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REACTION KINETICS OF INTERMEDIATES OF THE PHOTOSYNTHETIC CHAIN BETWEEN THE TWO PHOTOSYSTEMS

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SUMMARY

1. Measurements are reported of light-induced changes in absorbance and in the yield of chlorophyll *a* fluorescence of intact algae. Most experiments were done with the red alga *Porphyridium aerugineum*. In alternating non-saturating illumination with light mainly absorbed by Photosystem 1 and 2, respectively, a periodical oxidation and reduction of the components of the photosynthetic chain, P700, cytochrome *f*, plastoquinone and Q, the primary electron acceptor of Photosystem 2, was observed.

2. Lags observed in the oxidation and reduction kinetics agreed with the concept that the midpoint potentials of these compounds decrease in the order given above. The largest difference was observed between plastoquinone and cytochrome *f*; the kinetics of Q and plastoquinone were similar and suggested only a small potential difference between these compounds.

3. Relative pool sizes of 15:1.8:1 electron equivalent were observed for plastoquinone, cytochrome *f* and P700. Quantum efficiencies suggested the same high rate of electron flow through plastoquinone and cytochrome *f*. The kinetics of these components indicated that the only large pool in the chain between the two light reactions is that of plastoquinone.

4. Difference spectra obtained under conditions where all the above-mentioned components were alternately reduced and oxidized gave no evidence for cytochrome *b* participation in the chain between the two light reactions.

INTRODUCTION

During the last years, various investigations have contributed considerably to a more detailed understanding of the reactions in the photosynthetic chain and the nature of the primary and secondary electron transport processes in photosynthesis (reviews are given in refs. 1–3). Nevertheless, there are still several uncertainties and conflicting views, *e.g.* about the inclusion of compounds like *b*-type cytochromes in the chain, and about the identity and properties of the primary electron acceptors

Abbreviations: Q, the primary electron acceptor of Photosystem 2; P700, chlorophyllous pigment absorbing at 700 nm, primary electron donor of Photosystem 1; DCMU, 3 (3,4-dichlorophenyl)-1,1-dimethylurea.

and of other components of the chain and even the general concept of the so-called Z scheme is not accepted by all investigators (*e.g.* ref. 4).

Many of these studies have been carried out with isolated chloroplasts and with subchloroplast particles, usually from spinach. Although there are obvious advantages in working with subcellular systems, they suffer the disadvantage that the isolation procedure and further treatments during the preparation may induce artifacts.

In this paper we shall report a quantitative study of the kinetics and pool sizes of intermediates in the reaction chain between the two primary light reactions in intact cells of red algae. An advantage of red algae, compared to the more usually employed green algae, is a better optical separation of the two pigment systems. Most experiments were done with *Porphyridium aerugineum* which has the added advantage of a low absorption in the α-region of cytochromes, due to the absence of phycoerythrin.

MATERIALS AND METHODS

Porphyridium aerugineum, *Porphyridium cruentum* and *Chlorella vulgaris* were grown, as described elsewhere[5], in the media given by refs. 5, 6 and 7 (MC medium). The algae were harvested by centrifugation and resuspended in fresh growth medium, usually to give an absorbance of 0.4/mm at 680 nm, and transferred to 1-mm quartz vessels.

Measurements of changes in absorbance were performed by means of an apparatus described earlier[8]. Automatic timers provided fixed cycles of illumination. For measurements in the ultraviolet region the light source was a deuterium or a xenon arc; suitable glass and liquid filters served to minimize stray light from the monochromator and spurious effects due to the actinic illumination. The actinic light was filtered by means of a combination of interference and absorption filters. For *P. aerugineum* System 1 light was obtained by means of the following filter combinations: Schott RG 665, 3 mm, combined with AL 679 or 683 to give a band with maximum at 680 or 683 nm, respectively; Schott BG 12, 2 mm, and Corning 4-96 (440 nm); BG 12, 1 mm, BG 38, 4 mm and Corning 5-56 (430 nm). System 2 light (618 nm) was obtained with AL 618, RG 610, 2 mm and an interference filter with cut-off above 644 nm. A Balzers Calflex C filter was added to each filter combination. The intensities of actinic light given are those incident at the place of the vessel. The signal-to-noise ratio of the measurements was enhanced by means of a signal averager (Nuclear Chicago Model 7100 data retrieval computer).

