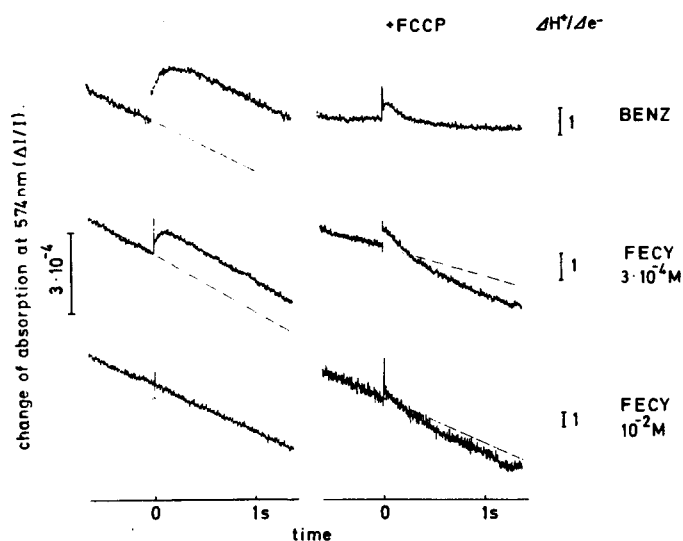


Fig. 3. Absorption changes of cresol red at 574 nm induced by a single-turnover flash of light at $t = 0$. Left: in the absence of FCCP. Right: in the presence of FCCP ($1 \mu\text{M}$). Standard reaction medium except for electron acceptors and for no buffer, pH 8. Average over 110 flashes, repetition rate 0.5 cycles/s.

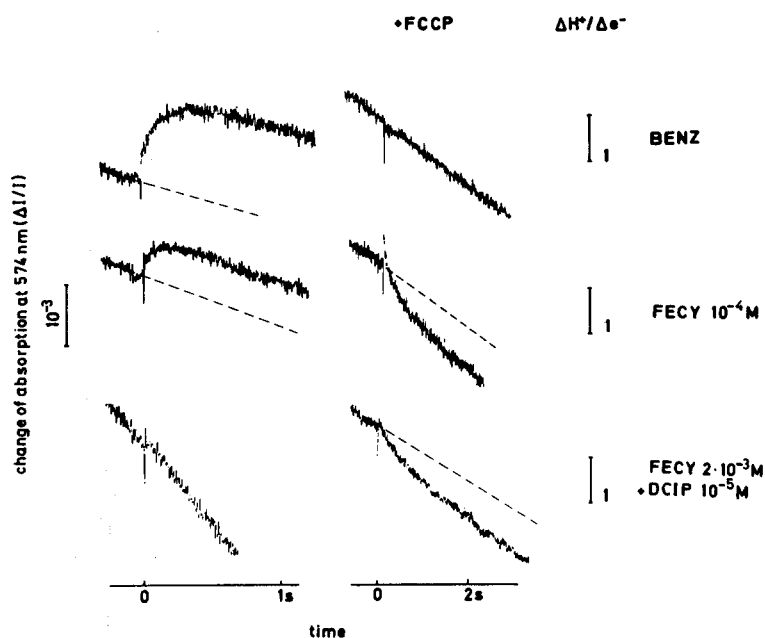


Fig. 4. Absorption changes of cresol red at 574 nm induced by a single-turnover flash of light at $t = 0$. Conditions as in Fig. 5 except for reduced repetition rate: 0.1 cycles/s and for the presence of DCIP.

of one of the two protons from the outer phase reflects the protolytic properties of the terminal electron acceptor, which is reduced at the outer side of the membrane. The latter conclusion agrees with other independent evidence, showing the accessibility of the reducing site of light reaction I to large antibodies from the outer side [14–16].

A comparison of the two lower traces (left) in Figs 3 and 4, respectively, shows a change in proton uptake from the outer phase. While it is $1 \text{ H}^+/\text{e}^-$, if ferricyanide accepts from light reaction I, it is zero, if ferricyanide at higher concentrations or with DCIP accepts between the two light reactions. This result is inserted into the 4th column of Table I.

