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THE 520 nm ABSORBANCE CHANGES IN *SCENEDESMUS OBLIQUUS* AND ITS RELATION TO PHOTOSYSTEM I

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SUMMARY

The kinetics (region of seconds) of the light-induced 520 nm absorbance change and its dark reversal have been studied in detail in the wild type and in some pigment and photosynthetic mutants of *Scenedesmus obliquus*. The following 5 lines of evidence led us to conclude that the signal is entirely due to the photosystem I reaction modified by electron flow from Photosystem II.

Gradual blocking of the electron transport with 3(3,4-dichlorophenyl)-1,1-dimethylurea resulted in diminution and ultimate elimination of the biphasic nature of the signal without reducing the extent of the absorbance change or of the dark kinetics. On the contrary, blocking electron flow at the oxidizing side of plastoquinone with 2, 5-dibromo-3-methyl-6-isoprophyl-*p*-benzoquinone or inactivating the plastocyanin with KCN, prolonged the dark reversal of the absorbance change apart from abolishing the biphasic nature of the signal.

Action spectra clearly indicate that the main signal (I) is due to electron flow in Photosystem I and that its modification (Signal II) is due to the action of Photosystem II.

Signal I is pH independent, whereas Signal II demonstrates a strong pH dependence, parallel to the O_2-evolving capacity of the cells.

Chloroplast particles isolated from the wild type *Scenedesmus* cells demonstrated in the absence of any added artificial electron donor or acceptor and also under non-phosphorylation conditions the 520 nm absorbance change with approximately the same magnitude as whole cells. The dark kinetics of the particles were comparatively slower. Removal of plastocyanin and other electron carriers by washing with Triton X-100 slowed down the kinetics of the dark reversal reaction to a greater extent. A similar positive absorbance change at 520 nm and slow dark reversal was also observed in the Photosystem I particles prepared by the Triton method.

Mutant C-6E, which contains neither carotenoids nor chlorophyll *b* and lacks Photosystem II activity, demonstrates a normal signal I of the 520 nm absorbance

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PQ, plastoquinone.

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change. This latter result contradicts the postulate that carotenoids are the possible cause of the 520 nm absorbance change.

INTRODUCTION

Since Duysens' [1] original observation on the light induced absorbance change near 520 nm in *Chlorella* many investigators have studied similar absorbance changes (variously referred to as 520, 525, 518 and 515 nm change) in green algae and in the chloroplasts of higher plants [2–8]. Although the 520 nm change is one of the most prominent light-induced absorbance changes occurring in plants and seems to be intimately related to the photosynthetic activity [9–11], no generally satisfactory explanation of its origin has been presented to date.

Carotenes [12] and chlorophylls [11, 13] were considered as the primary pigments that could cause absorbance changes at wavelengths of 520 and 475 nm. Complete loss of the absorbance change upon extraction of carotene from chloroplasts and restoration with the addition of pure β-carotene (c.f. ref. 14), and proportional increase in the extent of absorbance change with reference to the carotene content in mutant barley leaves [15] led some of the previous investigators to conclude that some carotenoid causes the absorbance change. The availability of a carotenoid-less mutant initiated our experiments to either reconfirm or abolish this assumption.

Pratt and Bishop [8] after a thorough study of the wild type and several mutant strains of *Scenedesmus* under aerobic and anaerobic conditions concluded that the biphasic 520 nm light-induced change is related mainly to Photosystem I. Their measurements were conducted with an apparatus recording absorbance changes in the region of seconds.

More rapid absorbance changes at 515 and 518 nm have also been observed after excitation with short light flashes of 1–3 μs duration [9–11, 16–22]. On the basis of their studies with spinach chloroplasts, Witt and his coworkers proposed that the production of an electrical field across the thylakoid membrane is responsible for the 515 nm change [23].

