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PRIMARY REACTIONS, PLASTOQUINONE AND FLUORESCENCE YIELD IN SUBCHLOROPLAST FRAGMENTS PREPARED WITH DEOXYCHOLATE

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SUMMARY

1. In subchloroplast fragments prepared with the detergent deoxycholate the primary reactions of Photosystem II could be studied at room temperature, because the secondary reactions were largely or completely inhibited.

2. The main quencher of chlorophyll fluorescence in these particles was the photosynthetically active pool of plastoquinone in its oxidized form. Its photoreduction in the presence of artificial electron donors was accompanied by a shift of a chlorophyll *a* absorption band. Its reoxidation in the dark was very slow, even in the presence of ferricyanide.

3. Of all the artificial electron donors tested $MnCl_2$ was by far the most efficient.

4. Measurements at room temperature of the C550 absorbance change confirmed its correlation with the primary electron acceptor. Its difference spectrum was broader and its extinction coefficient correspondingly lower than at liquid-N_2 temperature. In chloroplasts the C550 concentration was about 1 : 360 chlorophylls.

5. In the dark C550 was largely in the reduced state and its oxidation by plastoquinone took place in the presence of an artificial electron donor only, suggesting that the redox potential of C550 was increased by accumulated positive charges at the donor side of the reaction center.

6. The free radical 1,1′-diphenyl-2-picrylhydrazyl oxidized C550 directly in a 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-insensitive reaction. A DCMU-insensitive oxidation of C550 was observed at high ferricyanide concentrations as well, but probably in this case an endogenous electron donor was oxidized, which in turn oxidized C550 via the back reaction of the photochemical reaction.

7. The oxidized form of the primary electron donor, $P680^+$, accumulated in the light in the presence of deoxycholate and a low ferricyanide concentration. In chloroplasts the P680 concentration was about 1 : 360 chlorophylls.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPPH, 1,1′-diphenyl-2-picrylhydrazyl; C550, component responsible for absorption changes at 540 and 550 nm, correlated with the primary electron acceptor of Photosystem II; P700, P680, primary electron donors of Photosystems I and II, respectively.

8. The P 680 absorption difference spectrum and electron spin resonance could be explained by the oxidation of a chlorophyll *a* dimer. Repeated deoxycholate treatments progressively changed the spectra to those of a monomer. The monomer was still photochemically active.

9. A new interpretation of the difference spectrum of P700 is proposed: it may be the same as that of the difference spectrum of P680 if the bleaching at 700 nm is attributed to a band shift.

INTRODUCTION

Subchloroplast fragments enriched in Photosystem II have been prepared by a variety of detergents and mechanical methods [1] and some of them have been purified to a considerable extent [2, 3]. Although their composition has provided important clues to the structure of the photosynthetic apparatus, they have as yet not yielded new information on the primary reactions in Photosystem II.

We have studied subchloroplast fragments prepared with the negatively charged detergent, deoxycholate, according to the method of Bril et al. [4]. Until now, no photochemical activity of these particles has been reported. We found that they can be reactivated with artificial electron donors and acceptors. We have measured changes of absorbance, chlorophyll fluorescence yield, delayed fluorescence, and electron spin resonance induced by selective reactivation of parts of the electron-transport chain. In this way the primary donor and acceptor of Photosystem II could be studied.

METHODS

Subchloroplast fragments were prepared with sodium deoxycholate [4]. Briefly, spinach chloroplasts were prepared in 50 mM Tris buffer, pH 8.2, 0.4 M sucrose. Then deoxycholate and KCl were added to yield final concentrations of 100 μg chlorophyll/ml, 0.4 % deoxycholate and 0.15 M KCl. In some experiments this suspension was used without further treatment. Most experiments were done with the pellet obtained by a 30-min centrifugation at 10 000 $\times g$ ("deoxycholate 2 particles"), resuspended in Tris buffer with 0.04 % deoxycholate and 15 mM KCl. Sometimes the sediment was washed once with 0.04 % deoxycholate, 0.15 M KCl to obtain "deoxycholate 2W particles". The yield of material in the 10 000 $\times g$ pellet varied from 0 to 100 %, the rest being recovered in the supernatant. This was dependent on the batch of spinach, which was obtained from local shops. An incubation period of up to 15 min before centrifugation was introduced when necessary (not with winter-grown spinach) and only preparations with a 30 to 60 % yield of deoxycholate 2 particles were used.

Simultaneous measurements of light-induced absorbance changes at two wavelengths or at one wavelength, together with fluorescence yield, were done with the double split-beam apparatus used in ref. 5, which is based on the same principle as the single version described in ref. 6. Unless otherwise indicated the optical path length was 1 mm. The optical half band width was 1.6 nm.

Absorbance changes in the red region of the spectrum were measured with an appropriate combination of a Schott RG glass filter and a Balzers B40 interference filter (half band width about 12 nm) in front of the photomultiplier. Fluorescence artefacts never exceeded the noise level in the experiments reported.

2,6-Dichlorophenolindophenol reduction and 1,1′-diphenyl-2-picrylhydrazyl (DPPH) reduction were measured with an Aminco–Chance spectrophotometer in the split-beam mode. On this apparatus C550 was measured in the dual-wavelength mode, set at 540–550 nm. The spectrum was usually checked by measuring the 540–530 nm and the 560–550 nm difference as well. The optical path length was 1 cm and the half band width 2.7 nm.

Except for Fig. 1 the fluorescence measurements were carried out with weak modulated measuring and strong continuous actinic light, about 0.5 and 100 nEinstein $\cdot$ $cm^{-2} \cdot s^{-1}$, respectively, both of wavelengths between 400 and 600 nm.

Delayed fluorescence was measured simultaneously with prompt fluorescence in a Becquerel phosphoroscope with excitation and measurement on the same side of the 1-mm cuvette and a light-dark cycle of 2 ms as described earlier [7]. The decay of delayed fluorescence was measured by averaging the amplified dc signal from 900 cycles in 38μs intervals with a Nuclear Chicago model 7100 signal averager and was corrected for the time dependence of the chopper aperture, which was determined by measuring the fluorescence in a weak continuous light in the same way. Prompt and delayed fluorescence were measured through a Schott RG 665 and Balzers AL 680 filter.

Unless otherwise indicated, optical measurements were done with a chlorophyll concentration of 83 μM, determined according to Arnon [8].

Light-induced electron spin resonance changes were recorded with a Varian E3 spectrometer with 100 KHz modulation at a microwave power of 5 mW and frequency of 9.53 GHz. Samples with a chlorophyll concentration of about 1 mM were contained in an aqueous solution cell with an optical path length of 0.2 mm. Actinic illumination was provided by the visible emission from a Philips SP1000 mercury lamp.

Although sodium tetraphenylboran is an analytical reagent for K^+ its activity as an artificial donor to Photosystem II [9] was not hampered by the presence of K^+. This was checked in control experiments from which these ions were excluded.

All measurements were done at room temperature.

RESULTS

Separation of the photosystems

No clear-cut separation of the Photosystems I and II was obtained. The chlorophyll *a* to *b* ratio is not a good criterion in this respect, because deoxycholate tends to solubilize chlorophyll *b* preferentially. Deoxycholate apparently damages the cytochromes as well, because the reduced (with dithionite) minus oxidized (with ferricyanide) difference spectra as measured on the Aminco–Chance were decreased by detergent treatment.

As will be described below, the C550 absorbance change discovered by Knaff and Arnon [10] could be measured quantitatively in deoxycholate particles and its correlation with the primary electron acceptor of Photosystem II [11] was confirmed. Therefore it could be used to determine the concentration of System II reaction centers.

The maximal amplitude of the P700 absorbance change was used to determine the concentration of System I reaction centers.

Table I shows the distribution of C550 and P700 over the different fractions obtained as described in Methods. The C550/P700 ratio was very reproducible and never exceeded 2 in deoxycholate 2 particles from summer-grown spinach, although C550 concentrations of up to 1 : 200 chlorophylls were found. With winter-grown spinach the separation of the photosystems was usually much better.

