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LIGHT-INDUCED CHANGES OF FLUORESCENCE AND ABSORBANCE IN SPINACH CHLOROPLASTS AT −40 °C

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SUMMARY

1. Spinach chloroplasts were stored in the dark for at least 1 h, rapidly cooled to −40 °C, and illuminated with continuous light or short saturating flashes. In agreement with the measurements of Joliot and Joliot, chloroplasts that had been preilluminated with one or two flashes just before cooling showed a less efficient increase in the yield of chlorophyll *a* fluorescence upon illumination at −40 °C than dark-adapted chloroplasts. The effect disappeared below −150 °C, but reappeared again upon warming to −40 °C. Little effect was seen at room temperature in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), added after the preillumination

2. Light-induced absorbance difference spectra at −40 °C in the region 500–560 nm indicated the participation of two components, the socalled 518-nm change (P518) and C-550. After preillumination with two flashes the absorbance change at 518 nm was smaller, and almost no C-550 was observed. After four flashes, the bands of C-550 were clearly visible again

3. The fluorescence increase and the absorbance change at 518 nm showed the same type of flash pattern with a minimum after the second and a maximum at the fourth flash. In the presence of 100 μM hydroxylamine, the fluorescence response was low after the fourth and high again after the sixth flash, which confirmed the hypothesis that the flash effect was related to the so-called S-state of the electron transport pathway from water to Photosystem 2.

4 The kinetics of the light-induced absorbance changes were the same at each wavelength, and, apart from the size of the deflection, they were independent of preillumination. Flash experiments indicated that the absorbance changes were a one-quantum reaction. This was also true for the fluorescence increase in dark-adapted chloroplasts, but with preilluminated chloroplasts several flashes were needed to approximately saturate the fluorescence yield.

5. The results are discussed in terms of a mechanism involving two electron donors and two electron acceptors for System 2 of photosynthesis.

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

INTRODUCTION

During the last years, important information about the primary, and also about some secondary electron transport reactions in photosynthesis has been obtained by studies at low temperatures Many of these have been performed at the temperature of liquid N_2, but interesting results have also been obtained with material subjected to a less drastic temperature lowering. Studies[1-3] on the kinetics of chlorophyll fluorescence of chloroplasts and algae cooled to temperatures between 0 and −40 °C, indicate that electron transport between the primary electron acceptor (Q) of Photosystem 2 and the secondary acceptor pool (plastoquinone) is blocked in this temperature range. However, this does not imply that further cooling does not produce further changes in the properties of System 2, as has been shown *e.g.* by measurements of light-induced cytochrome b_{559} oxidation[4] and fluorescence changes[2]. Joliot and Joliot[5,6] observed that the rate of increase of the fluorescence yield at −40 °C was dependent upon the number of light flashes given prior to freezing, and gave evidence that the effect was related to the so-called S-state (see refs 7, 8) of the electron pathway from water to System 2. Recently, Vermeglio and Mathis[9] reported measurements of difference spectra at liquid N_2 temperature of chloroplast samples which had been subjected to various illuminations at 0 and −50 °C and explained their results by the assumption of more than one secondary electron donor to System 2.

In this paper we shall report comparative measurements on light-induced changes in absorbance and in fluorescence yield at −40 °C of spinach chloroplasts which had been subjected to various numbers of flashes before cooling The results will be discussed in terms of current theories about the primary and secondary reactions of Photosystem 2.

MATERIALS AND METHODS

Chloroplasts were obtained from spinach leaves by disruption at 0 °C in a blender in a solution containing 0.05 M *N*-tris (hydroxymethyl)methylglycine (Tricine), 0.01 M KCl, 0.002 M $MgCl_2$ and 0.4 M sucrose, pH 7.8. After filtration through nylon cloth, the chloroplasts were sedimented by brief centrifugation at 8000 × *g*, resuspended in the same buffer, and stored on ice in the dark for 1–3 h before use. The chlorophyll concentration, determined by the method of Whatley and Arnon[10], was $2 \cdot 10^{-3}$ M.

