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THE RELATIONSHIP BETWEEN Q, C-550 AND CYTOCHROME *b* 559 IN PHOTOREACTIONS AT —196° IN CHLOROPLASTS

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SUMMARY

Absorbance changes and fluorescence yield changes induced by irradiating spinach chloroplasts with red light at —196° were measured as a function of the redox potential of the chloroplast suspension. Absorbance changes at 546 nm indicate the photoreduction of C-550 and changes at 556 nm indicate the photooxidation of cytochrome *b* 559. The changes of fluorescence yield indicate the photoreduction of Q, the fluorescence quencher of chlorophyll *a* in Photosystem II. The titration curves for all three changes were essentially the same and showed the same midpoint potential. In other experiments as well, it was found that when C-550 is in the reduced state the fluorescence yield of the chloroplasts is high and the low-temperature photooxidation of cytochrome *b* 559 is blocked. These data indicate that C-550 may be equivalent to Q and that cytochrome *b* 559 serves as the electron donor for the photoreduction of C-550 at low temperature.

INTRODUCTION

KNAFF AND ARNON recently reported that cytochrome *b* 559 is photooxidized by Photosystem II[1] and that a new compound, C-550, is photoreduced by Photosystem II[2], both reactions occurring at temperatures down to —189°. They suggested, on the basis of these findings, that Photosystem II consists of two sequential photochemical reactions, Photosystem IIb, which reduces C-550 and forms a strong oxidant which can oxidize water and Photosystem IIa which oxidizes cytochrome *b* 559 and reduces ferredoxin[3]. Photosystem I, which is responsible for cyclic photophosphorylation, is assumed to be independent of Photosystem II.

We confirmed that the photoreduction of C-550 and the photooxidation of cytochrome *b* 559 at —196° were mediated by Photosystem II but suggested that the same photoreaction is responsible for both processes, *i.e.* that cytochrome *b* 559 is oxidized by Z^+, the strong oxidant of Photosystem II, and thus serves as the electron donor for the photoreduction of C-550[4]. We also suggested on the basis of the chemical reduction of C-550 by dithionite but not by ascorbate that C-550 might be related to Q, the fluorescence quencher of Photosystem II. In the present paper, we present evidence to support both of these suggestions.

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

METHODS

Spinach chloroplasts were prepared by methods described previously[5] and suspended in sucrose–Tris–NaCl buffer (0.4 M sucrose, 0.05 M Tris–HCl, 0.01 M NaCl, pH 7.8). Broken chloroplast fragments were obtained by suspending chloroplasts in water at 0° for 40 min. The large fragments obtained by centrifugation at 1000 × *g* for 5 min were discarded and the smaller fragments obtained by centrifugation at 10000 × *g* for 10 min were suspended in sucrose–Tris–NaCl buffer. The redox potential of the chloroplast suspension was established under anaerobic conditions according to the methods of CRAMER AND BUTLER[5]. Argon gas (Air Products) was passed through a manganese oxide column and a wash bottle with $CrSO_4$ before going into a 50-ml titration vessel. A combination platinum Ag–AgCl electrode (Instrumentation Lab 15020) was inserted into the titration vessel through a silicone rubber stopper to measure the redox potential.

The potential of the Ag–AgCl reference electrode was calibrated against a saturated quinhydrone electrode at 0° and found to be + 188 mV. This value was used to convert the potential measurement to the scale based on the hydrogen electrode.

25 ml of the reaction medium, pH 7.0, consisting of 0.4 M sucrose, 0.1 M phosphate, 0.04 M $MgCl_2$ and 0.01 M glucose *plus* the redox buffer, 5 mM EDTA and 50 μM $FeCl_3$, $E_{m_7} = +120$ mV, were bubbled with the purified argon at room temperature overnight. The following redox buffers were then added: 10 μM anthraquinone 2-sulfonate, $E_{m_7} = -225$ mV; 10 μM 2-hydroxy-1,4 napthoquinone, $E_{m_7} = -145$ mV; and 5 μM 1,4-napthoquinone, $E_{m_7} = +60$ mV. 2 mg of glucose oxidase (Mann, specific activity about 170 units) were added to further insure anaerobiosis and the mixture was stirred magnetically at room temperature for at least 1 h. Chloroplasts, equivalent to 3.5 mg chlorophyll, were placed in a Thunberg tube with 0.01 M glucose and 0.3 mg glucose oxidase and the tube was evacuated and filled with argon 3 times and kept in darkness at room temperature for 60 min. The chloroplasts and the redox buffer in the titration vessel were then cooled in an ice bath and the chloroplast suspension was transferred with a syringe to the titration vessel. The stream of argon gas was adjusted to blow across the surface of the chloroplast suspension to avoid the foaming and possible chloroplast damage which results from bubbling. The suspension was stirred magnetically. After the potential had become stable, the titrant was added in aliquots of 1 to 10 μl with a gas tight microsyringe through a septum in a sidearm. The reductant was 0.25 M methyl viologen in 0.05 M phosphate, pH 7.0, reduced by hydrogen with paladium on asbestos as catalyst. The oxidant was $K_3Fe(CN)_6$, 0.01 M or 0.1 M. Time intervals in between points were 30 to 50 min.