TABLE I

SEPARATION OF PHOTOSYSTEMS

Chloroplasts were from summer-grown spinach treated with sodium deoxycholate as described in ref. 4, with inclusion of a 15-min incubation period. Samples taken before centrifugation (chloroplasts), from the 10 000 × *g* pellet (deoxycholate 2) and from the 144 000 × *g* pellet (deoxycholate 1) are compared. Deoxycholate 2W was prepared as deoxycholate 2 using deoxycholate 2 as the starting material instead of chloroplasts. For P700, an extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ at 700 nm was used [12]. C550 was measured as described in the text and its concentration determined using an extinction coefficient of 2.2 $mM^{-1} \cdot cm^{-1}$ for the difference 540 minus 550 nm.

	Chloroplasts	Deoxycholate 2	Deoxycholate 2W	Deoxycholate 1
Yield (chl_{total})	100 %	50 %	30 %	5 %
Chl *a*/chl *b*	2.7	2.0	2.3	5.1
Chl_{total}/C550	360	310	250	> 1500
Chl_{total}/P700	450	550	830	260
C550/P700	1.25	1.8	3.3	< 0.17

Deoxycholate 2 particles did not evolve O_2 and even with artificial electron donors they reduced dichlorophenolindophenol at a very low rate, about 10 μmoles $\cdot h^{-1} \cdot$ mg $chlorophyll^{-1}$ or less (measurements as described in ref. 7). Without additions no significant light-induced absorption changes could be measured between 240 and 800 nm, except for a small change due to P700 oxidation which was largely irreversible. The chlorophyll fluorescence yield was almost invariant and the intensity of delayed fluorescence was very low, compared to intact chloroplasts.

Plastoquinone reduction

In the presence of an artificial electron donor for Photosystem II a slow light-induced fluorescence increase was observed, concomitant with a decrease of delayed fluorescence, as shown in Fig. 1. This effect was very sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). It is apparently the same as reported earlier for System II particles prepared with digitonin [7] and with Triton X-100 [13], but in deoxycholate 2 particles the fluorescence increase is much larger and almost irreversible.

These changes were accompanied by absorbance changes in the ultraviolet and red region of the spectrum. The light-induced difference spectrum (Fig. 2) shows the reduction of plastoquinone [14] and a band shift around 680 nm, which may be due to a chlorophyll *a*. This band shift was kinetically indistinguishable from the plastoquinone reduction. The positive band was often distorted and much smaller

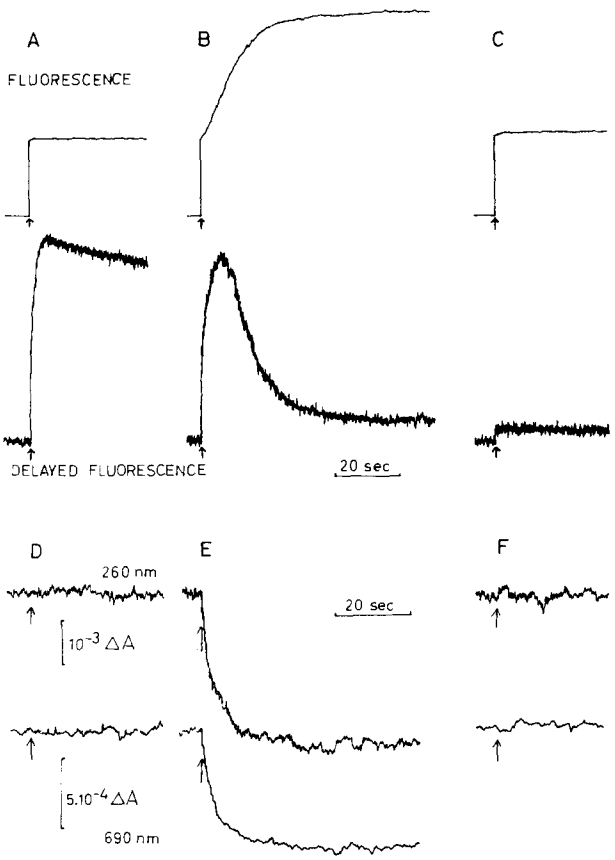

Fig. 1. Light-induced changes of prompt and delayed fluorescence in deoxycholate 2 particles. A, without additions; B, with 5 μM hydrazobenzene; C, with 5 μM hydrazobenzene+1 μM DCMU; and light-induced absorbance changes: D, without additions; E, with 1 mM hydroxylamine; F, with 1 mM hydroxylamine+1 μM DCMU. Hydroxylamine oxidation itself does not cause absorption changes at these wavelengths. The arrows mark the onset of actinic light.

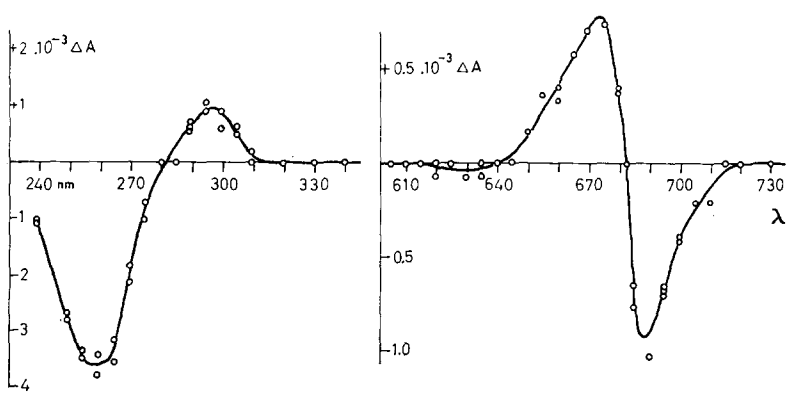

Fig. 2. Light minus dark difference spectrum of the absorption change of which the kinetics are shown in Fig. 1E. At wavelengths between 320 and 610 nm no significant changes occurred.

than the negative one, apparently because of an additional bleaching at 670 nm with similar kinetics. The above data suggest that the fluorescence quenching is due to the oxidized form of plastoquinone, which can be reduced via Photosystem II if an electron donor is supplied. The reduced form is apparently not reoxidized by Photosystem I. This is confirmed by the fact that almost no dark reduction of photooxidized P700 was observed, whether or not plastoquinone was reduced, provided an electron donor was used which did not reduce $P700^+$ directly (e.g. tetraphenylboran). Even in the presence of ferricyanide the reoxidation of plastoquinone was very slow. The maximal extent of the plastoquinone reduction was usually about 1 : 50 chlorophylls. The total pool was several times larger but, as its reduction with dithionite did not increase the fluorescence yield any further, it is evidently only the photochemically active pool which quenches fluorescence.

Plastoquinone reduction was reversibly inhibited by higher deoxycholate concentrations. It could not be measured before centrifugation (in the presence of 0.4 % deoxycholate) or in deoxycholate 2W particles, and in deoxycholate 2 particles the ability to reduce plastoquinone appeared slowly after resuspension of the pellet, reaching its maximum up to 1 h later. Sometimes, especially when winter-grown spinach was used, it did not appear at all. In this work "deoxycholate 2 particles" are operationally defined as particles from the $10\,000 \times g$ pellet which exhibit photoreduction of plastoquinone upon addition of an electron donor.

TABLE II

RELATIVE REQUIREMENT FOR DIFFERENT ELECTRON DONORS IN THE PHOTOREDUCTION OF PLASTOQUINONE

For each electron donor, the approximate concentration in μM which supports 30 % of the maximal rate of plastoquinone reduction in strong actinic light is given. All donors yielded the same maximal rate at high concentrations. The fluorescence increase and 690-nm bleaching were measured because, in the ultraviolet, absorbance changes due to the oxidation of the donor occur in most cases.