Measurements of fluorescence and of absorbance changes were done in a single-beam spectrophotometer. Fluorescence was excited with a band around 630 nm, obtained by means of a Balzers B40, Schott AL and two long-wave cut-off interference filters. Except where noted, the same light was used as actinic light to bring about absorbance changes. Fluorescence was measured at 692 nm with an EMI 9659 extended photomultiplier, facing the rear side of the vessel and equipped with a Schott AL 692 interference and Corning 4-77 and Schott RG 665 glass filters. The same photomultiplier, equipped with suitable interference and glass filters to transmit the measuring but to cutt off the actinic light was used for measurements of absorbance changes. Light flashes from a xenon flash tube were used as preillumination and in some experiments also as actinic illumination. The light was filtered by a filter combination transmitting between 500 and 630 nm to obtain a reasonably homogeneous illumination throughout the sample. The duration of the flash was 8 μs at one-third of the peak. The

peak intensity was 50 W · cm^{-2}. Some experiments were done with a flash of 15 μs, peak intensity 16 W · cm^{-2}.

Just before each measurement, aliquots of the chloroplast suspension were mixed with appropriate amounts of glycol and buffer to obtain a suspension of the desired chlorophyll concentration containing 50 % (v/v) glycol, transferred in darkness to a 1-mm vessel, illuminated with various numbers of flashes, given at 1 2-s intervals, and rapidly cooled. Cooling started within 5 s after the last flash and it took about 10 s to lower the temperature from about 15 to −40 °C. The sample was usually cooled to about −50 °C and slowly warmed or cooled to the desired temperature to obtain temperature equilibrium, needed for stable readings.

RESULTS

Fluorescence kinetics

Fig. 1 shows the kinetics of fluorescence of spinach chloroplasts at −40 °C Recording a was obtained with dark-adapted chloroplasts. Recording b was measured after two preilluminating flashes, given before cooling. It can be seen that the fluorescence increase upon illumination was much slower after the latter pretreatment. Recordings e and f show control experiments performed at room temperature. These recordings demonstrate that the high concentration of glycol, needed to prevent crystallisation upon cooling, did not significantly change the yield and kinetics of fluorescence, in contrast to observations of Fraçkowiak *et al.*[11] on intact cells of *Chlorella*.

The effect of preillumination at room temperature upon the fluorescence kinetics at −40 °C is the same as reported by Joliot and Joliot[5,6], apart from the different time scale due to a lower actinic intensity in our experiment. We also confirmed the observation[5,6] that, with dark-adapted chloroplasts, a flash given at −40 °C gave a

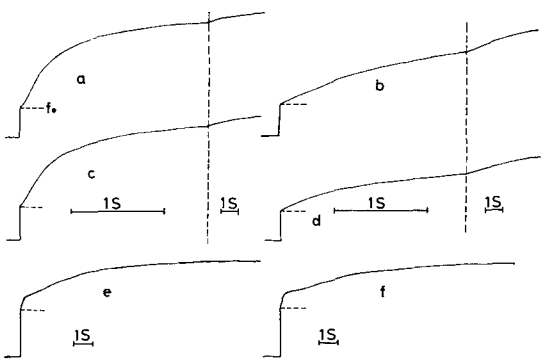

Fig. 1. Time curves of chlorophyll fluorescence in spinach chloroplasts. The vertical line segments mark the onset of the excitation light (630 nm, 1.0 nEinstein · cm^{-2} · s^{-1}) Chlorophyll concentration 1 mM. Recordings a–d, temperature −40 °C; a and c, without preillumination; b and d, after two preilluminating flashes given before cooling; a and b, no additions; c and d, in the presence of 3 mM potassium ferricyanide. Curves e and f were recorded at room temperature (no preillumination), without (e) and with glycol (f), at two times higher sensitivity than the other recordings. Further experimental details see Materials and Methods.