A similar but comparatively slower absorption change at 520 nm was also reported to occur upon the addition of salt to chloroplast suspensions in the dark [14]. Because of the discrepancy in the interpretation of the 520 nm signal in whole algal cells (*Scenedesmus*, region of seconds) and chloroplasts of higher plants (region of μs) experiments with whole cells and particles as well as with mutants of *Scenedesmus* were conducted.

MATERIALS AND METHODS

Algal culture

Cultures of *Scenedesmus obliquus* strain D_3 were grown and synchronized as described previously [24, 25]. Mutant strains were grown heterotrophically on a nitrate medium supplemented with 0.5 % glucose and 0.1 % yeast extract [26] and were maintained at 30 °C in Erlenmeyer flasks kept in the darkness on a rotating shaker. Wild type cells were harvested in the light phase of the synchronous culture at the

time of maximal photosynthetic capacity and quantum yield [22], and heterotrophic cultures after 3–5 days of growth.

Preparation of chloroplast particles

Chloroplast fragments from the algal cells were prepared by shaking cells equal to 500 μl packed cell volume in 20 ml nutrient medium together with 48 ml of glass beads (0.7 mm $\varnothing$) in a Vibrogen Cell-Mill (Bühler, Tübingen) for 3 min at 1 °C. Whole cells and cell wall materials were removed by centrifugation at 600 g for 3 min and the supernatant containing chloroplast fragments was used without further purification.

Photosystem I-enriched particles were obtained by further fractionating the chloroplast particles with Triton X-100. Chloroplast fragments were incubated in 1 % Triton at a final chlorophyll concentration of 100 μg/ml for 60 min at 1 °C. Particles sedimenting between 50 000 and 150 000 g were used.

Measurements

Chlorophyll was extracted from the cells with hot methanol and its concentration was determined as described previously [27].

Absorbance changes at 520 nm were measured using the Aminco DW-2 spectrophotometer (split-beam mode). The response time of the spectrophotometer was in the millisecond range and the total signal measured over a period of about 2 s. Cells were suspended in the nutrient medium at a final chlorophyll concentration of 30 μg/ml. Unless otherwise mentioned red light (620–740 nm, $3 \cdot 10^4$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1}) was used as actinic illumination (about 2 s). The photomultiplier was shielded against stray light using a Corning 9882 glass filter. The resulting absorbance change was normally biphasic. The biphasic nature of the signal could be abolished by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), subsequent illumination or other treatments. The resulting signal was referred to as signal I. Its amount was determined as the integrated area under the curve. The difference between the areas under the mono- and biphasic signals was referred to as signal II. See page 6 for a further explanation.

RESULTS

An example of a typical light-induced 520 nm absorbance change in the wild type strain of *Scenedesmus* (autotrophic and heterotrophic) cells is shown in Fig. 1. These results with *Scenedesmus* are similar to those obtained by Chance and Strehler [2] with *Chlorella*. Usually the first illumination (2 s) caused a biphasic signal that could be kinetically separated into an initial rapid phase followed by a dip and a second slow phase that reached its maximum after about 0.5–1.0 s. This dip and the slow increase of the second phase were found to be less pronounced in heterotrophic cells (Fig. 1b). In the dark the whole signal decayed with a half time of 10 ms. However, a second and subsequent illumination immediately after the dark decay of the signal completely removed the biphasic nature without affecting the total level of the absorbance change.

The light-induced absorbance change in *Scenedesmus* cells as a function of wavelength is shown in Fig. 2. The presence of a typically higher positive signal with its maximum around 520 nm and of a comparatively smaller negative signal around