$MnCl_2$	< 1
Tetraphenylboran	1.5
Hydrazobenzene	5
Ascorbate	6
Ferrocyanide	9
Diphenylhydrazine	30
Hydroxylamine	50
sym-Diphenylcarbazide	90
Phthaloylhydrazine	100
H_2O_2	8000

The relative efficiency of several known electron donors to Photosystem II is compared in Table II. The concentrations required to support 30 % of the maximal rate of plastoquinone reduction in strong actinic light are given. The maximal rate was independent of the electron donor used and it was apparently not saturated by an incident light intensity of about 100 nEinstein $\cdot$ $cm^{-2} \cdot s^{-1}$ of wavelengths between 400 and 600 nm. In most preparations the half time of the change was about 1 s at this intensity, so the quantum yield of the plastoquinone reduction was very low. With $MnCl_2$ the rate was always maximal and dropped abruptly to zero when the

donor was exhausted. Electron donation to System II by ferrocyanide has not been reported before but in these particles, which did not rapidly reduce ferricyanide, it is clearly seen to be an efficient donor. With all electron donors at any concentration, plastoquinone reduction was abolished by 1 μM DCMU. With the most efficient donors, especially at low light intensity, the fluorescence rise was not strictly correlated with plastoquinone reduction: there was in addition a smaller variation of fluorescence yield, which was correlated with C550, as will be discussed below.

C550 reduction

After addition of ferricyanide to deoxycholate 2 particles the photoreduction of C550 could be observed. As light-induced cytochrome reactions, 515-nm absorption change, pigment bleaching or scattering changes did not occur, the only absorbance changes that interfered with the measurement of C550 were those of P700, the difference spectrum of which was the same as reported by Ke in ref. 15 (see Fig. 15). Where necessary far-red background light was applied to minimize these changes. The difference spectrum of C550 shown in Fig. 3 still contains a slight P700 contribution.

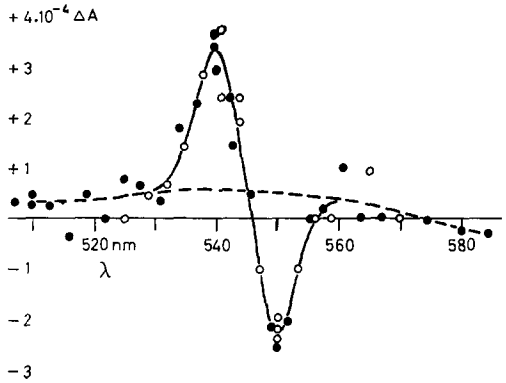

Fig. 3. Light minus dark difference spectrum caused by C550. Open circles: measured with the Aminco Chance apparatus in the split-beam mode, as described in Methods, with a chlorophyll concentration of 83 μM; additions: 100 μM ferricyanide and 10 μM tetraphenylboran. Solid circles: measured with the double split-beam apparatus in the presence of weak far-red background light ($\lambda > 695$ nm); optical band width 1.0 nm; optical path length 2.3 mm; chlorophyll comcentration 360 μM; additions: 100 μM ferricyanide and 20 μM tetraphenylboran. The dashed line represents residual P700 oxidation, checked at 700 nm.

Apart from this it reflects a band shift centered at 545 nm, just as the difference spectrum at −196 °C [16]. This suggests that the position of the absorption band (546 nm) and the distance of its light-induced shift (2 nm) are the same at room temperature and at −196 °C. The difference spectrum is 2.5 times broader than at −196 °C. This would then be explained by a band sharpening of about 2.5 times upon cooling to −196 °C. If the absorption band is a Gaussian curve with the same area under the curve in both cases, the maximal difference at room temperature, 540–550 nm should be about 5.5 times smaller on a chlorophyll basis than the maximal difference at −196 °C. A comparison of our results with measurements at −196 °C, e.g. those reported in ref. 17, shows that this is indeed true. So the amount of active C550 at 20 °C after deoxycholate treatment is probably the same as in intact chloro-

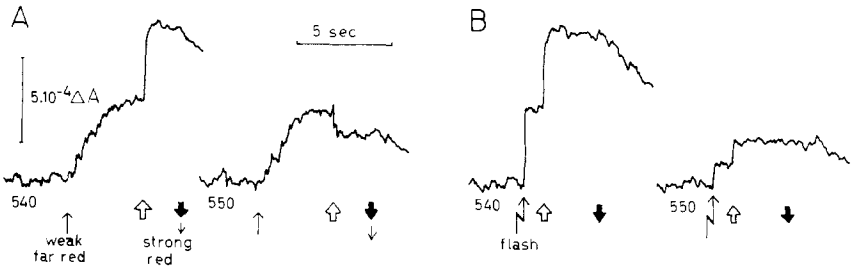

Fig. 4. Simultaneous measurement of absorption changes at 540 and 550 nm with the double split-beam apparatus as in Fig. 3. After an illumination with a weak beam of $\lambda > 695$ nm in A and with a xenon flash, 8 μs at one-third of the flash peak, $650 < \lambda < 695$ nm in B, the changes were saturated with strong red light. Upward and downward arrows indicate actinic light on and off, respectively. The difference between the 540- and 550-nm change was due to C550 and their mean to P700.

plasts at −196 °C. Deoxycholate 2 particles had about 15 % more C550 on a chlorophyll basis than chloroplasts (Table I).

As the C550 absorbance change was very small, the quantum yield of its photoreduction was difficult to measure directly. Therefore the photoreduction of C550 was compared to the photooxidation of P700 by simultaneous measurement of absorbance changes at 540 nm and at 550 nm. The P700 change was the same at both wavelengths and the C550 change was of equal magnitude at 540 and 550 nm but of opposite sign (see Fig. 3), so the difference was due to C550 and the mean to P700. Fig. 4A shows that far-red light was relatively ineffective in reducing C550: a weak beam of wavelengths longer than 695 nm which saturated the P700 change to about 70 % did not cause more than 15 % reduction of C550. The 100 % values were determined by subsequent strong red illumination. Fig. 4B shows that a non-saturating 8-μs xenon flash, filtered by a combination transmitting between 650 and 695 nm, which oxidized 50 % of P700 also caused 45 to 55 % reduction of C550 in a fresh sample. So in red light, the reduction of C550 in deoxycholate 2 particles has the same quantum yield as the oxidation of P700.

Fig. 5 illustrates the relation between fluorescence, plastoquinone and C550 in deoxycholate 2 particles. Simultaneous measurement of plastoquinone reduction and fluorescence yield with hydroxylamine as electron donor and with strong actinic light (Fig. 5A) shows an approximately linear relationship between fluorescence yield and the extent of plastoquinone reduction, while there is only a small C550 change. After addition of ferricyanide (Fig. 5C), the initial fluorescence yield was significantly lower than without additions (shown in Fig. 6A) and the light-induced C550 reduction was accompanied by a small fluorescence increase. DCMU, which inhibited plastoquinone reduction and the concomitant fluorescence increase completely (Fig. 1), did not affect this increase but, as with C550, blocked its reversal in the dark (Fig. 5C). The fluorescence quenching then is due to plastoquinone only. From these data it may be inferred that C550 is largely in its reduced state in deoxycholate 2 particles, and that it is oxidized in the dark by ferricyanide. To find the relationship between fluorescence and the redox state of plastoquinone in the presence of oxidized C550, the donor-supported fluorescence increase was measured in the presence of a low ferricyanide concentration and strong actinic light. In the dark,