Changes in the yield of chlorophyll *a* fluorescence were measured in the same apparatus. Fluorescence was excited by a beam of modulated light, filtered by Balzers B40-564, Calflex C and Schott BG 38, 4 mm. The intensity ($5 \cdot 10^{-2}$ nano-Einstein$\cdot$cm$^{-2}\cdot$sec^{-1}) was too weak to affect significantly the yield of fluorescence. Changes in the yield were brought about by the same (non-modulated) actinic beams that were used to bring about changes in absorbance. The detecting apparatus responded only to modulated light, and thus only recorded changes in the yield of chlorophyll *a* fluorescence excited by the modulated beam (see ref. 9). Fluorescence was filtered by AL 679 and RG 665, 2 mm.

Absorbance of algal suspensions was measured with a Cary 14 spectrophoto-

meter equipped with a scattered light attachment or with a Zeiss PMQ II absorption spectrophotometer equipped with opal glass. The apparent absorbance at 740 nm was subtracted from the absorbances measured at other wavelengths in order to correct for light scattering.

All experiments were performed at room temperature (21–23°).

RESULTS AND INTERPRETATION

Kinetics of Q, plastoquinone, cytochrome f and P700

Fig. 1 shows the kinetics of absorbance and fluorescence changes in *P. aerugineum* in alternating illumination with light mainly absorbed by System 1 and by System 2, respectively (to be called "System 1" and "System 2 light"). As shown by Tracings a–d, System 1 caused an oxidation of P700 (measured as an absorbance decrease at 703 nm), cytochrome *f* (measured at 553 nm), plastoquinone (260 nm) and Q, the primary electron acceptor of Photosystem 2, measured by the yield of chlorophyll *a* fluorescence. System 2 light caused a reduction of these four components of the electron transport chain. Similar kinetics were observed with *P. cruentum*.

In order to check whether the absorbance changes at 260 nm were due to plastoquinone, we measured the absorption difference spectra, shown in Fig. 2. The spectra had a maximum at about 260 nm and an isosbestic point at 277 nm, and were very similar to those obtained earlier with blue-green[10] and green algae[11] and with spinach chloroplasts[12]. The shape is similar to that of oxidized *minus* reduced plastoquinone.

As was earlier observed with *Anacystis nidulans*[10], 3(3,4-dichlorophenyl)-1,1

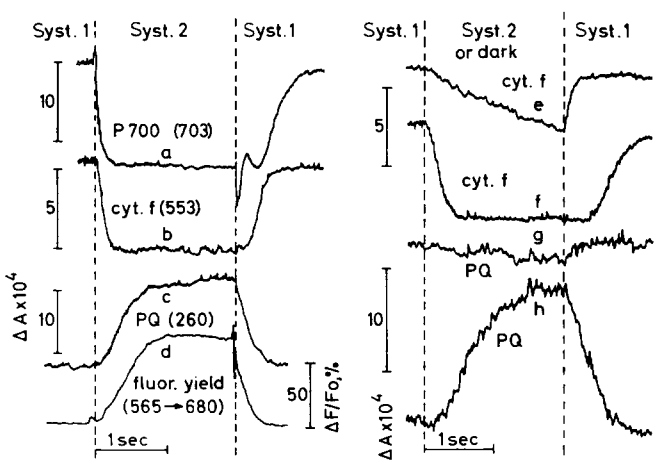

Fig. 1. Kinetics of absorbance changes and of the yield of chlorophyll *a* fluorescence in *P. aerugineum* in alternating 2-sec System 1 and 2 light. The wavelength of measurement is given between parentheses; fluorescence was excited by weak light of 565 nm (see MATERIALS AND METHODS). The actinic illumination was: left hand row: System 1, 430 nm, 1.7 nanoEinsteins·cm^{-2}·sec^{-1}, except tracing b, 680 nm, 2.7; System 2, 618 nm, 2.2; right hand row: System 1, 683 nm, 1.3; System 2, 618 nm, 1.1. For tracings e and g System 2 light was replaced by darkness. The tracings are the average of 10–40 experiments. An upward moving trace indicates a decrease of absorbance or an increase of fluorescence yield. The rapid spikes in tracings a and d are fluorescence artifacts. The absorbance at 680 nm was 0.4. Curve a, P 700 (703 nm); b, cytochrome *f* (553 nm); c, plastoquinone (260 nm); d, fluorescent yield (565→680 nm); e and f, cytochrome f; g and h, plastoquinone.

dimethyl urea (DCMU) inhibited the reduction of plastoquinone by System 2, but not the oxidation by System 1. The difference spectrum of *P. aerugineum* under these conditions was similar to that of Fig. 2.