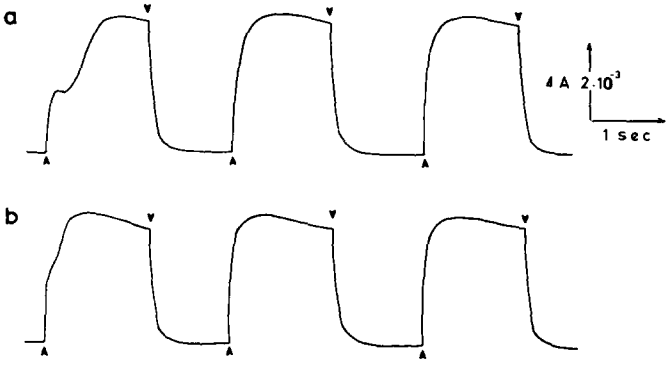

Fig. 1. Light-induced absorbance changes at 520 nm in wild type *Scenedesmus* cells at 23 °C. a, autotrophic; b, 2 day-old heterotrophic cells. Absorbance changes were measured using an Aminco DW-2 spectro-photometer (split-beam mode). Cells were suspended in the nutrient medium at a final chlorophyll concentration of 30 μg/ml. Red light (620–740 nm) at $3 \cdot 10^4$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1} served as actinic illumination. Arrows indicate actinic light on (▲) and off (▼).

480 nm confirm some of the earlier reports for *Chlorella* [1, 28, 29]. In Fig. 2 the spectrum of only the signal I part (see below) is shown; that for the signal II part has the same but somewhat broader maximum.

Modifications of the kinetics of 520 nm changes in autotrophic cells of *Scenedesmus* upon treatment with various electron transport inhibitors is shown in Fig. 3. Blocking the electron transport at the reducing side of PQ with 10 nM DCMU did not show any reduction in the total level of the absorbance change. On the other hand, the second slow phase was found to be accelerated. Increasing the concentration to 1 μM completely abolished the biphasic nature of the signal (Fig. 3a). Very high concentratons, 5–10 μM, caused a small decrease in the total level of the absorbance

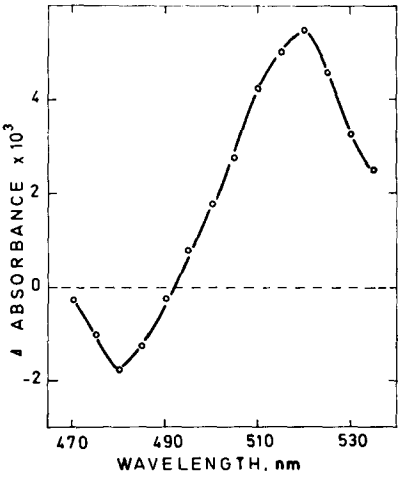

Fig. 2. Spectrum for the signal I part of the light-induced absorbance change in *Scenedesmus* cells. The measuring wavelength had a half band width of 3 nm. Other conditions were as indicated in Fig. 1.

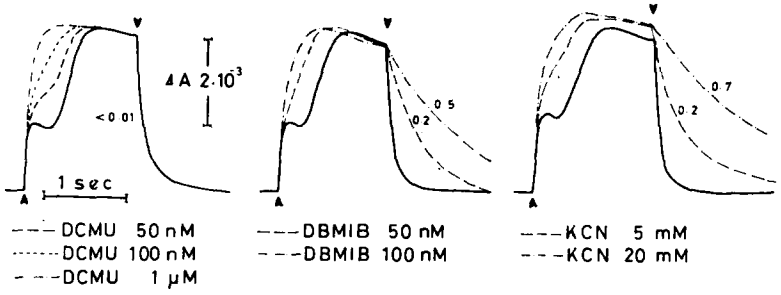

Fig. 3. Changes in the kinetics of the 520 nm absorbance change of *Scenedesmus* cells upon treatment with different concentrations of DCMU, DBMIB and KCN. Cells were incubated at 23 °C for 5 min at the indicated concentrations of the added substances before measurements. Other conditions as listed in Fig. 1.