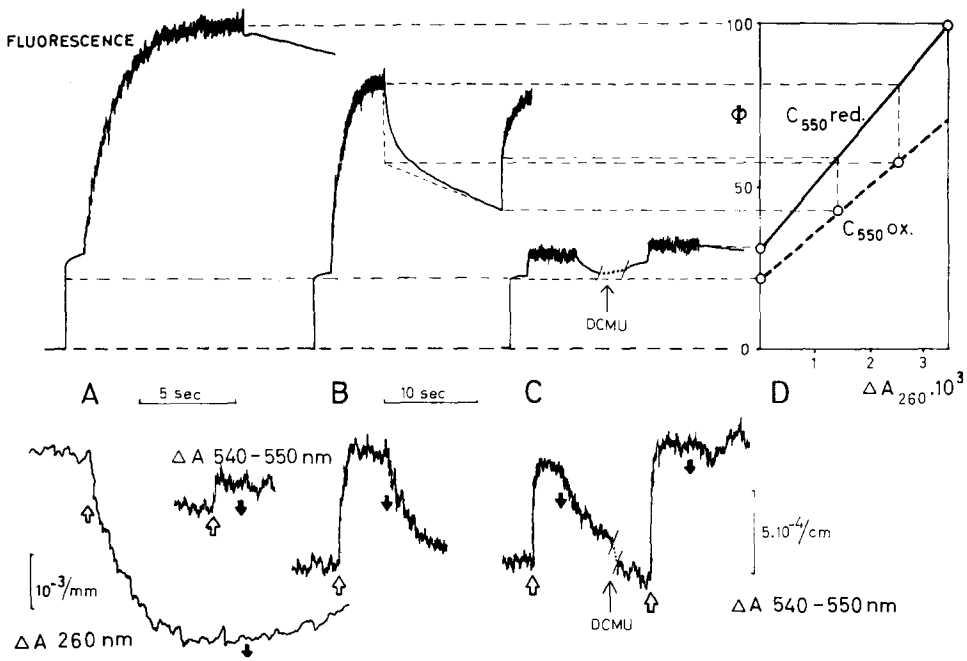

Fig. 5. Simultaneous measurements of fluorescence yield and absorption changes of plastoquinone with the double split-beam apparatus and C550 measurements under the same conditions on the Aminco–Chance in the dual-wavelength mode. Additions: A, 1 mM hydroxylamine; B, 20 μM ferrocyanide and 20 μM ferricyanide; C, 20 μM ferricyanide, 1 μM DCMU. Open and closed arrows indicate beginning and end of actinic-light period, respectively. In the fluorescence measurements actinic-illumination periods are evident from the increased noise level. In D the relation between fluorescence yield and extent of plastoquinone reduction is given in the presence of reduced C550 (continuous line) and of oxidized C550 (dashed line).

C550 was reoxidized much faster than the plastoquinone pool, giving rise to biphasic fluorescence kinetics (Fig. 5B). The contribution of each component to this fluorescence yield decrease could be roughly estimated. This is indicated in the fluorescence diagram in Fig. 5D, which summarizes the data from Fig. 5A, B and C. The exact values for percentage fluorescence quenching and amount of plastoquinone reduced depended on the particular batch of particles, so all measurements had to be done with the same batch. The C550 measurements (bottom row in Fig. 5) were done with the same concentrations of particles and additions, but still they are not strictly comparable because of the necessarily very heterogeneous illumination in the 1-cm cuvette.

C550 oxidation

It is remarkable that in the absence of artificial electron donors reduced C550 did not reduce plastoquinone. The most efficient electron donors of Table II, $MnCl_2$ and tetraphenylboran, caused a DCMU-sensitive oxidation of C550 by plastoquinone in the dark.

This is shown in Fig. 6. Addition of ferricyanide caused an oxidation of C550, which, as mentioned above, was largely in the reduced state in deoxycholate 2 particles

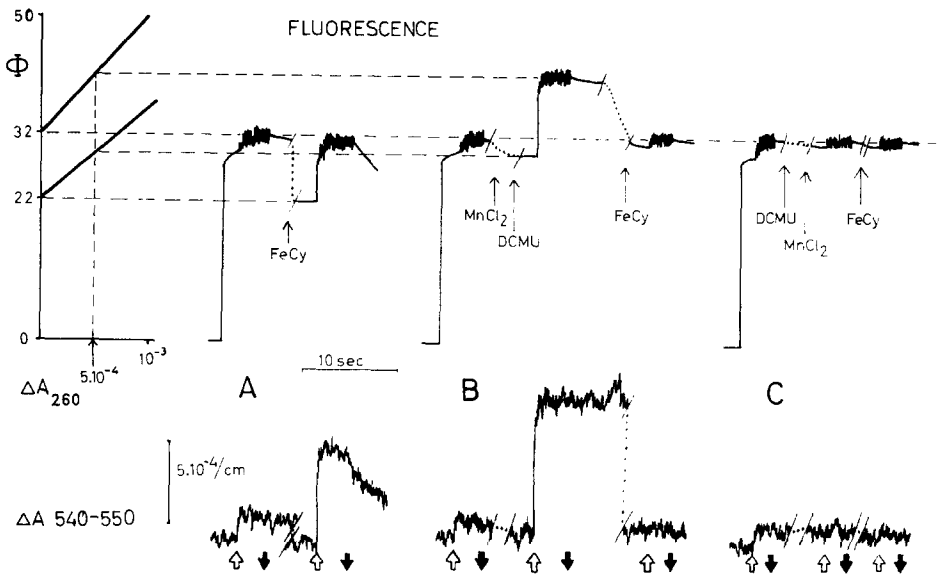

Fig. 6. Measurements as in Fig. 5 with the same batch of particles on the same day. Where indicated 50 μM ferricyanide, 10 μM $MnCl_2$ and 5 μM DCMU were added. The diagram on the left side is part of the diagram in Fig. 5D.

(Fig. 6A). This oxidation was accompanied by a fluorescence yield decrease and was shown to be DCMU-sensitive in Fig. 5C. Fig. 6B shows, that addition of $MnCl_2$ caused a much smaller decrease of the fluorescence yield, but C550 evidently became oxidized because subsequent illumination caused its photoreduction. The photoreduction was accompanied by the expected fluorescence increase, if DCMU was added before illumination to prevent plastoquinone reduction. So the addition of $MnCl_2$ caused an oxidation of C550, but the concomitant fluorescence decrease was nearly compensated by a fluorescence increase which was not related to C550. The original fluorescence yield was restored by subsequent addition of ferricyanide (in the presence of DCMU). These data strongly suggest that the increased fluorescence level was due to reduced plastoquinone. The DCMU-sensitivity of the plastoquinone reduction, concomitant with C550 oxidation (Fig. 6C), implies that at least one of the two electrons needed to reduce a plastoquinone molecule came from C550 and the C550 absorption change can thus be calibrated to that of plastoquinone. With the aid of the fluorescence diagram of Fig. 5D the amount of plastoquinone reduced was estimated at 1/7 of the active plastoquinone pool (as indicated on the left side of Fig. 6). If $\Delta\varepsilon$ 260 is 14 $mM^{-1} \cdot cm^{-1}$ [14] a differential extinction coefficient for the 540 minus 550 nm difference of 2.2 ± 0.2 cm^{-1} per mM plastoquinone reduced, is obtained.