We measured the protolytic reactions in the internal phase of the inner chloroplast vesicles by the following indirect method: As shown under Experimental the contribution of the internal phase to the total buffering capacity of a chloroplast suspension is below 10 % at a pH around 8. Thus, the absorption changes of cresol red will indicate the net proton production or consumption from both sides of the membrane if the absorption changes are measured at a time scale long enough for the equilibration for pH changes across the functional membrane. We increased the velocity of equilibration by adding the proton permeability-increasing agent FCCP. Under these conditions the equilibration occurred in less than 1 s.

The absorption changes of cresol red in the presence of FCCP, which indicate the net proton production at both sides of the membrane, are depicted in the right column of Figs 3 and 4, respectively. It is obvious from a comparison of the two upper traces (right) in each of Figs 3 and 4, that there is no net production of protons if O_2 via benzylviologene is the terminal electron acceptor, while $1 \text{ H}^+/\text{e}^-$ is produced in the presence of ferricyanide (low concn). This is inserted into the 5th column of Table I. Comparison with the proton uptake at the outer side of the membrane shows: with benzylviologene there is a release of two protons per electron into the inner phase, since two protons are taken up from the outer one and the net production is zero, and with ferricyanide (low concn) again two protons are released into the inner phase as the uptake from the outer phase is one and the net production one, too.

So there is no change in the proton release into the inner phase, if benzylviologene is substituted by ferricyanide (low concn) as terminal electron acceptor. This corroborates the above conclusion that the one proton-uptake site, which is deactivated with ferricyanide (low concn) reflects the protolytic properties of the terminal electron acceptor which is reduced at the outer side of the membrane.

When ferricyanide in higher concentrations (10 mM) was present as electron acceptor between the light reactions, the indicator cresol red became partly oxidized which resulted in a loss in indicator sensitivity (see scale bar in the lower right trace in Fig. 3). Since the oxidation reaction was rather slow, calibration of the signals was still possible. The oxidation did not occur with ferricyanide at lower concentration plus DCIP (see scale bar in the lower right of Fig. 4). It is obvious from the lower traces in Figs 3 and 4 that there is a net production of $1 \text{ H}^+/\text{e}^-$ if ferricyanide acts as electron acceptor between the two light reactions. As there is no proton uptake from the outer phase under these conditions, $1 \text{ H}^+/\text{e}^-$ is the proton release into the inner phase.

So when moving from lower ferricyanide concentrations to higher ones, which accept between the light reactions, two sites of protolytic reactions are de-

activated lying on different sides of the membrane. Only one of the four protolytic reaction sites is active even at high ferricyanide concentrations, a site of proton release into the inner phase.

These results on the protolytic reactions have been derived from the extent of the pH-indicating absorption changes of cresol red, while the kinetics have been left out of consideration (see Discussion).

The protolytic reactions have been measured under repetitive excitation with flash light. The repetition frequency was 0.5 Hz (Fig. 3) and 0.1 Hz (Fig. 4), respectively. In the absence of FCCP the first frequency will induce a considerable acidification of the inner phase. The question arises whether our results depend on this acidification. The agreement between the results which have been obtained at frequencies differing by a factor of 5 clearly demonstrates that the influence of the internal acidification was negligible in our experiments.

DISCUSSION

The above experiments have led to the identification of four protolytic reaction sites in the functional membrane of photosynthesis: two proton-uptake sites at the outer side of the membrane, in agreement with prior studies [3], and two sites of proton release into the inner phase. One of these sites is attributable to the reduction of the terminal electron acceptor, which occurs at the outer side of the membrane.

The interpretation of the remaining sites depends largely on our knowledge on the acceptor site of ferricyanide (high concn) and ferricyanide plus DSCP, respectively, between the two light reactions. As mentioned above, ferricyanide can accept electrons from plastoquinone (W. Haehnel, cited in ref. 32) but before the inhibition site of the electron blocker dibromothymoquinone [32]. This inhibitor does not block cyclic electron flow around light reaction I mediated by redox cofactors [32]. On the other hand, cyclic electron transport is blocked by CN^- which interacts with copper in plastocyanin but not with the iron in cytochrome *f* [35]. These results may be interpreted as follows: Cyclic electron flow around light reaction I involves plastocyanin. Dibromothymoquinone blocks electron transport at a site located between plastoquinone and plastocyanin. Ferricyanide (high concn) can accept electrons between plastoquinone (included) and plastocyanin (excluded).