change (data not shown). The normal dark decay was observed in all the cases. Blocking the electron transport after PQ with 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) also caused changes in the light phase of the signal similar to those observed in DCMU-treated cells; however, this treatment delayed the dark decay to a greater extent (Fig. 3b). This effect was also found to be proportional to the concentration of DBMIB. Cells treated with 50 nM or 100 nM DBMIB showed a dark decay with a half time (0.2 and 0.5 s, respectively) much slower than that of the control (10 ms). Similar changes were also observed when the electron transport between the two systems was blocked by inactivation of plastocyanin with KCN (Fig. 3c). Upon treatment with 5 and 20 mM KCN the half reversal time was delayed to 0.2 and 0.7 s, respectively. All these experiments showed that when only Photo-

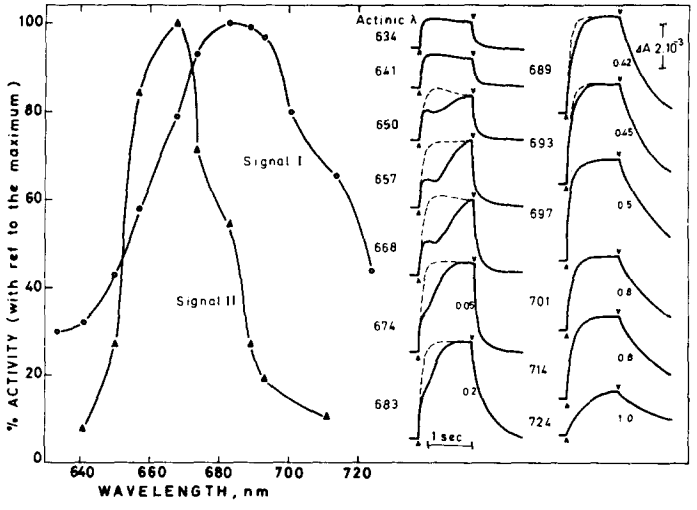

Fig. 4. Action spectra for the signal I and II portion of the 520 nm absorbance change in *Scenedesmus* cells. Monochromatic light was isolated by using appropriate narrow-band interference filters(Schott, Mainz). Part of the cuvette at the side of actinic illumination was filled with clear glass to avoid differential absorption by chlorophylls at the red region of the spectrum. Light intensity was maintained at $5.1 \cdot 10^3$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1} at all wavelengths. For other details refer to the text.

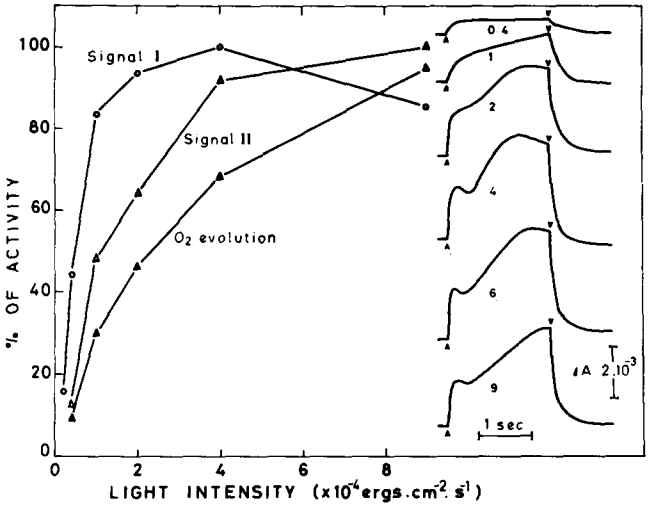

Fig. 5. Kinetics of the 520 nm change in *Scenedesmus* cells as a function of the intensity of the actinic illumination (620–740 nm). Conditions for measurements as listed in Fig. 1. Numbers along the traces indicate the intensity of the actinic light ($\cdot 10^{-4}$ erg $\cdot$ cm^{-2} $\cdot$ s^{-1}).

system I was functional, the biphasic nature of the signal was completely abolished, but without any apparent reduction of the magnitude of the absorbance change. A normal functioning electron transport system from PQ to Photosystem I is required for the fast dark decay. Such a transformation from a bi- to a monophasic signal was also shown in *Scenedesmus* cells under anaerobic conditions [8]. For convenience, that portion of the absorbance change seen in the presence of a functional Photo-