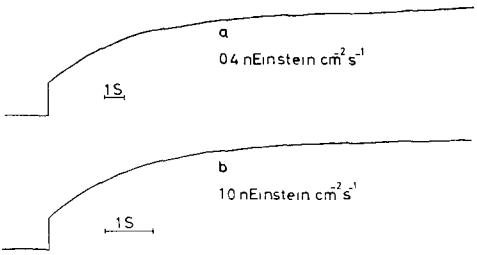

Fig. 2. Fluorescence kinetics at −40 °C, at two different intensities. Excitation light 630 nm. Curve b was recorded at 2.5 times lower sensitivity. Note the different time scales for the two recordings. Further conditions as for Recording b of Fig. 1.

fluorescence increase which was only little less than the maximum increase obtained in continuous light. Chloroplasts preilluminated with two flashes before cooling showed a much smaller fluorescence increase in a flash. With dark-adapted, as well as with preilluminated chloroplasts, the rates of fluorescence increase were linearly proportional to the intensity of illumination (Fig. 2).

The difference in fluorescence kinetics between dark-adapted and preilluminated chloroplasts gradually disappeared at temperatures below −90 °C and was completely absent below −150 °C (Fig. 3). The rapid lowering of the rate of fluorescence increase below −90 °C for non-preilluminated chloroplasts has been noted before[3,4]. The effect of cooling was reversible: after warming to −40 °C the differences in kinetics were the same as before.

Since 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) presumably inhibits between Q and plastoquinone[12,13], one might expect that a similar effect of preillumination could be observed at room temperature in the presence of this inhibitor. Fig. 4, however, shows that the fluorescence kinetics were only slightly different in the presence of DCMU with preilluminated and dark-adapted chloroplasts. Sometimes the differences were even smaller or of opposite sign and often no difference could be observed at all. DCMU was always added after the preillumination. As DCMU does

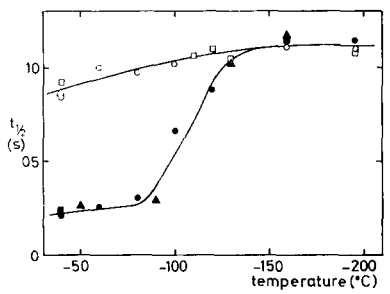

Fig. 3. Half-time of the fluorescence increase in continuous light (630 nm, 1.0 nEinstein · cm^{-2} s^{-1}) as a function of temperature. Solid symbols: no preillumination; open symbols: preillumination two flashes. Different symbols refer to different chloroplast preparations. The ordinate scale applies to the measurements with circles and triangles. The half-times of the sample represented by squares were about 40% longer than shown here, and are normalized for sake of clarity. Chlorophyll concentration 0.5 mM.

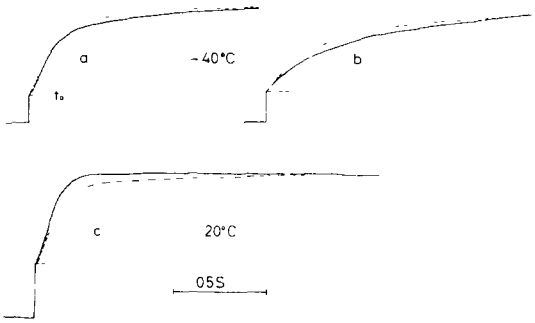

Fig 4. Fluorescence recordings in the presence of DCMU Recordings a and b, −40 °C. a, no preillumination, b, preillumination two flashes; ———, no DCMU, - - -, 50 μM DCMU, added 1 s after the last flash, if any, and 4 s before cooling Curves c, 20 C, 50 μM DCMU, ———, no preillumination; - - -, preillumination two flashes Excitation light 472 nm Chlorophyll concentration 0 025 mM

not inhibit O_2 evolution[12], these experiments suggest an effect of preillumination (and thus of the S-state) upon a secondary reaction at the reducing side of System 2 At −40 °C, DCMU did not inhibit the effect of flash preillumination. Both with and without[6] preillumination it gave a small enhancement of the rate of fluorescence increase (Fig. 4)

Absorbance changes

In order to obtain more specific information about the mechanism underlying these phenomena and about the photochemical reactions occurring under these conditions, we have measured light-induced absorbance changes at −40 °C in the region 500–560 nm By using the measuring light as a background, it was checked that its intensity was too low to cause any noticeable change of the fluorescence kinetics. Most experiments were done in the presence of 3 mM potassium ferricyanide to minimize possible effects due to Photosystem 1. Essentially the same absorbance