When the potential had been stable for several minutes, a 0.3-ml sample (132 μg chlorophyll per ml) was withdrawn with a syringe and injected into the measuring cuvette under a stream of argon. The cuvette was immersed in liquid nitrogen in the optical dewar[6] with argon over the sample. After the sample was thoroughly frozen, air did not affect the redox state of the components.

Absorption spectra were measured with a single-beam spectrophotometer which consisted of a Cary Model 14 monochromator, tungsten lamp, sample compartment, and a logarithmic photometer on line with a PDP 8/I computer[7]. Each single-beam spectrum was measured 4 times and the spectral data were added together to improve the signal-to-noise ratio by a factor of 2. The single-beam spectra were stored in the

computer or punched out on paper tape for later computation of difference spectra. Absolute spectra are presented as the difference between the single-beam spectrum of the sample and that of a blank consisting of frozen buffer. The spectral curves were plotted with an X–Y recorder directly from the computer.

Absorption spectra were measured with a 600-nm short-pass filter (Optics Technology) placed in front of the phototube (EMI 9558C) to reduce stray light from the chlorophyll fluorescence. The relative fluorescence intensity of the sample at —196° was determined by replacing the 600-nm short-pass filter with a 695-nm interference filter (15 nm half-width), setting the monochromator at 650 nm with a 0.5-mm slit (instead of the 0.2-mm slits used for absorbance measurements), and measuring the photocurrent with a given phototube voltage. Fluorescence was measured in the narrow spectral region around 695 nm to maximize the fluorescence of variable yield from Photosystem II. The fluorescence around 720 nm is very high at liquid nitrogen temperature but there is relatively little fluorescence of variable yield in this region. Light-induced changes of absorbance and fluorescence yield were determined by recording the absorption spectra and the relative fluorescence intensity before and after irradiating the sample at —196° with red (645 nm) actinic light 10^3 μW/cm^2 for 30 sec. In the titration curves the relative amount of C-550 photoreduced was calculated from the absorbance difference between 546 and 543 nm while the relative amount of cytochrome *b* 559 photooxidized was calculated from the absorbance difference at 556 nm from an estimated baseline level in the low-temperature light-*minus*-dark difference spectrum.

Test titrations with FMN in the redox buffers gave reversible titration curves which had the expected midpoint potential of —200 ±5 mV. The titrations were made by measuring either absorbance or fluorescence on an aliquot of the FMN solution frozen to —196° as a function of the redox potential established at 20°.

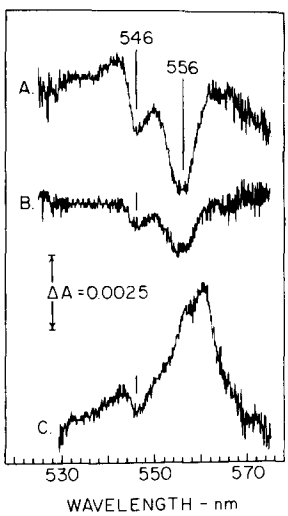
The fluorescence data in Fig. 5 were obtained by measuring the fluorescence ($\lambda > 680$ nm) from a chloroplast suspension (12 μg chlorophyll per ml) excited by 10^3 μW/cm^2 of blue light (Unitron microscope lamp through 2 cm of 20 % $CuSO_4$ and a 480-nm interference filter). The phototube was connected to an oscilloscope and the time course of the fluorescence signal following the onset of irradiation was recorded from the oscilloscope trace to determine the initial fluorescence signal when the shutter was first opened. The data plotted are the initial fluorescence intensities obtained at the onset of irradiation as a function of dark time after the previous irradiation.