Under our experimental conditions (flash light) ferricyanide (high concn) as electron acceptor between the light reactions was more effective than light reaction I which oxidizes plastoquinone with a half time of 10 ms. Thus we may conclude, that the direct or indirect oxidation of plastohydroquinone by ferricyanide (high concn) takes less than 10 ms. The apparent time resolution of the pH indicator cresol red in our above experiments was some 10 ms (see below). So we would not expect the dye to catch protolytic reactions which may follow the rapid reduction of plastoquinone and its rapid reoxidation by ferricyanide. Thus for experiments on protolytic reactions at a time resolution of about 100 ms we may regard ferricyanide (high concn) as a non-proton-binding substitute for the proton-binding intrinsic electron carrier plastoquinone as electron acceptors between the two light reactions.

We have shown that two protolytic reaction sites are deactivated if ferricyanide at higher concentrations starts to accept electrons at the above specified site between the light reactions. Since, at the time resolution of our experiments, this was

equivalent to substituting plastoquinone by the non-proton-binding ferricyanide, we may conclude: one deactivated site of proton uptake from the outer phase is associated with the reduction of plastoquinone at the outer side of the membrane, the site of proton release into the inner phase is associated with the oxidation of plastoquinone at the inner side of the membrane.

One site of proton release into the inner phase has still to be interpreted. This site was active even if the electron transport chain was shortened down by ferricyanide (high concn) (see Table I). The best candidate for this site is the oxidation of water, which then has to be visualized as to occur at the inner side of the membrane.

Thus our results on light-reaction activity, electric potential generation and protolytic reactions (see Table I) are compatible with the vectorial alternating e^- -H-transport system which is illustrated in Fig. 5. This scheme found independent support from other results, which have been cited in the Introduction.

There is one argument which may shed doubt on the above interpretation: The kinetics of proton binding from the outer phase as indicated by cresol red (Figs 3 and 4) was slow as compared with the expected reduction kinetics of the electron carrier plastoquinone and the terminal acceptor, respectively. Since protolytic reactions in water are among the most rapid reactions, there are only two possibilities

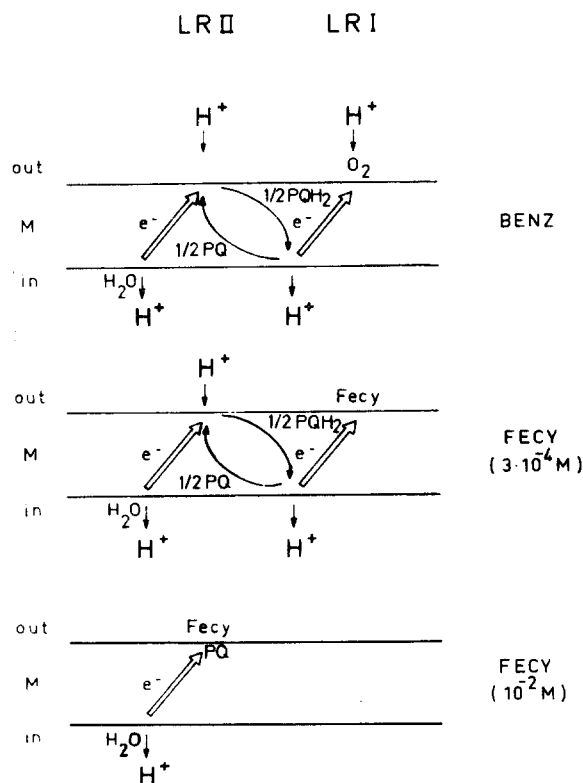


Fig. 5. Vectorial electron transport scheme compatible with the experimental results summarized in Table I. For details see text. Electron acceptors as in Fig. 4. LR I and LR II: light reactions I and II, respectively; PQ, plastoquinone.

for this kinetic discrepancy, either there is a diffusion barrier between the redox-reaction site and the outer water phase, or our above conclusions are wrong. Preliminary evidence for the existence of such a diffusion barrier for protons has already been reported [3]. These studies have been continued to characterize the chemical nature of this barrier which is rate limiting for protolytic reactions with the outer phase. The results will be reported in a subsequent paper in this series.

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