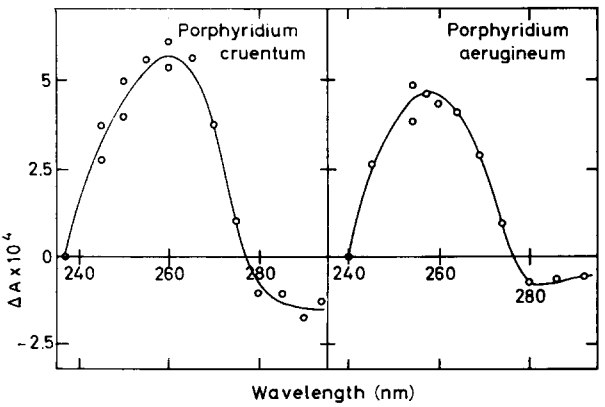

Fig. 2. Steady-state absorption difference spectra (System 1 light *minus* 2 light) obtained under conditions of alternating illumination similar to those of Fig. 1. For *P. cruentum*, a band of 560 nm was used for System 2 illumination. The absorbance at 680 nm was 0.3 and 0.2, respectively.

Comparison of Tracings a–d of Fig. 1 indicates that in System 2 light P700 becomes reduced first, cytochrome *f* somewhat more slowly, and that plastoquinone reduction was markedly retarded and that it was still largely oxidized when most of the cytochrome was already reduced. The kinetics of the reduction of Q, as reflected by the fluorescence yield were similar to those of plastoquinone, but the reduction of Q was slightly retarded. Upon System 1 illumination there was a marked lag in P700 and cytochrome *f* oxidation (*cf.* ref. 13), which persisted until most of the plastoquinone was oxidized. Curves e–h allow a comparison with the kinetics of cytochrome *f* and plastoquinone in alternating System 1 light and darkness. Upon darkening cytochrome *f* was slowly reduced, while plastoquinone apparently remained completely or almost completely oxidized in the dark.

The recordings of Fig. 1 were obtained at intensities of light which were below saturation for photosynthesis. For this reason one may assume that "dark" reactions were not rate limiting, and that the components of the chain between the two light reactions were in equilibrium with each other throughout the experiment. The kinetics thus reflect the oxidation–reduction potentials of the various compounds, and are in agreement with the usually accepted order of these compounds in the photosynthetic chain, the oxidation–reduction potential of P700 being the highest one, and that of Q the lowest one, of these four components. The largest gap in oxidation–reduction potential apparently exists between cytochrome *f* and plastoquinone: Curves f and h show that plastoquinone was still 90 % oxidized while cytochrome *f* was about 90 % reduced.

Comparison of the kinetics of chlorophyll *a* fluorescence and of the absorbance changes at 260 nm indicated that Q was always reduced somewhat more slowly than plastoquinone by System 2 light, but oxidized somewhat faster by System 1 light, suggesting that the midpoint potential of Q is slightly more negative than

that of plastoquinone. A non-linear relation between the redox level of Q and the fluorescence yield of chlorophyll *a*, as described by Joliot and coworkers[14,15], would bring the curves for these two components more closely together, but an analysis based upon the average of several recordings, such as those of Fig. 1, indicated that this would not account for all of the difference.

Fig. 3 compares the intensity dependence of the oxidation–reduction level of plastoquinone and of the fluorescence yield of chlorophyll *a*. The steady state levels, reached after one or a few seconds of illumination with orange light (610–625 nm, mainly absorbed by System 2) were plotted. The orange light was alternated with blue (System 1) light in order to keep the algae in "Pigment state 1" (formerly called State Q', refs. 9, 16–18).