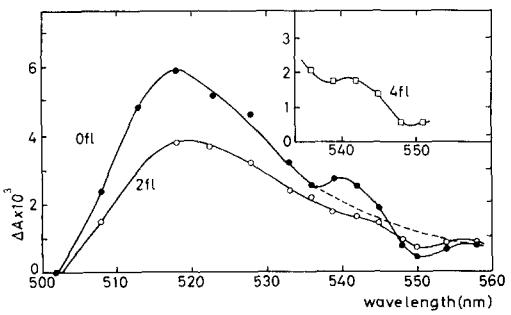

Fig. 5 Absorption difference spectra (light *minus* dark) at −40 °C, measured 80 ms after a flash of light in the presence of 3 mM ferricyanide ●, no preillumination, ○, preillumination with two flashes, given before cooling. Insert· preillumination with four flashes The points of the two main spectra represent the averaged and normalized (at 539 nm) results obtained with about five different samples. Chlorophyll concentration 1 mM Except for the spectrum of the insert, 15-μs flashes were used.

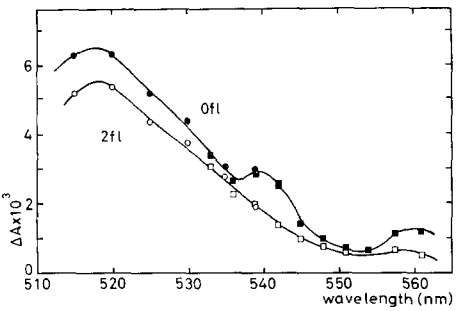

Fig 6. Absorption difference spectra obtained as for Fig 5, except that 1 s of continuous actinic light (630 nm, 1 0 nEinstein cm^{-2} s^{-1}) was used. Open symbols, preillumination with two flashes, solid symbols, no preillumination Circles and squares were obtained with different samples.

changes, however, occurred in the absence of ferricyanide. Ferricyanide had little effect upon the fluorescence kinetics at −40 °C, except that the fluorescence increase, especially in preilluminated chloroplasts, was smaller (Fig. 1, Recordings c and d, *cf* ref. 6).

Figs 5 and 6 show difference spectra of absorbance changes at −40 °C. The difference spectrum induced by flash illumination of dark-adapted chloroplasts, showed maxima near 518 and 540 nm and a shallow minimum near 550 nm (Fig 5). The main band is presumably due to the well-known 518-nm change (ref. 14) and the spectrum in the region 540–555 nm is apparently composed of a band shift due to C-550 (refs 15, 16), and the flank of the 518-nm band. After two preilluminating flashes C-550 was largely absent in the difference spectrum and the 518-nm band was about 35 % lower. After a preillumination with four flashes, the bands of C-550 were clearly visible again. Similar results were obtained with continuous illumination (Fig. 6), but sometimes the bands of C-550 were less clearly discernible than shown here

The effect of the number of preilluminating flashes upon the changes in absorbance and fluorescence yield is shown in Fig. 7. The flash patterns for fluorescence[5,6]

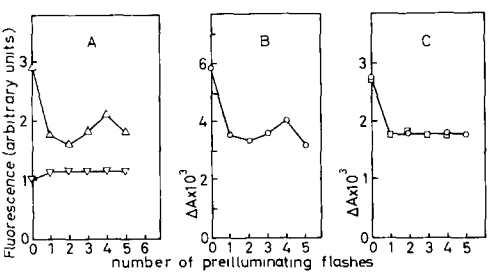

Fig 7. Increase of fluorescence yield and of absorbance in the presence of 3 mM ferricyanide, measured 80 ms after a single flash given at −40 C Abscissa, number of preilluminating flashes given before cooling. Chlorophyll concentration 0 5 mM (A) and 1 mM (B and C), respectively A, relative fluorescence yield. A weak excitation beam (630 nm) was used to monitor the fluorescence before (▽–▽) and after (△–△) the flash B, absorbance increase at 518 nm; C, at 539 nm Triangles, circles and squares refer to different preparations of chloroplasts