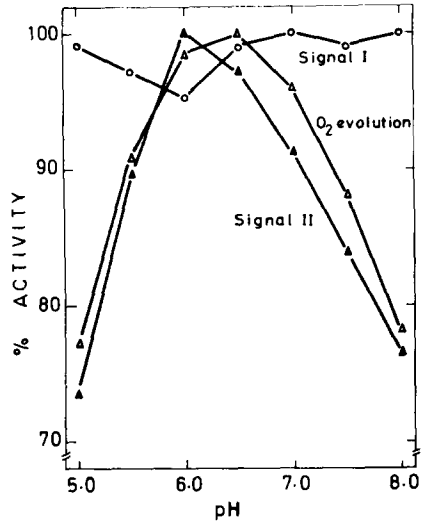

Fig. 6. Dependence of pH on the signal I and II portion of the 520 nm change in *Scenedesmus* cells. Conditions for measurements as in Fig. 1. Cells were suspended Sørensen's NaH_2PO_4/K_2 HPO_4 buffer adjusted to the required final pH.

system I (on eliminating Photosystem II by DCMU treatment), is hereafter referred to as signal I, and differences in the level of the signal, i.e., before and after the DCMU treatment, as signal II; these values were calculated by determining total areas under the respective curves.

Similarly, transformation of the biphasic nature of the kinetics of the 520 absorbance change was also observed under illumination with different wavelengths of light. When cells were irradiated with wavelengths of light that are more specific for Photosystem II, the typical biphasic signal (though the total level of the extinction was comparatively less) was observed (Fig. 4). A more prominent dip and second slower phase was seen at wavelengths around 660–670 nm. Gradual reduction in the level of the dip and acceleration of the second phase, ultimately leading to a monophasic signal, was observed with increasing wavelengths of light (Fig. 4, insert). Signals of relatively higher amplitude were obtained in the region of 680–714 nm, i.e. wavelengths of light more specific for Photosystem I. Similar higher activation with 710–720 nm light was also observed in *Chlorella* [29]. Simultaneously, the half time of the dark decay increased in the far-red region of the spectrum. Up to about 200 times slower dark decays were observed after illumination with lights above 700 nm. The action spectra for the signal I and II portion of the 520 nm change, as computed from the kinetics, are shown in Fig. 4. The maxima observed for signal II and I portion of the 520 nm change coincide with many of the action spectra reported for the reactions of Photosystems II and I.

Changes in the 520 nm absorbance change as a function of the intensity of actinic light are shown in Fig. 5. The typical signal (insert figure) was developed even at $2 \cdot 10^4$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1}. Higher intensities only slowed the second phase of the signal. As demonstrated in Fig. 5, the signal I portion was found to saturate even at lower intensities, whereas signal II required relatively higher intensities; this response was found to parallel the light intensity dependence of O_2 evolution by whole cells.

Photosynthetic O_2 evolution is highly pH dependent. To check the possible relation of the 520 nm change to the O_2 evolution, the influence of the external pH

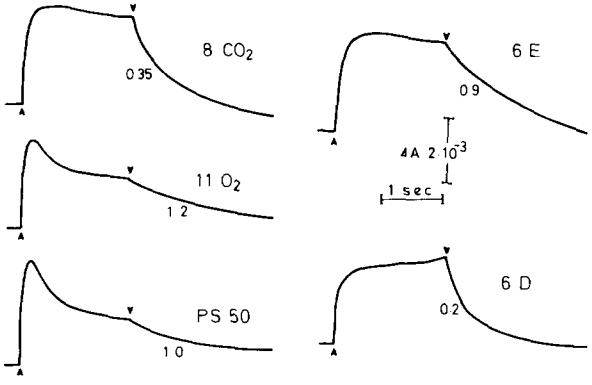

Fig. 7. Comparison of the light-induced absorbance changes at 520 nm in various photosynthetic (PS-8, PS-11 and PS-50) and pigment (C-6E, C-6D) mutants of *Scenedesmus*. Cells at a final concentration of 10 μl packed cell vol/ml were suspended in the nutrient medium. All mutants were grown heterotrophically for a period of 3–5 days. Conditions of measurements as indicated in Fig. 1.