At low intensities, the fluorescence yield rapidly increased with increasing intensity, followed by a much slower increase above about 1 nanoEinstein·cm^{-2}·sec^{-1}. At this intensity, the redox level of Q (and thus the closure of the traps of System 2) is probably adjusted to give a balanced rate of oxidation and reduction (*cf.* ref. 15), determined by the relative absorption of orange light by the two pigment systems. Experiments with a broad band of more intense light (520–650 nm) showed a monotonic increase of the fluorescence yield with the intensity, up to a level not far from that observed in saturating light in the presence of DCMU. At these intensities the electron transport in the chain presumably becomes rate limiting, causing a further accumulation of reduced Q. The fluorescence yield in moderately strong blue light, which was within rather wide limits independent of the intensity, was assumed to represent complete oxidation of Q. The reduction level of plastoquinone appeared to be saturated at lower intensities than that of Q. No evidence was obtained for a further reduction at still higher intensities; at increasing intensities

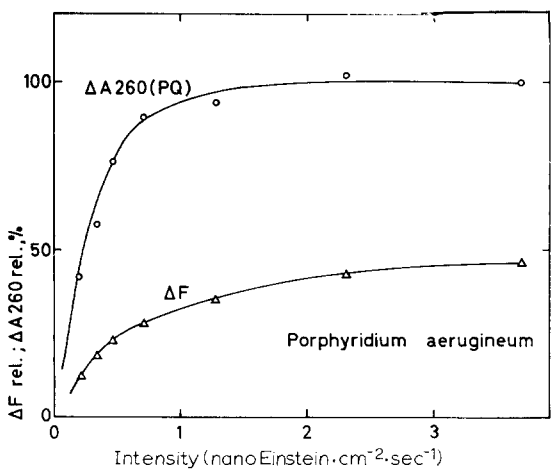

Fig. 3. Relation between the oxidation–reduction level of plastoquinone and the yield of chlorophyll *a* fluorescence. The algae were subjected to alternating cycles of 7 sec System 1 light (440 nm, 1.2 nanoEinsteins·cm^{-2}·sec^{-1}) and 1–5 sec of System 2 light (618 nm) of various intensities. The relative maximum sizes of the absorbance decreases at 260 nm or of the changes in fluorescence yield reached during the System 2 illumination are plotted as function of the light intensity. The increase in fluorescence yield is plotted as percentage of the maximum increase in strong light with DCMU. The maximum yield in strong light with DCMU was 2.8 times the lowest yield without DCMU.

the absorbance change at 260 nm even decreased somewhat in size. We do not know if this represented a lower level of reduction of plastoquinone or whether the effect was caused by interfering absorbance changes due to some other compound.

Analogous results have been obtained by us with spinach chloroplasts[19].

Apart from the observed difference in redox level at high light intensities it may be stated that our observations indicate a small difference in oxidation–reduction potential between Q and plastoquinone. The results will be discussed further below.

Pool sizes and quantum requirements

Since the algae absorb relatively strongly in the ultraviolet region, we have attempted to apply a correction for the so-called "flattening" or "sieve" effect[20] when converting the absorbance changes into concentration changes of intracellular compounds. It has been shown[20] that the flattening effect causes a reduction of the absorbance when the pigments are contained in particles, especially at wavelengths of strong absorbance, and that this reduction is even stronger for (small) absorbance differences than for the absorbance itself[8].

An approximate correction was made by comparing the absorption spectrum of a suspension of *P. aerugineum* (corrected for scattering) with that of one which had been sonicated under N_2 for 3 min. Longer sonication times were found to give no further changes in the spectrum. The same method has been used for Chlorella[21]. For the absorbance we obtained a flattening factor of 0.80, 0.97 and 0.98 at 260, 553 and 703 nm, respectively. This corresponds, for spherical particles, to a factor of 0.63, 0.95 and 0.97, respectively[8], for absorbance differences at these wavelengths, which means that the absorbance changes of plastoquinone, cytochrome *f* and P700, respectively, should be divided by these numbers for a calculation of the amounts of compounds which react.