The photooxidation of cytochrome *b*559 at liquid-N_2 temperature is limited by the amount of reducible C550 [11]. If one C550 molecule is reduced for each cytochrome *b*559 oxidized, and assuming a differential extinction coefficient at 556 nm of 19 $mM^{-1} \cdot cm^{-1}$ for the cytochrome, we estimate a differential extinction coefficient of 12 $mM^{-1} \cdot cm^{-1}$ for the 543 minus 547 nm difference caused by C550 reduction at −196 °C. At room temperature this would be 5.5 times smaller, i.e. 2.2 $mM^{-1} \cdot$

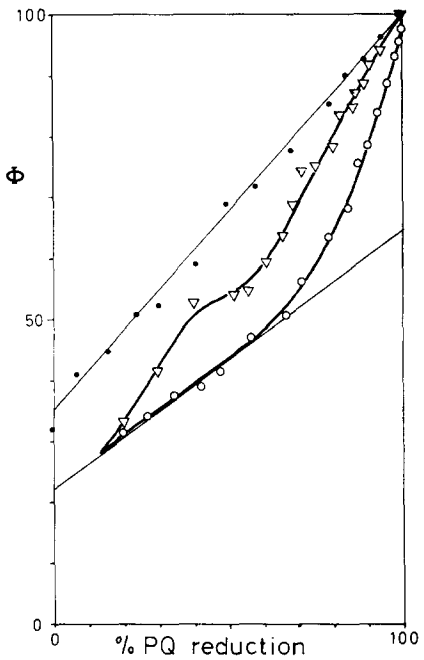

Fig. 7. Diagram of fluorescence yield versus plastoquinone reduction as in Fig. 5D, obtained from kinetic curves as in Fig. 5A. Dots: 1 mM hydroxylamine, strong actinic light. Circles: 10 μM $MnCl_2$, weak actinic light. Triangles: 10 μM $MnCl_2$, strong actinic light. Strong and weak actinic light were about 80 and 4 nEinstein $\cdot$ $cm^{-2} \cdot s^{-1}$ of wavelengths between 400 and 600 nm.

cm^{-1} for the 540 minus 550 nm difference. So the measured value appears to be correct, implying that C550 probably supplied only one of the two electrons needed to reduce a plastoquinone molecule. The other electron may have come from an intermediate electron carrier, which equilibrates with C550 in a one-electron reaction and with plastoquinone in a two-electron reaction, as has been postulated recently on other grounds [18, 19].

With an efficient electron donor, especially at low light intensity, plastoquinone was reduced first and reduced C550 accumulated gradually as the plastoquinone pool was exhausted (circles in Fig. 7). With the less-efficient donor hydroxylamine, at high light intensity, C550 was largely in the reduced state from the start (dots in Fig. 7 and Fig. 5A). The more complicated kinetics in intermediate cases (Fig. 7, triangles) may have been caused by the presence of the electron carrier between C550 and plastoquinone mentioned above, which does not influence the fluorescence yield [19].

In the presence of DCMU, C550 could be oxidized by the free radical DPPH at a concentration of only 3 μM (Fig. 8). The difference spectrum of DPPH reduction has a broad negative band between 500 and 600 nm. With tetraphenylboran as electron donor, a DCMU-insensitive net photoreduction of DPPH could be measured (Fig. 8B). As the reaction required a System II electron donor, and was abolished by incubating the particles for 5 min at 70 °C, it is apparently driven by Photosystem II. The DCMU-insensitivity of the reaction was not specific for deoxycholate 2 particles;

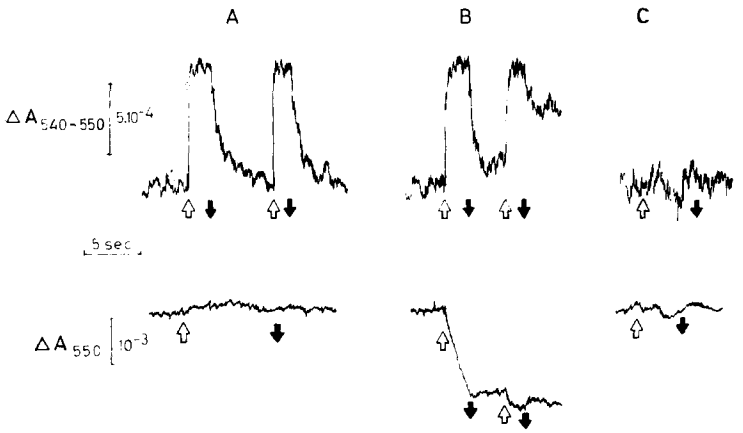

Fig. 8. C550 absorption changes and DPPH reduction in the presence of 5 μM DCMU. Additions: A, 3 μM DPPH; B, 5 μM DPPH and 5 μM tetraphenylboran; C, the same as B after keeping the particles for 5 min at 70 °C. The contribution of DPPH reduction to the 540- minus 550-nm difference was negligible.

also in System II particles, prepared with digitonin and with Triton X-100, DPPH was found to be a very efficient DCMU-insensitive electron acceptor, but in intact chloroplast with water as electron donor its reduction proceeds mainly via Photosystem I and is DCMU-sensitive (van Gorkom, H. J. and van der Linden, I. K., unpublished). With deoxycholate particles, $MnCl_2$ could also be used as an electron donor but then the extent of the reduction of DPPH was smaller, probably because of a simultaneous reoxidation of the reduced DPPH. In the same concentration range where DPPH oxidized C550, it also quenched the chlorophyll fluorescence: 5 μM DPPH decreased the fluorescence yield in the presence of oxidized C550 and oxidized plastoquinone by 50 %.

Neither α-benzyl-α-bromo-malodinitrile nor $HgCl_2$ caused any oxidation of C550 so their reported DCMU-insensitivity (ref. 20 and 21, respectively) could not be confirmed in deoxycholate particles.

Ferricyanide, at concentrations above 1 mM, was also found to cause an oxidation of C550 in the presence of DCMU. However, this reaction was inhibited by the presence of tetraphenylboran. The slow ferricyanide photoreduction in the presence of tetraphenylboran as an electron donor, measured at 390 nm where pigment bleaching (see below) was negligible, was fully DCMU-sensitive. The decay of delayed fluorescence in the ms time range (Fig. 9) was approximately first-order with a half-time of 350 μs. As the primary acceptor was not rapidly reoxidized this decay time probably reflects a deactivation step at the donor side of the reaction center. The emission was abolished by DCMU, but addition of a high ferricyanide concentration restored the delayed fluorescence which then had a half-time of 900 μs. So there was not only reoxidation of the primary acceptor, but also an inhibition of the deactivation at the oxidizing side of the reaction center. We conclude that ferricyanide probably cannot oxidize the primary acceptor in the presence of DCMU, but oxidizes an endogenous electron donor which in turn causes an oxidation of the primary acceptor via the back reaction of the photochemical reaction.

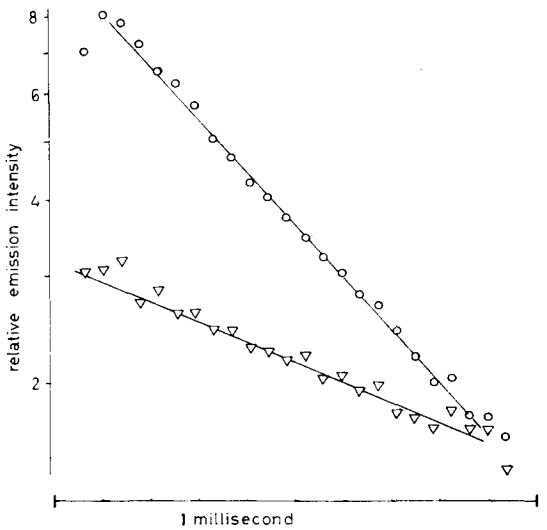

Fig. 9. Semilogarithmic plot of the decay curve of delayed fluorescence measured with a phosphoroscope (see Methods). Circles: no additions. Triangles: 5 μM DCMU and 10 mM ferricyanide. With DCMU alone the emission intensity was less than 0.5 on this scale.