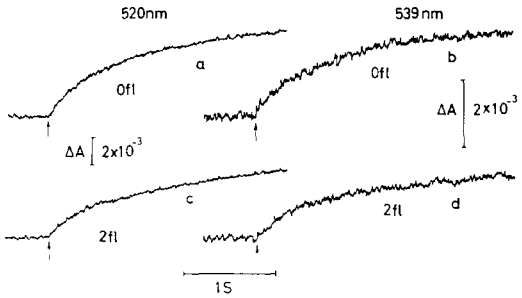

Fig. 8. Kinetics of absorbance changes at −40 °C at 520 and 539 nm. Recordings a and b, no preillumination; c and d, preillumination two flashes Conditions as for Fig. 6 Arrows mark the onset of actinic light.

and the increase in absorbance at 518 nm were similar, and showed a minimum after the second and a maximum after the fourth flash. A second maximum was not observed at 539 nm, but the spectrum of Fig. 5 (insert) indicates that the flash dependence of C-550 was of periodicity four, like that of the other phenomena. With 100 μM hydroxylamine, added 15 min before preillumination, the rate of fluorescence increase in continuous light at −40 °C was the same without preillumination and after two flashes; it was lower after four, and higher again after six flashes. Since hydroxylamine is known to delay by two flashes the flash pattern of O_2 evolution[17], these results confirm the hypothesis[5,6] that the flash effect is in some way related to the formation of S-states in the pathway to water.

In contrast to those of fluorescence, the kinetics of the absorbance changes, at least during the first two seconds, were the same without preillumination and with two flashes, although the sizes were different. Within the error of measurement, this was true at all wavelengths tested (Fig. 8). Even at several times higher intensities than used for Figs 6 and 8, the absorbance changes at 518 nm were smaller with than without preillumination, and the spectrum showed little C-550 after preillumination with two flashes. Apart from the S-shape in the fluorescence induction curve which may be due to energy transfer between photosynthetic units[18] the time courses for fluorescence and absorbance changes were about the same for not too dense samples of dark-adapted chloroplasts. The absorbance changes were not inhibited by 50 μM DCMU, added after the preillumination. At − 196 °C, the absorbance changes at 542 nm were the same with two flashes and without preillumination

The absorbance changes induced by a single flash were only little less than those brought about by strong continuous actinic light both with dark-adapted and preilluminated chloroplasts. Fig. 9 shows the effect of repetitive flashes. The second flash gave only a small increment of the absorbance at 539 (or 518) nm compared to the first one, and subsequent flashes had virtually no further effect This indicates that the changes are a one-quantum reaction. This was also true for the fluorescence yield with dark-adapted chloroplasts, but preilluminated chloroplasts needed several flashes to approximately saturate the fluorescence yield (Fig. 9B). Like the experiments with continuous light, this indicates that, with preilluminated chloroplasts, each reaction center has to react more than once in order to give the maximum yield of fluorescence, but apparently the absorbance changes reflect a one-quantum reaction.

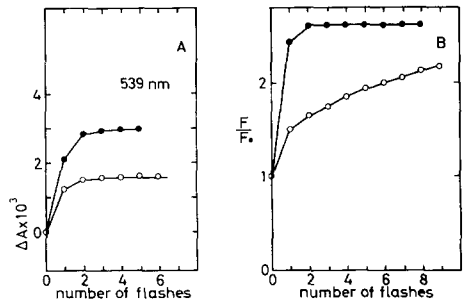

Fig. 9 Absorbance increase at 539 nm (A) and relative fluorescence yield (B) as a function of the number of flashes given at −40 °C, measured 1 s after the flash. The flashes were given at 1 2-s intervals. ●, no preillumination; ○, preillumination two flashes. Conditions as for Fig. 1 (Recordings c and d), except B chlorophyll concentration 0 5 mM. Fluorescence was measured in the same way as for Fig. 7.