Using these corrections, we calculated pool sizes of the constituents of the chain from the highest absorbance changes under appropriate conditions (optimum

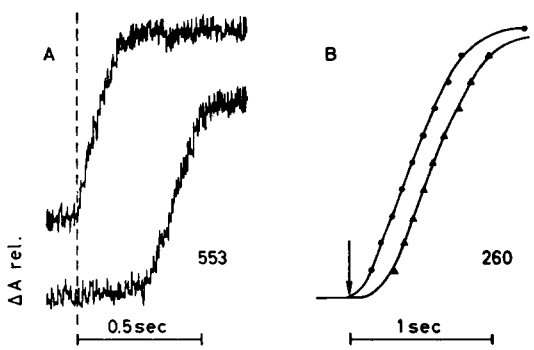

Fig. 4. (A) Kinetics of cytochrome *f* oxidation in far-red light (700 nm, 2.7 nanoEinsteins·cm^{-2}·sec^{-1}). Upper tracing: after 3 sec darkness following previous far-red illumination; lower tracing: after immediately preceding System 2 illumination (618 nm, 1.4 nanoEinsteins·cm^{-2}·sec^{-1}). The dashed line gives the time of onset of far-red light.
(B) Kinetics of plastoquinone reduction in System 2 light (618 nm, 1.4 nanoEinsteins·cm^{-2}·sec^{-1}). For clarity, the tracings were plotted from hand-smoothed recordings. Circles: after 5 sec darkness following previous System 1 illumination; triangles: after immediately preceding System 1 illumination. System 2 light was turned on at the arrow. The curves are not drawn to the same scale as those of A.

intensities of System 1 and System 2 light for plastoquinone and cytochrome *f*, alternating high light intensity and darkness with DCMU for P700). The relative pool sizes of P700, cytochrome *f* and plastoquinone in *P. aerugineum* were found to be 1, 1.8, and 15 electron equivalents, on basis of molar extinction coefficients of 73 (equal to that of chlorophyll *a* in acetone–water), 15.7 (ref. 22) and 14 $mM^{-1} \cdot cm^{-1}$ (ref. 23) at 703, 553 and 260 nm, respectively. The ratio for P700:cytochrome *f* is lower than the earlier reported[24] value which was based on measurements in the soret band of cytochrome, uncorrected for flattening.

An independent estimate of the relative pool sizes of some of the constituents of the chain by methods similar to those applied to chloroplasts[25,26] was made from the kinetics of plastoquinone reduction. Fig. 4B shows the absorbance decrease at 260 nm in *P. aerugineum* in orange light, after preceding far-red illumination strong enough to oxidize nearly all cytochrome *f* and most of the P700. The figure also shows the kinetics after an intervening dark time of 6 sec, which sufficed to give "spontaneous" re-reduction of P700 and cytochrome *f*, but did not change the oxidation state of plastoquinone. The delay of reduction of plastoquinone in the right as compared to the left hand curve apparently reflects the pool sizes of P700, cytochrome *f* and other possible intermediates. Comparison of the time (and thus the number of quanta) needed to effect 50 % reduction of plastoquinone, at which time P700 and cytochrome *f* are completely reduced already, gives a ratio of the combined pool sizes of P700 and cytochrome *f* to that of plastoquinone of 1:5.8, in good agreement with the directly measured ratio of 1:5.4 (see above). Moreover, one should take into account that the ratio obtained in this way will be somewhat too low, because P700 probably was not completely oxidized in the preceding far-red light and because there occurs also a "dark" reduction of P700 and cytochrome. The good correspondence between these data may suggest the absence from the chain of high potential compounds other than P700 and cytochrome *f*.

Fig. 4A shows analogous experiments on cytochrome oxidation in far-red light, (a) after preillumination with System 2 light and (b) after darkness. In the first experiment plastoquinone was in the reduced state at the onset of far-red light, in the second one it was in the oxidized state. It can be seen that these experiments would suggest a higher ratio (about 1:2.5) between the cytochrome and plastoquinone pools than is indicated by the other experiments. This effect is probably caused by side reactions, such as cyclic electron transport in System 1, which tend to delay cytochrome oxidation (*cf.* ref. 13).