of the reaction mixture was determined. Fig. 6 demonstrates the effect of pH on the levels of the signal I and II portions of the 520 nm change. A reduction of about 25 % in the signal II level was observed at pH 5.0 and 8.0 as compared with that at pH 6.0 The close relationship between the O_2-evolution reaction and the signal II portion of the 520 nm change is also quite apparent from these measurements. Changes in the signal I portion were much less apparent in the whole pH range measured. A similar independence for part of the 520 nm change was also reported by Witt et al. [23]

The characteristics of the 520 nm change were also examined in some of the pigment and photosynthetic mutants of *Scenedesmus*. None of the mutants examined showed the typical biphasic signal (Fig. 7). Mutant C-6E, which is devoid of even traces of carotene and chlorophyll *b* also exibited a 520 nm signal of the same magnitude as observed in wild types when calculated on the basis of chlorophyll *a* content. Mutant C-6D which develops carotene precursors, low levels of non-cyclic carotenes, and chlorophyll also demonstrated the 520 nm change, but of lesser magnitude than that seen in the wild type. Other photosynthetic mutants lacking either the O_2-evolving system (PS-11), or the CO_2-fixation reaction (PS-8), or blocked at the cytochrome *f* site (PS-50) [30], demonstrated a rapid increase upon illumination followed by a reduction in the level of about 30–40 %. The dark decay was found to be much delayed in most of the mutants. Comparatively lower values were seen only in the mutants PS-8 and in C-6D.

Experiments were also performed with isolated chloroplast particles prepared according to the procedure indicated under Methods. Such particles showed very low electron transport from $H_2O \rightarrow$ NADP or methylviologen without the addition of plastocyanin and ferredoxin [26], but comparatively higher rates of individual partial reactions of photosynthesis. The 520 nm signal in *Scenedesmus* chloroplast particles (Fig. 8), resembled more closely those reported for spinach chloroplast particles [17, 31]. But in *Scenedesmus* chloroplast particles the dark decay kinetics were comparatively slower. This dark decay was further delayed when plastocyanin and other redox-carriers were removed with 1 % Triton. Photosystem I particles prepared by Triton method exhibited (on a chlorophyll basis) a slightly lower level of the 520 nm change (Fig. 8).

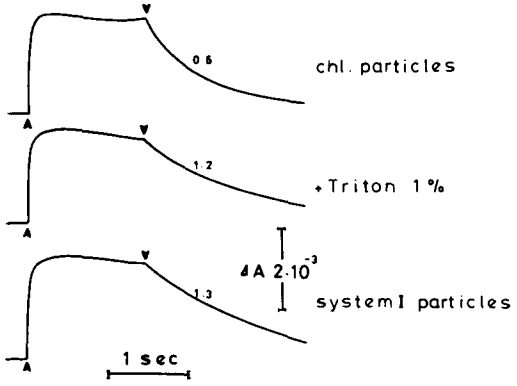

Fig. 8. Absorbance changes at 520 nm in chloroplast particles prepared from *Scenedesmus* cells. Chloroplast particles and photo system I-enriched particles were prepared as described in Methods. Conditions for measurements as in Fig. 1.

DISCUSSION

The light-induced absorbance changes measured in this study are identical with those initially reported by Duysens [1] and some others [2, 3, 8] for intact algae. The kinetics in the time region of seconds (Figs 1, 2) and the wavelengths-dependence clearly identify it as the light-induced absorbance change originally described as the 520 nm change. Measurements with isolated chloroplasts, mainly carried out by Witt and his coworkers, covered only the very fast initial reaction in the millisecond region and its decay kinetics. Although there can be no doubt that those measurements deal with the same phenomenon, one has to be aware of the difference between the slow in vivo measurements in whole algae and the in vitro flash-induced changes in isolated chloroplasts (cf. Pratt and Bishop [8]).