RESULTS

A typical light-*minus*-dark difference spectrum showing the absorbance changes caused by irradiating chloroplasts at —196° is shown in Fig. 1, Curve A. The bleaching at 556 nm is due to oxidation of cytochrome *b* 559 and the bleaching at 546 nm with the concomitant increase at 543 nm is due to reduction of C-550. The chloroplast sample used to obtain Curve A was poised at a redox potential of +55 mV prior to freezing and the absorbance changes observed are maximal. At potentials above 400 mV the cytochrome *b* 559 is fully oxidized and shows no additional light-induced change but the absorbance changes due to photoreduction of C-550 remain[4]. The cytochrome *b* 559 involved in the low temperature photooxidation appears to be the high potential one reported by BENDALL[8] to have a midpoint potential of +370 mV.

The sample used to obtain Curve B was poised at —67 mV prior to freezing. In this case the light-induced changes of C-550 and cytochrome *b* 559 are less than those seen in Curve A. Curve C, which is the difference spectrum between the two nonirradiated samples, —67 mV *vs.* +55 mV, shows that C-550 is partially reduced chemically at —67 mV, so less is available for the photoreduction. The absorption band at 560 nm in Curve C is due to the reduction of cytochrome b_6. The shoulder on the short wavelength side of that band indicates the presence of a low-potential cytochrome *b* 559 such as that reported by FAN AND CRAMER[9]. At potentials lower than —150 mV the chemical reduction of C-550 is complete and there are no light-induced absorbance changes; even the photooxidation of cytochrome *b* 559 is blocked at low potential.

Fig. 2 shows the light-induced absorbance changes of C-550 and cytochrome *b* 559 at —196° as well as the light-induced fluorescence-yield changes at —196°C as a function of the redox potential established at 0° prior to freezing. The light-induced fluorescence-yield changes, plotted as the ratio of the intensity of chlorophyll fluorescence after the actinic irradiation to the intensity of fluorescence before irradiation, indicates the low-temperature photoreduction of Q, the quencher of chlorophyll fluorescence in Photosystem II. The intensity of chlorophyll fluorescence in the



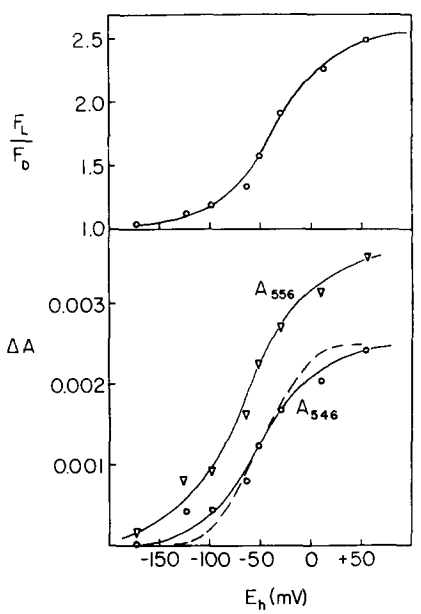


Fig. 1. Curves A and B, light-*minus*-dark difference spectra of chloroplast samples (0.3-ml samples contained 40 μg chlorophyll) irradiated with 645 nm light. The redox potential prior to freezing was +55 mV for Curve A and —67 mV for Curve B. Curve C, difference spectrum of the dark —67 mV sample *vs.* the dark +55 mV sample.

Fig. 2. Lower: absorbance changes at 546 nm, calculated as the absorbance differences between 546 and 543 nm, and at 556 nm, calculated as the difference from an estimated baseline level, resulting from irradiation with 645 nm light at —196° as a function of the redox potential of the chloroplast suspension prior to freezing. Dashed curve, theoretical plot of the Nernst equation for a 1-electron transition with a midpoint potential of - 50 mV. Upper: change of fluorescence yield at —196° due to the irradiation at —196° as a function of the redox potential. F_D and F_L are the intensities of fluorescence measured at 695 nm before and after irradiation, respectively.

nonirradiated samples increases with decreasing potential as Q becomes reduced chemically so that the increase of fluorescence due to photoreduction is less. As expected, titration curves for chemical reduction of C-550 and for the fluorescence yield are complementary to the titration curves for the light-induced changes.