From the rates of the absorbance changes and the absorbed light intensities we also calculated quantum requirements for cytochrome *f* and plastoquinone oxidation and reduction, using the same flattening corrections and molar extinction coefficients as mentioned above. The lowest quantum requirement for the oxidation of cytochrome *f*, calculated from the steepest part of the curves was found to be 1.9 *hν*/equiv at 680 nm, for the reduction: 2.3 *hν*/equiv at 618 nm. A correction was made for the spontaneous dark reduction. The corresponding numbers for plastoquinone were 1.4 and 1.7, respectively. Even at the time of maximum rate of cytochrome *f* reduction or oxidation, these reactions do not reflect the total rate of electron flow, as P700 and plastoquinone are also reduced and oxidized simultaneously, albeit at lower rates (see Fig. 1). Therefore, it may be stated that the quantum requirements for cytochrome and plastoquinone are in good agreement with each

other, and that they suggest the same high rate of electron transfer through both compounds.

Participation of b-type cytochromes

The results discussed above demonstrate that under conditions of alternating System 1 and System 2 illumination, like those of Fig. 1, both the primary electron acceptor, Q and donor P700, as well as the low potential component of the chain, plastoquinone and the high potential component cytochrome *f* are periodically oxidized and reduced. This implies that other components of the chain, if any, should change their redox state in the same manner, since, being part of the chain, they should rapidly enter into equilibrium with the above mentioned intermediates.

Since *b*-type cytochromes have often been proposed as intermediates of the chain between the two light reactions (*e.g.* refs. 27–29, see also refs. 30, 31) we have measured the difference spectrum in the α-band region of these cytochromes, in order to test this hypothesis.

Fig. 5 shows the result of such an experiment. The spectrum, obtained upon alternating System 1 and 2 illumination, shows the oxidation of only one cytochrome, cytochrome *f*, with an α band at 552–553 nm (probably closely related to Porphyra cytochrome 553, ref. 22), and gives no evidence for the participation of either cytochrome b_{559} or b_{563}. In a control experiment with the same regime of illumination, the oxidation and reduction of plastoquinone was observed, as in Fig. 1.

A similar experiment with *Chlorella vulgaris* also failed to give evidence for cytochrome *b* participation (Fig. 6). The spectrum, although complicated by other changes, shows a clear cytochrome *f* band at 553 nm. It gives no indication for the reaction of a comparable amount of cytochrome *b*.

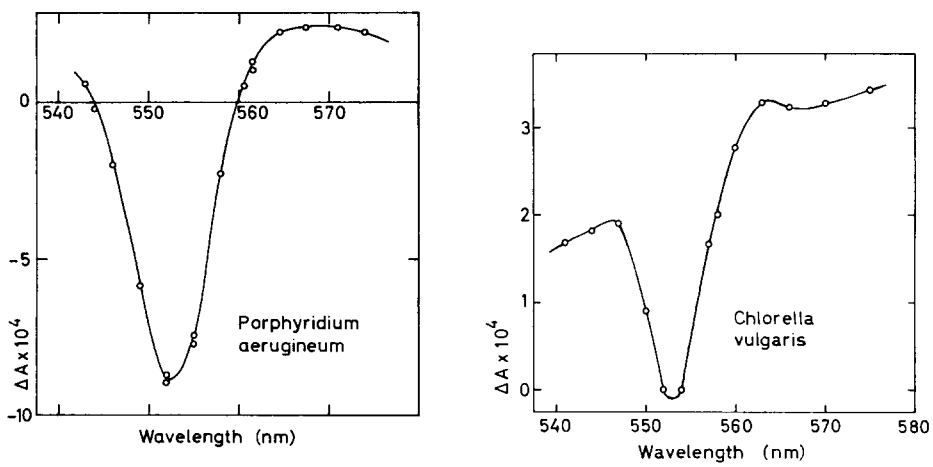

Fig. 5. Absorption difference spectrum (System 1 *minus* System 2) of *P. aerugineum* in alternating 2-sec System 1 (683 nm) and 2 (618 nm) light. The intensities were 4.5 and 2.3 nanoEinsteins·cm^{-2}·sec^{-1}, respectively. The absorbance at 680 nm was 0.4.

Fig. 6. Absorption difference spectrum (System 1 *minus* System 2) of *Chlorella vulgaris* in alternating 5-sec System 1 (700 nm) and 2 (643 nm) light. The intensities were 2.1 and 1.9 nanoEinsteins·cm^{-2}·sec^{-1}, respectively. The absorbance at 680 nm was 0.6.