As stated already, no significant effects of ferricyanide upon the light-induced absorbance changes were observed at −40 °C. No evidence for light-induced cytochrome b_{559} and cytochrome *f* oxidation was observed either with or without 3 mM ferricyanide and with or without preillumination with one or two flashes. Without ferricyanide, and with a several times higher actinic intensity than used for the other experiments, a small decrease in absorbance at 556 nm was observed with chloroplasts preilluminated with two flashes, suggesting the photooxidation of some cytochrome b_{559}. Measurements of light-induced absorbance changes at −196 °C indicated that cytochrome b_{559} was in the oxidized state in the presence of 3 mM ferricyanide (Visser, J. W. M., personal communication). The same was probably true for cytochrome *f*. The chemically-induced difference spectrum (ferricyanide–hydroquinone[19]) indicated that the chloroplasts contained 4–5 mmoles of high-potential cytochrome b_{559} per mole of chlorophyll. Without ferricyanide, at least before addition of glycol, both cytochromes appeared to be in the reduced state, as indicated by the absence of cytochrome bands in the difference spectrum with hydroquinone.

DISCUSSION

The periodicity four, observed with flash preillumination for the fluorescence kinetics at −40 °C indicates, as pointed out by Joliot and Joliot[5,6], that these kinetics are influenced by the so-called S-state, the number of positive charges accumulated in the pathway from water to System 2. This conclusion is supported by the effect of hydroxylamine, reported here. The same mechanism that influences the fluorescence is apparently also responsible for the periodicity observed in the absorbance changes induced by illumination, but the kinetics of the absorbance changes were not affected in the same way as those of fluorescence.

There are two main hypotheses to explain the differences in fluorescence kinetics at −40 C of dark-adapted and preilluminated chloroplasts. According to the first one, the net efficiency of quantum conversion in the reaction center is lower after preillumination than with dark-adapted chloroplasts. At first sight, this might seem an attractive hypothesis: a similar one has been used to explain the low yield of fluores-

cence increase at −196 °C (ref. 20) One could assume that the low yield is due to a high rate of the back reaction of primary reactants, which reaction competes with a less efficient reaction with a second donor or acceptor needed to stabilize the reaction products. Schematically this could be written *e g.* by

$$DPQ \overset{h\nu}{\rightleftharpoons} DP^{+}Q^{-} \longrightarrow D^{+}PQ^{-}$$

where P denotes P680 (ref 21), Q the primary acceptor of System 2, and D a secondary electron donor. Accumulation of charge in the reaction center by preillumination then could, in principle, explain the enhanced rate of back reaction of P and Q or lower rate of the secondary reaction by a reasoning similar to that advanced by Van Gorkom and Donze[22]. Another mechanism that might lower the yield of photochemical conversion, by triplet formation, has been reported by Duysens *et al.*[23].

However, the identical kinetics of the absorbance changes with and without preillumination is difficult to explain by the low yield hypothesis. This also applies to the results of flash experiments (see also Fig 9). The absorbance change induced by a flash at 518 nm in preilluminated chloroplasts was 65–85 % of that obtained in continuous light. This would not necessarily contrast to the relatively smaller increase of fluorescence in one flash since this difference could in principle be explained by energy transfer between reaction centers or photosynthetic units[18], but it is difficult to explain why a second or at least a third flash, each converting in succession the majority of the remaining traps, would fail to bring the fluorescence yield to almost the maximum level. More evidence against the hypothesis was recently given by Joliot and Joliot[6].

The second hypothesis, which we prefer in view of the above reasoning, would involve at least two electron acceptor and two donor molecules per reaction center. In dark-adapted chloroplasts, only one donor and acceptor are converted in the light at −40 °C, but in chloroplasts preilluminated with two flashes, a second electron donor and acceptor molecule also become oxidized and reduced, respectively, upon illumination. If it is assumed that, like at room temperature[24] the fluorescence yield is, at least mainly, determined by the redox state of the primary acceptor Q, the kinetics of fluorescence indicate that Q and the other acceptor become reduced at about the same rate The size of the second electron acceptor pool is obviously much smaller than that of plastoquinone[25] and may be equal to that of Q Cytochrome b_{559} is apparently not involved as second electron donor, as is indicated by the difference spectra and by the observation that the effect of preillumination was not basically altered by the addition of ferricyanide, which presumably oxidizes the cytochrome The lowering of the rate of fluorescence increase by more than a factor of two after preillumination, and the large number of flashes needed to approximately saturate the fluorescence yield in preilluminated chloroplasts suggest a lowering of the photochemical efficiency after the first quantum conversion, perhaps by charge accumulation and by partial closure of the traps, as reflected by the enhanced fluorescence.