The dashed curve in Fig. 2 is a plot of the Nernst equation for a 1-electron transition with a midpoint potential of —50 mV. The experimental data for the absorbance change at 546 nm fits closely to a Nernst equation for a 0.8 electron change. We tend to ascribe the deviation from a 1-electron process to inadequate redox equilibration with the medium. A titration curve on broken chloroplasts (Fig. 3) gave results which were much closer to a 1-electron transition (the straight line in Fig. 3) but the midpoint potential appeared to be +25 mV.

In any given experiment, the titration curves for the photoreduction of C-550, the photooxidation of cytochrome *b* 559, and the light-induced fluorescence-yield change were always similar and showed the same midpoint potential but there was some variation in midpoint potential between different titration runs. In particular a hysteresis-like phenomenon appeared between titrations made in the reducing and oxidizing directions. The midpoint potentials in the oxidizing titration were always more positive by as much as 100 mV. The reasons for this hysteresis effect are unknown but may be related to inadequate equilibration. Better reversibility was obtained with the broken chloroplasts preparation used for Fig. 3. The data, however,

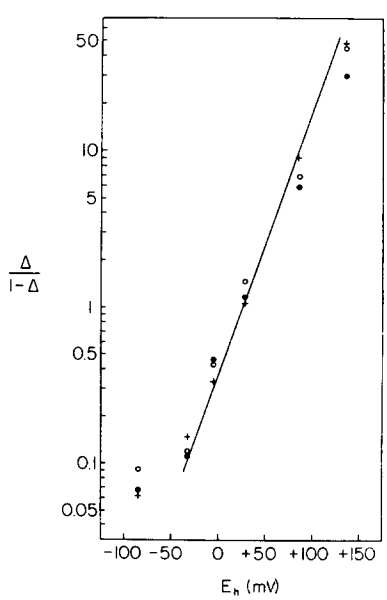


Fig. 3. Semilog plot of $\Delta/1-\Delta$ where Δ is the fraction of the component available for the light-induced absorbance change at —196° as a function of the redox potential of a suspension of chloroplast fragments established prior to freezing. Light-induced absorbance changes at 546 nm (○) and 556 nm (●) and the light-induced fluorescence yield changes (+) are shown. Log $(\Delta/1-\Delta)$ is equivalent to log (C-550 oxidized/C-550 reduced). The straight line is the semilog plot of the Nernst equation for a 1-electron transition with a midpoint potential of +25 mV.

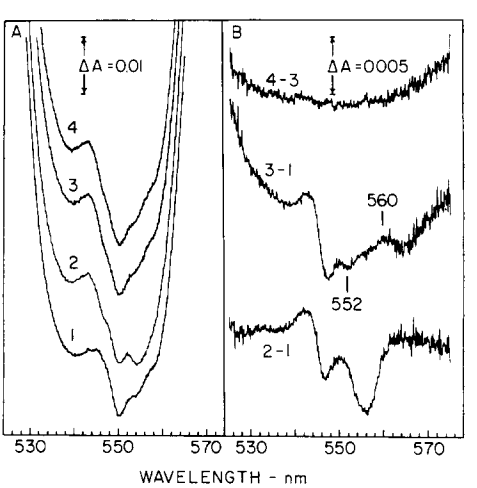


Fig. 4. A. Absolute spectra of chloroplast samples at —196°. Curves 1 and 2, spectra measured before and after irradiation with 645 nm light at —196°. Curves 3 and 4, spectra of sample irradiated during the freezing process before and after a subsequent irradiation at —196°. B. Difference spectra between the absolute spectra in A.

are presented to show the similarity between the measurements of absorbance at 546 and 556 nm and fluorescence yield rather than to establish precisely the value of the midpoint potential.