A detailed analysis of the 520 nm absorbance changes under in vivo conditions provided evidence that the signal is caused mainly by Photosystem I reaction, and the apparent biphasic nature of the kinetics is a result of the partial compensation of the signal by Photosystem II. At least five lines of evidence are suggested here for support of this hypothesis. Firstly, blocking the electron transport between the two photosystems at different levels with DCMU, DBMIB or KCN caused a gradual disappearance of the biphasic nature of the signal without reducing the extent of the total absorbance change. The DCMU- or DBMIB-insensitivity of the 520 signal was also reported by some of the earlier workers [13, 32]. Secondly, mutant PS-11, which lacks Photosystem II activity but retains a functional Photosystem I, demonstrated a normal level of the 520 nm signal. Thirdly, activation of Photosystem I alone with specific monochromatic light (690 nm) completely removed the biphasic nature of the signal. The second phase was found to be much more pronounced at wavelengths specific for Photosystem II (640–660 nm). The action spectrum for signal I coincides with the Photosystem I reaction of photosynthesis. Fourthly, signal II of the 520 nm change reacts more similarly to the course of O_2 evolution and its reaction to pH, as seen from the light saturation curves. Independence of the 520 nm change to external pH and temperature is also reported [23]. Finally, Photosystem I-enriched particles prepared from *Scenedesmus* cells in the absence of phosphorylation and normal electron transport demonstrated the 520 nm change more or less of the same magnitude as observed in whole cells. Similar high levels of 520 nm change were also shown in Photosystem I particles obtained from spinach chloroplasts by the digitonin method [17] or by sonication technique [31]. A similar conclusion was also drawn by Pratt and Bishop [8] after studying the 520 nm absorbance change under anaerobic conditions in wild type and mutants of *Scenedesmus*. Transformation of cells to anaerobic conditions eliminated the biphasic nature of the signal.

The present results disagree with some of the earlier reports [12, 14, 15] where the 520 nm change was postulated as a result of change in β-carotene [14] or carotene complex. These postulations were based on the observation that the 520 nm signal was completely removed upon extraction of carotene from the chloroplasts and its restoration upon the addition of pure β-carotene. To verify this under more physiological conditions mutant C-6E that lacks even traces of carotene but has a normal amount of chlorophyll *a*, was used. Even though this mutant has no photosynthesis but good photo-reduction, it possesses the 520 nm signal of the same magnitude as observed in wild type when calculated on a chlorophyll basis.

All the data presented here led us to postulate that the entire 520 nm absorbance change is most likely an oxidation process of possibly a chlorophyll-protein complex associated with Photosystem I and which is reduced through Photosystem II in the dark. Further evidence for this hypothesis was also obtained from the kinetics of the dark decay. Blocking the electron transport either after plastoquinone with DBMIB or at plastocyanin with KCN caused no reduction in the net level of the absorbance change but reduced the dark reversal to a greater extent. Similarly, when Photosystem I is specifically activated with monochromatic light, in the absence of enough reductants the dark reversal of the signal was much delayed because of the lack of sufficient reductants supplied by Photosystem II. Such delay in the dark reduction was also shown for cytochrome *f* after illumination with 701 nm light, whereas a normal rapid reduction occurred in DCMU-treated cells [33]. Also the removal of plastocyanin and further electron carriers in *Scenedesmus* particles by washing with Triton further delayed the dark reversal kinetics, which again indicates a necessity of the electron transport from Photosystem II for its dark reduction.

In this connection it is of interest to note a similarity between the kinetics and the time range of the 520 nm absorbance change and the fluorescence induction (Kautsky effect) in whole cells. In both cases the first illumination caused a biphasic signal due to a delay in establishing a perfectly coupled electron transport between the two Photosystems.

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