The striking correlation at −40 °C between the preillumination responses of the 518-nm change and the fluorescence, shown in Fig. 7, indicates that the 518-nm change was mainly driven by System 2. This experiment was done in the presence of ferricyanide, which probably lowered the activity of System 1 by partial oxidation of P700 (see also ref. 26), but a similar pattern was observed for the 518-nm change

also without ferricyanide. It has been concluded that the 518-nm change is an electrochromic shift, due to a charge separation across the thylakoid membrane[14]. However, with preilluminated chloroplasts, the different kinetics in continuous light of the 518-nm change and the fluorescence increase do not seem to agree with the electrochromic hypothesis. The same applies to the different responses to a series of flashes given at −40 °C Moreover, the 518-nm change was smaller in preilluminated than in dark-adapted chloroplasts, even after several seconds of strong actinic light, whereas one might expect it to be as large, or even larger. Therefore, it appears that the absorbance change reflects a more specific change in or near the reaction center Similar shifts of pigment absorption in the purple bacterium *Rhodopseudomonas spheroides* have been found[27–29] to occur over too large distances (1–10 nm) to be explained by a direct effect of an electric field.

At first sight, the flash dependence of C-550 (Figs 5, 6) would seem to agree with the hypothesis of Joliot and Joliot[5], involving different primary acceptors, Q_1 and Q_2, both quenchers, which function in States S_0 and S_1, and in States S_2 and S_3, respectively. Q_1 then would be identical to C-550. However, this hypothesis is not very attractive to explain the other results, since it would predict the possibility of two light steps starting from S_1 (present in dark-adapted chloroplasts mainly) and perhaps from S_3, but not from S_2 (present after one flash). Therefore, the assumption that Q_1 functions as acceptor in all S states, and Q_2 only in S_2 and S_3 would explain the slow fluorescence kinetics in States S_2 and S_3 in a much simpler way. In a more recent paper[6] it was suggested that Q_2 would be reduced, but less efficiently than Q_1 in States S_0 and S_1, the opposite would occur in States S_2 and S_3. Reduction of Q_1 would be accompanied by a large fluorescence increase, reduction of Q_2 by a small one. At present we feel that there is insufficient evidence for the occurrence of more than one quantum conversion in States S_0 and S_1 (see *e.g.* Fig 9); further experimentation will be needed to distinguish between the various models. It should be noted that recent experiments with dithionite-treated chloroplasts in our laboratory[30] and the results obtained with DCMU, which at room temperature seems to inhibit the reduction of Q_2 ,suggest that there is only one primary acceptor for System 2 Also, the disappearance of the preillumination effect below −150 °C may indicate that the effect is due to a secondary reaction, which is inhibited at low temperature. The observation that C-550 was largely absent in the difference spectrum obtained after two flashes, whereas Q_1 would be photoreduced in any case, suggests that Q_1 cannot be simply equated with C-550, but that a more complicated relation between C-550 and the primary acceptor of System 2 exists

Finally, it is interesting to compare our results with those of Vermeglio and Mathis[9], who measured difference spectra at −196 °C of chloroplasts that had been illuminated at −50 °C. They found cytochrome b_{559} to be in the oxidized state when chloroplasts had received one or two flashes before freezing, and observed the bands of C-550 irrespective whether a preillumination had been applied or not Unless the measurements at −196 °C do not represent conditions at higher temperatures, and oxidation–reduction reactions occur when the material is cooled from −50 to −196 °C, one must assume that these differences are due to differences in the material used (*e.g.* age and preparation) or in the illumination conditions. Vermeglio and Mathis[9] used 5 s of white light, presumably of much higher intensity than that used in our experiments Anyway, it is clear that the scheme given by these authors, with

cytochrome b_{559} and another compound as electron donors, is too simple to explain the results discussed in this paper.

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