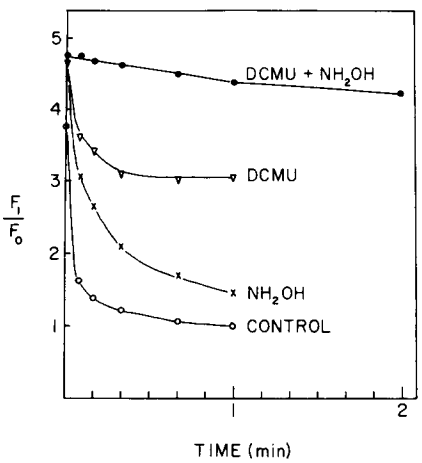
The similarity between the three titration curves shown in Figs. 2 and 3 indicates that the three processes are closely related. The agreement between the titration curves for the light-induced absorbance changes of C-550 and the light-induced fluorescence-yield changes gives strong support to the suggestion that C-550 is analogous to Q or is closely allied to it functionally. The correspondence between the photooxidation of cytochrome *b* 559 and the photoreduction of C-550 indicates that at —196° cytochrome *b* 559 serves as the electron donor to Photosystem II for the photoreduction of C-550 and that when C-550 is reduced chemically there is no electron acceptor available for the photooxidation of cytochrome *b* 559. Cytochrome *b* 559, however, is not an obligate electron donor for the photoreduction of C-550 since the latter reaction still proceeds when cytochrome *b* 559 is oxidized chemically[4].

Other methods for obtaining C-550 in the reduced state at low temperatures were sought in an effort to find conditions where the fluorescence yield might not be correlated with the reduction state of C-550 or where the photooxidation of cytochrome *b* 559 at —196° might not require the concomitant photoreduction of C-550. When chloroplasts suspended in the normal sucrose–Tris–NaCl buffer are irradiated with red light at room temperature in the absence of an electron acceptor, Q becomes fully reduced in the light and cytochrome *b* 559, which is normally reduced in the dark, remains reduced in the light[10]. This condition can be frozen in by irradiating chloroplasts with red light during the initial part of the cooling process. Fig. 4A shows the absolute spectra of a chloroplast sample frozen in darkness, before and after irradiation at —196°, and of a sample irradiated during the cooling process, before and after a subsequent irradiation at —196°.

Difference spectra between the various curves in Fig. 4A are presented in Fig. 4B. Curve 2-1 shows the normal light-induced spectral changes obtained at —196°. The difference spectrum between the sample irradiated during cooling and that frozen in complete darkness (Curve 3-1) shows that the C-550 is reduced in the preirradiated sample. The fluorescence yield of the preirradiated sample was high and could not be increased further by irradiation at —196°. The difference spectrum also shows a slight oxidation of cytochrome *f* (giving a minimum at 552 nm) and some reduction of cytochrome b_6 (giving a maximum at 560 nm), both of which should be expected since Photosystem I mediates these reactions at room temperature[10], but no change of cytochrome *b* 559. The absolute spectrum of the preirradiated sample (Curve 3) confirms that cytochrome *b* 559 remained reduced. Irradiation of the preirradiated sample at —196° (see Curve 4-3) produces no spectral changes. The photooxidation of cytochrome *b* 559 at —196° is blocked by the prior photoreduction of C-550 in agreement with the data obtained at low redox potential.

BENNOUN[11] showed that the dark oxidation of QH is blocked in the presence of NH_2OH and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) so that the high fluorescence yield which obtains during the irradiation of chloroplasts is maintained after irradiation ceases. Fig. 5 shows the influence of NH_2OH and DCMU on the decay of fluorescence yield at room temperature as a function of the time in darkness after an irradiation. The high fluorescence yield which is maintained after irradiation in

the presence of NH_2OH and DCMU can be restored to the F_0 level by adding a small amount of ferricyanide. To obtain the data for Fig. 6, chloroplasts with and without NH_2OH *plus* DCMU were irradiated at room temperature for 30 sec with red light and placed in darkness 1 min before freezing to —196°. Absorption spectra were measured before and after irradiation at —196°. Fig. 6 shows the light-induced difference spectra for the control chloroplasts (Curve A) and for the chloroplasts in the presence of NH_2OH and DCMU (Curve B) and the chemically induced difference spectrum between the two chloroplast samples before the irradiation at low temperature (Curve C). The fluorescence yield at —196° of the chloroplasts with NH_2OH and DCMU was high and could not be increased further by irradiation.