P680, the primary electron donor of System II

At a higher deoxycholate concentration, in deoxycholate 2W particles and also in chloroplasts after addition of deoxycholate, ferricyanide elicited a reversible chlorophyll bleaching in strong actinic light (Fig. 10). In some preparations 10 μM ferricyanide was sufficient and the change was unaffected by an increase of the concentration to 50 mM. In contrast to the irreversible light-induced pigment bleaching induced by ferricyanide, this reversible change was abolished by the most efficient

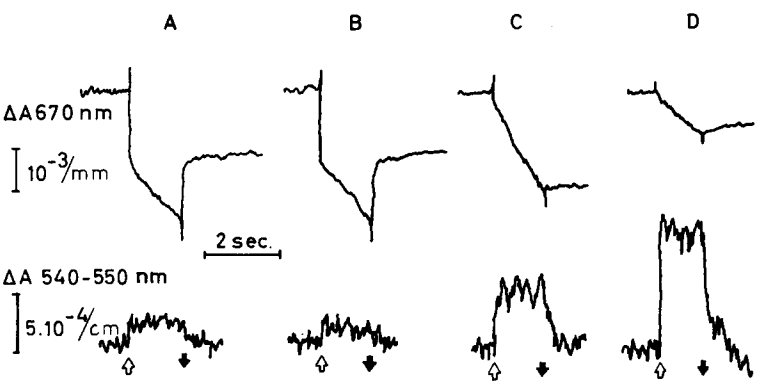

Fig. 10. Chlorophyll and C550 absorption changes in deoxycholate 2W particles in the presence of 500 μM ferricyanide. Additions: A, none; B, 10 μM DCMU; C, 20 μM tetraphenylboran; D, 3 μM DPPH. The chlorophyll absorption change did not contribute significantly to the 540- minus 550-nm measurements which were carried out at the same chlorophyll concentration but in a 1-cm cuvette and so at a much lower average actinic intensity. At this intensity the chlorophyll absorbance changes could not have been measured.

electron donors for Photosystem II and by heat treatment (5 min, 70 °C), so the reversible bleaching appeared to be an oxidation by Photosystem II. It was DCMU-insensitive so it required only one photoact. These properties suggest that it is the reaction-center chlorophyll of System II, P680. As the measurement can be repeated almost indefinitely in the presence of DCMU (until the irreversible pigment bleaching has destroyed the sample) the only decay route is apparently the back reaction with the primary acceptor. This may explain why almost no C550 reduction could be measured at the lower light intensity in C550 measurements (bottom row in Fig. 10). But also at high light intensity no fluorescence-yield increase, reflecting C550 reduction,

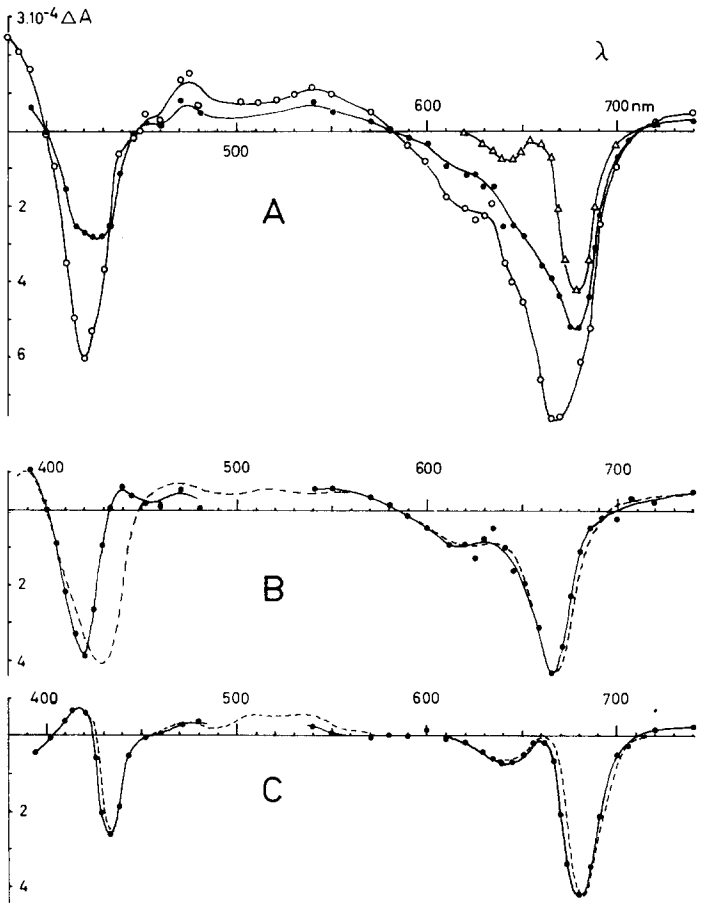

Fig. 11. Light minus dark difference spectra in the presence of ferricyanide. Only the rapidly reversible change is plotted. Each point is the average of three or four measurements. (A) Triangles, chloroplasts at 2 mM chlorophyll after incubation with 2.5 % deoxycholate, measured with an optical path length of 0.1 mm; in this case 5 mM ferricyanide was required for complete oxidation of P700; dots, deoxycholate 2 particles after 2 days at −20 °C, 200 μM ferricyanide; circles, deoxycholate 2W particles, 200 μM ferricyanide. (B) and (C) 665-nm and 680-nm components accounting for the spectra shown in A. The dashed line in B is the difference spectrum of the oxidation of chlorophyll *a* to its π-cation radical as reported by Borg et al. [23]. The dashed line in C is the difference spectrum of "chlorophyll a_{II}" as reported by Döring et al. [24].

could be measured, so if this interpretation is correct, it confirms the hypothesis [22] that $P680^+$ is a fluorescence quencher.

The only efficient inhibitor for the P680 change found so far is DPPH, which abolished the change at a concentration of 5 μM. DPPH did not short-circuit P680 and the primary acceptor, since in the presence of DPPH, $C550^-$ accumulated in strong actinic light (Fig. 10D). Perhaps DPPH catalyses the reduction of $P680^+$ by an endogeneous electron donor.

The light-induced difference spectrum of the reversible chlorophyll bleaching was variable; three examples are shown in Fig. 11A. The red maximum shifted from 680 nm to 665 nm and the 420-nm peak increased with repeated deoxycholate treatments, with ageing of the chloroplasts prior to deoxycholate treatment and with prolonged storage of the particles. The spectra could be accounted for by two components, present in a varying ratio. As shown in Fig. 11B one component had peaks at 665 and 418 nm and was similar to the difference spectrum of the oxidation of chlorophyll *a* to its π-cation radical [23] (dashed line). The other component (Fig. 11C) had peaks at 680 and 434 nm and was similar to the difference spectrum of "chlorophyll a_{II}" as measured by Döring et al. [24]. This component could be isolated by keeping the chloroplasts at a high concentration during detergent treatment and measurement. In this case higher deoxycholate and ferricyanide concentrations were required as well, but the shape of the spectrum was dependent on the dilution of the chloroplasts only. The sum of both components, if extrapolated to infinite light intensity in a double reciprocal plot (Fig. 12), was quite reproducible, and if the con-

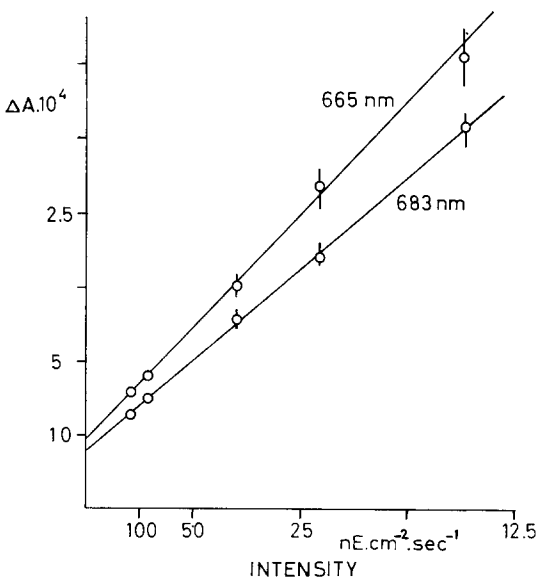

Fig. 12. Double reciprocal plot of the extent of the reversible chlorophyll bleaching versus the incident light intensity. Deoxycholate 2 W particles with 100 μM ferricyanide. The actinic light was filtered by a Balzers Calflex C+Corning CS 4-96, transmitting from 400 to 600 nm. Each point is the average of five measurements.

centration in chloroplasts was assumed to be 1:360 chlorophylls, a differential extinction coefficient of $75 \pm 5\ mM^{-1} \cdot cm^{-1}$ was found for both changes in their red maximum.