DISCUSSION

The data presented in this paper are in agreement with the concept that plastoquinone, cytochrome *f* and P700 are in the main pathway of electron flow from System 2 to System 1. They also suggest that there is only a small difference in oxidation–reduction potential between Q and plastoquinone and that the biggest gap in redox potential is between plastoquinone and cytochrome *f*. The high efficiency observed for plastoquinone oxidation and reduction and the kinetics of the other components indicate the absence of a second large pool in the chain, either of a compound with the same or one with a higher redox potential than that of plastoquinone. This indicates that plastoquinone is identical to the Pool "A" revealed by studies of fluorescence and oxygen evolution of algae and chloroplasts (*e.g.* refs. 32–35). For "A" it has likewise been concluded[35] that its redox potential is only slightly higher than that of Q.

The relative pool sizes of plastoquinone and P700 (15:1) in *P. aerugineum* agree well with those calculated by MARSHO AND KOK[26] for "Q + A" and P700 in chloroplasts (12:1). STIEHL AND WITT[12] found a ratio of 14:1 for chloroplasts. The amount of plastoquinone relative to that of cytochrome appears to be smaller, about 8–9, in *P. aerugineum* than in chloroplasts. With regard to the above numbers it should be noted, however, that the pool size of plastoquinone may have been underestimated, because the pool may have been not completely reduced by the System 2 light used. This may also be true for P700; DCMU and higher intensities than for cytochrome *f* were needed to approximately saturate the absorbance change at 703 nm. On the other hand, the molar extinction coefficient of 73 $mM^{-1} \cdot cm^{-1}$ used for P700 may be slightly too high, in view of recent measurements of T. Hiyama and B. Ke (B. Ke, personal communication), which indicate a number of 64–70 for detergent-prepared System 1 particles, lower than earlier reported[36].

The similarity of the kinetics and of the intensity dependence at low light intensities of the chlorophyll *a* fluorescence and of the redox state of plastoquinone is in fact rather surprising, since the latter compound is a two-electron and Q is presumably a one-electron acceptor[37]. When a two- and a one-electron acceptor, with about the same midpoint potential, are in redox equilibrium as in solution, the ratios of concentrations of the oxidized and the reduced forms should be different at high and low redox potentials, according to the Nernst equation. In this respect it is interesting to note that FORBUSH AND KOK[35] arrived at a low potential difference between Q and "A" on the basis of a model in which both Q and A are one-electron acceptors. The much lower intensity of System 2 needed for saturation of the reduction level of plastoquinone than of Q, which is suggested by Fig. 3, might be due to a local change in pH at high intensity which may affect a pH-dependent equilibrium[38] between Q and plastoquinone. Furthermore, it is possible that the yield of fluorescence with DCMU in strong light may deviate from that which would occur without DCMU when Q is completely reduced, *e.g.* because of a different pigment state[16–18] in this condition.

The absence of a cytochrome *b* band in the difference spectrum of Fig. 5 strongly suggests that cytochrome *b* does not form part of the chain between the two light reactions in Porphyridium and Fig. 6 suggests the same for Chlorella. Evidence for the reaction of a *b* cytochrome in Porphyridium was only found by us in light–

darkness spectra. This was most clearly seen in the presence of *N*-methylphenazonium methosulphate, which abolished the cytochrome *f* absorbance change. A weak band at 563 nm was observed, presumably due to the same *b*-type cytochrome as observed by NISHIMURA[29] in *P. cruentum*. Under these conditions light caused an oxidation of this cytochrome only; the absorbance changes in alternating System 1 and 2 light were smaller than those observed upon alternating light and darkness. From the above results we conclude, contrary to Nishimura, that cytochrome b_{563} probably either reacts in a cyclic or in a non-photosynthetic (possibly respiratory) chain.

Recently, JOLIOT and co-workers[39], on the basis of flashing light experiments, proposed the existence of a second, low potential electron acceptor for System 2. The evidence given above does not rule out the possibility that this compound may be identical to, or react with cytochrome b_{559}, since the low potential acceptor would not become reduced to a significant extent under our experimental conditions. Recent evidence[40] indicates that there is a low (negative) potential cytochrome b_{559}, as well as a high potential one, in spinach chloroplasts.

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