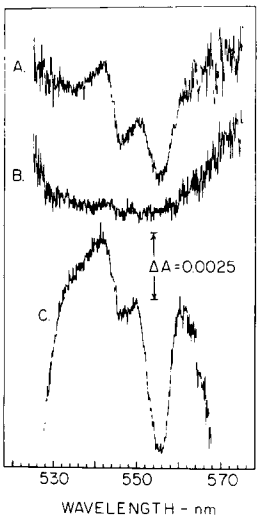


Fig. 5. Relative fluorescence intensity ($\lambda < 680$ nm) of chloroplasts (12 μg chlorophyll per ml) obtained at the onset of irradiation with 480 nm light (10^3 $\mu W/cm^2$) as a function of dark time after a previous 30-sec irradiation. The initial fluorescence intensity of dark adapted, normal chloroplasts is taken as unity. 25 mM NH_2OH and 10 μM DCMU were present where indicated.

Fig. 6. Curves A and B, light-*minus*-dark difference spectra of chloroplasts irradiated with 645 nm light at —196°. The chloroplasts were kept in the dark at room temperature for 1 min after a 30-sec irradiation with 480 nm light (as in Fig. 4) before freezing to —196°. Curve A, control chloroplasts with no additions. Curve B, chloroplasts with 25 mM NH_2OH and 10 μM DCMU. Curve C, difference spectrum between the dark sample with NH_2OH and DCMU and the control sample.

The chloroplast sample with NH_2OH and DCMU (Curve B) shows no light-induced changes at —196°. The difference spectrum between that sample and the control sample (Curve C) shows that C-550 remains reduced following an irradiation at room temperature in the presence of NH_2OH and DCMU. Again the reduced state of C-550 correlates with a high fluorescence yield of the chloroplasts.

The Curve C difference spectrum also shows that the cytochrome *b* 559 in the sample containing DCMU and NH_2OH is largely oxidized while that in the control sample is reduced. The amount of cytochrome *b* 559 relative to the amount of C-550 appears to be greater in the chemical difference spectrum (Curve C). We generally observe about twice as much cytochrome *b* 559 in ascorbate-*minus*-ferricyanide

difference spectra as we observe in the low-temperature light-induced difference spectra. Apparently there is more cytochrome *b* 559 than C-550 present but the amount of cytochrome *b* 559 which can be photooxidized at low temperature is limited to the amount of C-550 present. Assuming that one molecule of C-550 is reduced for each molecule of cytochrome *b* 559 oxidized by irradiation at low temperature, the change of the extinction coefficient due to the reduction of C-550 can be estimated relative to that for the oxidation of cytochrome *b* 559.

DISCUSSION

KNAFF AND ARNON, after discovering the Photosystem II mediated photoreduction of C-550 and photooxidation of cytochrome *b* 559 at low temperature, proposed that Photosystem II actually consists of two photoreactions, Photosystem IIb which reduced C-550 and makes an oxidant strong enough to evolve O_2 from water and Photosystem IIa which oxidizes cytochrome *b* 559 and reduces ferredoxin. Our results which show that the low temperature photooxidation of cytochrome *b* 559 requires the concomitant photoreduction of C-550 suggest that both redox reactions are mediated by the same photochemical reaction and that Photosystem II need not be separated into two systems. More recently ARNON and co-workers[12,13] have emphasized arguments from room-temperature experiments rather than low-temperature experiments in support of two Photosystem II photosystems. The results presented in this paper argue only that the low-temperature data is satisfied with just one photoreaction.

It should be recognized that not all photochemical reactions which occur at liquid nitrogen temperature are necessarily primary photochemical events that occur at room temperature as well. The low-temperature photooxidation of cytochrome *b* 559 may be an artifact brought on at low temperature because the strong oxidant of Photosystem II cannot receive electrons from water. It has been reported that cytochrome *b* 559 is closely associated with, or is a part of, the reaction center of Photosystem II[14] and, thus, should be in close proximity to the Photosystem II oxidant. The photooxidation of cytochrome *b* 559 by Photosystem II at room temperature can be induced by Tris treatment[13,3,4] since electron transport between water and primary oxidant of Photosystem II is inhibited under these conditions as well. Under normal conditions, however, the oxidizing power of Photosystem II is dissipated by splitting water and cytochrome *b* 559 appears to participate in the electron transport chain between Photosystem II and Photosystem I[10].

Of particular significance is the correlation between the fluorescence yield of chlorophyll and the reduction state of C-550. Under all conditions we have examined (these experiments include the effects of ultraviolet irradiation, heat treatment and lipase extraction in addition to the data reported here) this correlation has held up. Thus, we feel that C-550 is the same as, or is an excellent index of, Q and that it is the primary electron acceptor of Photosystem II.

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