The 665- and 680-nm changes were both DCMU-insensitive, had a very similar intensity dependence (Fig. 12) and were equally sensitive to electron donors. The decay time was in the order of 100 ms in deoxycholate 2W for both forms and less than 10 ms in chloroplasts after incubation with deoxycholate.

These absorbance changes are accompanied by electron spin resonance signals with the same *g* values as "Signal I" [25], which is due to the oxidized form of P700 (for review see ref. 26. and for more recent evidence ref. 27). After oxidation by ferricyanide of P700 to its full extent, determined previously by photooxidation in the absence of ferricyanide, a rapidly reversible signal occurred in strong actinic light. The band width of this signal, of which three examples are shown in Fig. 13, was broader than that of the $P700^+$ signal and appeared to depend on the preparation. The spectra are normalized to the same peak to peak amplitude. The ESR signal of particles containing only the 680-nm form of P680 (triangles) was identical to the $P700^+$ signal (drawn line). The ESR signal at −196 °C attributed by Malkin and Bearden [28] to $P680^+$ was similar to the $P700^+$ signal as well. On the basis of its band width the $P700^+$ signal has been attributed to the one-electron oxidation product of a chlorophyll *a* dimer [29, 30]. The same interpretation may apply to the ESR signal of the 680-nm form of P680. The absorption difference spectrum of the 665-nm form (Fig. 11B) could be attributed to a chlorophyll *a* monomer. If the 680-nm form is a dimer, the 665-nm form should be accompanied by a $\sqrt{2}$ times broader ESR

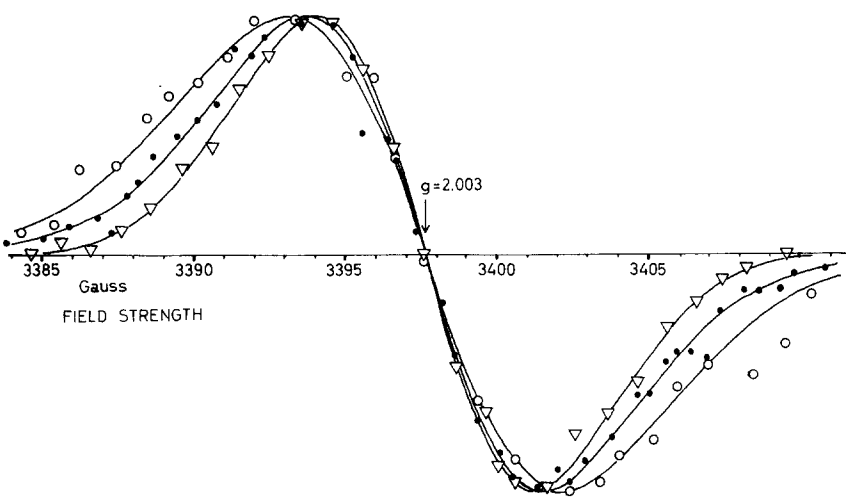

Fig. 13. Light-induced ESR spectra. Changes of the derivative ESR signal with 1.7 Gauss modulation amplitude were measured. At successive field strengths the change induced by 3 s illumination was recorded with a time constant of 1 s. Rapidly reversible change in three preparations of chloroplasts (1 mM chlorophyll) with 2.5 % deoxycholate and about 20 mM ferricyanide, in which P700 was completely oxidized. The absorption difference spectra of these preparations contained the 665-nm change (Fig. 11B) and the 680-nm change (Fig. 11C) in the following ratios: triangles 0 : 1, dots 1 : 1, circles 5 : 1. The drawn lines were calculated as explained in the text.

spectrum than the 680-nm form [29]. Curves predicted on this assumption (drawn lines in Fig. 13) show a reasonable fit to the ESR changes observed in different preparations (dots and circles).

DISCUSSION

Fluorescence yield

The chlorophyll fluorescence yield of Photosystem II is variable. In intact algae and chloroplasts this is mainly due to a quenching by the oxidized form of the primary acceptor [31], which can be interpreted as a decrease of the apparent lifetime of the excited state by the photochemical reaction itself. To explain the time lag between the reduction of the primary acceptor, the increase in fluorescence yield at room temperature [32] and the rate difference of C550 reduction and fluorescence increase at liquid-N_2 temperature [17], it has been proposed that the oxidized form of P680 also quenches fluorescence [22]. In addition the endogenous plastoquinone may quench fluorescence [33, 34] a fact which is not unlikely in view of the known quenching effect of quinones on chlorophyll fluorescence both in solution [35] and in chloroplasts [36]. In this work all three types of quenching were observed in deoxycholate particles: the main quencher was found to be the oxidized form of the photosynthetically active pool of plastoquinone, possibly in some way bound to chlorophyll *a*, as indicated by the absorption band shift near 680 nm. A smaller part of the variable fluorescence was correlated with the primary acceptor, but only if P680 was in the reduced state. Then the relationship of fluorescence yield, plastoquinone, and the primary acceptor in deoxycholate particles is given in the diagram of Fig. 5D. When P680 was in the oxidized form no fluorescence increase accompanying the reduction of the primary acceptor was seen.

C550

The measurement of C550 at room temperature in deoxycholate particles was not complicated by absorbance changes other than P700. The relationship of the absorption band shift around 545 nm to the primary electron acceptor inferred from experiments at low temperature [11], was found to hold at room temperature as well. The photoreduction of C550 is DCMU-insensitive, has about the same quantum yield as P700 for light which is absorbed about equally by both photosystems, but it has a much lower quantum yield for light which is absorbed by Photosystem I mainly. C550 is oxidized by plastoquinone but not in the presence of DCMU. The extinction coefficient of C550 at room temperature was found to be 2.2 $mM^{-1} \cdot cm^{-1}$ for the 540 minus 550 nm difference. The total amount of C550 was 1 : 360 chlorophylls in chloroplasts, in agreement with, for example, fluorescence measurements of Mauzerall and Malley [37]. In intact chloroplasts at room temperature reliable C550 measurements are difficult because the C550 absorbance change is almost an order of magnitude smaller than the cytochrome changes and almost twice as small as the P700 change in this spectral region. Our results indicate that the 540 minus 550 nm difference in such measurements appears to be larger than can be accounted for by C550 alone. Part of the change may be caused by another component, which is driven by Photosystem I [38] and depends on the membrane potential [39], but the reported difference spectra are too incomplete to allow an identification.

A more or less detailed explanation of the original inactivity of Photosystem II in deoxycholate particles is now possible. The primary acceptor is in the reduced state, but does not reduce plastoquinone, unless an electron donor for Photosystem II is supplied. The effect may be due to an increase of the redox potential of the primary acceptor, caused by accumulation of positive charge on intermediates at the donor side of the reaction center [40]. The accumulation of oxidized intermediates may in turn be explained by an inhibition of one or more steps in the water-oxidation process. The exceptional efficiency of $MnCl_2$ as an artificial electron donor in these particles (Table II) indicates that this inhibition may be due to removal of endogenous Mn^{2+}, which is known to play an essential role at the oxidizing side of the reaction center (see for example ref. 41). In addition plastoquinone seems to be quite inaccessible both to endogenous oxidation by photooxidized P700 and to added ferricyanide. This and its abnormally large quenching effect may be caused by a change in the membrane induced by deoxycholate. At higher deoxycholate concentration (during incubation and in deoxycholate 2W particles) no photoreduction of plastoquinone could be measured in the presence of electron donors.

P680

The reversible chlorophyll bleaching in the presence of ferricyanide (Fig. 10) was shown to be associated with an oxidation caused by Photosystem II activity, and the total amount of chlorophyll involved was estimated at one molecule per Photosystem II reaction center. This suggests that the bleaching reflects the oxidation of the primary electron donor of Photosystem II. The two components in the difference spectrum (Fig. 11) appeared to be two forms of the same electron carrier, and apparently the 665-nm form (Curve B) is a modification of the 680-nm form (Curve C) caused by the deoxycholate treatment. Therefore we propose that the difference spectrum of P680 in vivo is the one shown in Fig. 11, Curve C, which is the same as reported by Döring et al. [24]. In spite of its small extent and DCMU-sensitivity the absorption change measured by these authors can be attributed to the primary electron donor of Photosystem II as we have explained elsewhere [40]. This interpretation of the absorbance change measured by Döring et al. [24] is confirmed by the experimental evidence presented in this paper. Floyd et al. [42] have reported a reversible chlorophyll bleaching at 680 nm at −196 °C, and ascribed it to the primary donor of Photosystem II.

The oxidized minus reduced difference spectrum of the 665-nm form may be ascribed to the oxidation of a monomeric chlorophyll *a* molecule to its π-cation radical [23], except for the fact that its negative peak in the soret region is narrower and occurs at 10 nm shorter wavelength than expected. This identification is confirmed by the ESR signal accompanying the oxidation product.

The difference spectrum of the 680-nm form is clearly more complex. The width of the accompanying ESR spectrum suggests that it is due to the one-electron oxidation product of a chlorophyll *a* dimer [29]. The absorption spectrum of such a species is not known. Fig. 14 shows the difference spectrum (Curve A) which might be expected if the oxidation product of the dimer (Curve B) were one free chlorophyll *a* monomer (Curve C) plus one oxidized monomer (Curve D). In the red region this is a reasonable approximation to the measured difference spectrum of P680 (dotted line). The deviation from the expected curve below 430 nm, which was not seen in the

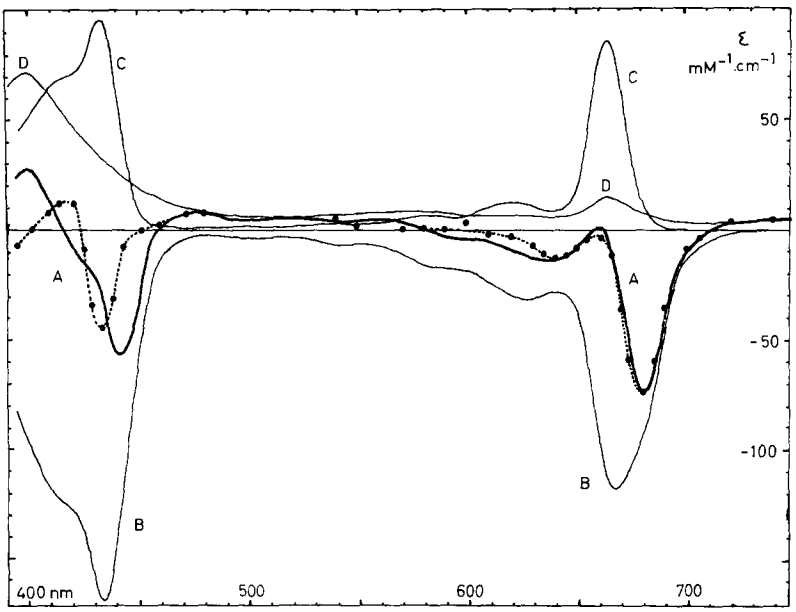

Fig. 14. Comparison of the measured difference spectrum of P680 photooxidation (dotted line) with the expected difference spectrum of the reaction $Chl_2 \rightarrow Chl + Chl^+$ (Curve A), which is composed of the disappearance of a dimer spectrum (Curve B) and the appearance of the spectra of a monomer (Curve C) and an oxidized monomer (Curve D). The dimer and monomer spectra used are those calculated by Sauer et al. [43] from measurements in solutions in chloroform. The oxidized chlorophyll spectrum is the one measured by Borg et al. [23] in a solution in dichloromethane.

monomer difference spectrum of Fig. 11B, may indicate that the oxidized dimer does not have the 399-nm absorption band of the oxidized monomer. The measured negative peak at 434 nm is again narrower and at shorter wavelength than expected, just as was found for the monomer in Fig. 11B. Therefore this deviation from the expected curve probably does not arise from dimer interaction in the oxidation product. Since it is situated at a longer wavelength than in the monomer, the simplest explanation would be a lower absorption than expected at the long wavelength side of the blue absorption band both of the monomer and of the dimer. It is possible that a better fit could be obtained with the corresponding spectra of pheophytin *a* instead of those of chlorophyll *a*. The C550 absorbance change could be due to a shift of a pheophytin *a* absorption band and might then be a shift of the P680 absorption spectrum upon reduction of the primary electron acceptor.

It may be noted that the interpretation of the 665- and 680-nm forms of P680 as given above agrees well with the extinction coefficient of 75 $mM^{-1} \cdot cm^{-1}$ in the red maximum of both forms. The assumption that the P680 concentration in chloroplasts is 1:360 chlorophylls thus appears to be correct. This means a ratio of P680 to C550 of 1:1, which is to be expected if P680 and C550 represent the primary donor–acceptor couple.

An interesting consequence of our interpretation of the difference spectra is that the dimeric form of P680 is apparently not a strict requirement for photoreactivity, because the monomer can still be photooxidized.

The possibility to accumulate $P680^+$ at room temperature in the presence of ferricyanide and deoxycholate can be explained as follows. $P680^+$ is not normally accumulated to any extent, presumably because of its rapid reduction by an electron donor Z. Z is not destroyed by deoxycholate because the effect of the detergent is reversible: in deoxycholate 2 particles, in which the deoxycholate concentration is small, the accumulation of $P680^+$ is again not observed. Probably Z is not oxidized by ferricyanide, unless its midpoint potential is decreased by deoxycholate. This is confirmed by the fact that at lower detergent concentration a higher ferricyanide concentration is required for accumulation of $P680^+$. The lowering of the midpoint potential of Z may be due to the electrostatic influence of the negatively charged detergent on the oxidizing side of the reaction center, where accumulated positive charges play an essential role [40].

P700

If both P700 and P680 are chlorophyll *a* dimers, one would expect their absorption difference spectra to be similar. Since the spectra are different, we assume that the more complicated spectrum of P700 contains an additional component. The difference between the two difference spectra (Fig. 15) then indicates that in the Photosystem I

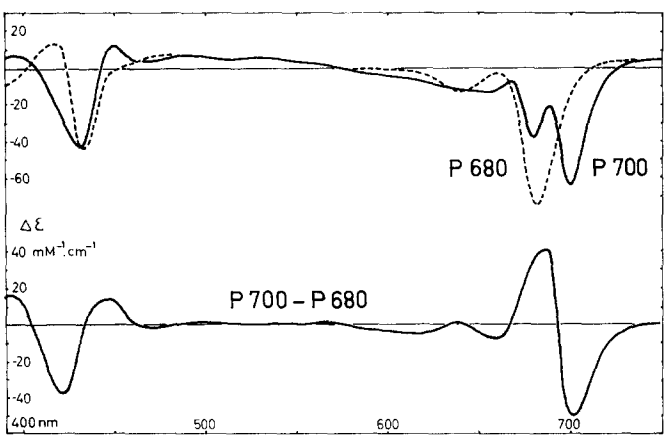

Fig. 15. Comparison of the difference spectra of P700 and P680. The P700 difference spectrum is the one reported by Ke in ref. 15.

reaction center the oxidation of the chlorophyll *a* dimer is accompanied by a shift of an absorption band near 700 nm. The difference between the P700 and P680 difference spectra in the blue region is more difficult to interpret: it may be related to the 700-nm band shift and would then indicate a broadening of the associated blue absorption band. The part of the difference spectrum that can be attributed to the photooxidation itself may then be the same as in Photosystem II. The negative maxima at 700 nm and at 680 nm are usually attributed to a bleaching and a band shift, respectively [44, 45], but Fig. 15 shows that the interpretation can be reversed. In our interpretation the direction of the band shift is to shorter wavelength, in analogy to P800 of photosynthetic bacteria [46].

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