Relationship of P700, Electron Spin Resonance Signal, and Photochemical Activity of a Small Chloroplast Particle Obtained by the Action of Triton X-100*

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ABSTRACT: The small chloroplast particle obtained with Triton X-100 has been studied regarding its light-induced electron spin resonance (esr) signal, light-induced absorbancy change of P700, and its ability to photoreduce nicotinamide–adenine dinucleotide phosphate (NADP). The particle contains both cytochromes *f* and b_6 in the ratio of 0.5 P700:1 cytochrome *f*:1 cytochrome b_6:50 chlorophylls. Of these components only the P700 undergoes a change upon illumination. A light-induced bleaching of the P700 takes place in the absence of any added cofactors. Comparison of the stability to heating and irradiation with visible light, as well as the response of the esr signal to varying light intensities and various addition, shows a correspondence between the esr signal and the P700 bleaching. Similar half-times for the decay of the P700 and esr signals are observed for the particle alone and in the presence of electron donors. The addition of the acceptor system (ferredoxin, ferredoxin–NADP reductase, and NADP) does not significantly change the magnitude of the P700 response, but does inhibit the subsequent decay of the signal in the dark. Addition of the donor system (ascorbate, 2,6-dichlorophenolindophenol, and plastocyanin) stimulates the decay of the P700 signal with some preparations. However, when the donor and acceptor systems are both present, there is a somewhat greater bleaching (oxidation) of the P700 followed by a very rapid decay. This rapid turnover of the P700 under these conditions indicates it is involved in the photochemical step preceding NADP reduction. This is substantiated by the similar response of the P700 signal and NADP photoreduction activity under various light intensities. The particle will also photoreduce NADP with only plastocyanin or *Euglena* cytochrome *f* as the donor molecule. In this case the photooxidation of plastocyanin or *Euglena* cytochrome *f* coupled to NADP reduction can be followed. Since the acceptor system alone does not influence the P700 signal and the signal is seen in the absence of added cofactors, it is unlikely that ferredoxin itself reacts directly with the photoexcited P700. Therefore, the electron-transfer sequence of photosystem 1 of chloroplasts appears to be plastocyanin → P700 (light) → X → ferredoxin → reductase → NADP. The compound X is as yet unknown.

The past 25 years have seen many studies on the effects of detergents upon chloroplasts (Kupke and French, 1960). Definitive experiments were recently performed by Boardman and Anderson (1964), who obtained two fragments from chloroplasts after treatment with digitonin. These fragments have been studied rather extensively (Anderson *et al.*, 1964, 1966; Anderson and Boardman, 1966; Wessels, 1965). The heavy particle obtained by this means has a lower chlorophyll a:chlorophyll b ratio and has more activity related to photosystem 2 (oxygen evolution) than does the small particle, which has a much higher chlorophyll a:chlorophyll b ratio compared to the original chloroplast and is enhanced in photosystem 1 activity (NADP[1] photoreduction with a donor system such as ascorbate-DPIP).

A similar fragmentation is obtained with Triton X-100, except that the large particle in this case does not evolve oxygen (Vernon *et al.*, 1966a,b). The small particle obtained with Triton is very reactive in photosystem 1 reactions and shows a greatly magnified light-induced absorbancy change at 430 and 700 nm (P700) as well as a large electron spin resonance (esr) signal which is also light dependent. All of these phenomena have been related to photosystem 1 since these responses are absent in chloroplast systems which do not have a functional photosystem 1. This paper reports an investigation of these three properties of the small particle obtained with Triton X-100 with a view toward a possible identification of the P700 and esr signals with the photochemically reactive chlorophyll a of the photosystem 1.

Methods

Chloroplasts were treated with 4.0% Triton X-100

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[1] Abbreviations used: DPIP, 2,6-dichlorophenolindophenol; DTNB, 5,5′-dithiobis(2′-nitrobenzoic acid); NADP, nicotinamide–adenine dinucleotide phosphate; PMS, *N*-methylphenazonium methosulfate; Fd, ferredoxin; PC, plastocyanin; NADP-Red, the reductase.

near 0° to produce the small particle, called the P-D10 particle, by the procedure previously described (Vernon *et al.*, 1966a). The particle was separated by differential centrifugation, sedimenting after 10 hr at 144,000*g*. A modified Beckman DB recording spectrophotometer was utilized for routine assays of NADP photoreduction (Zaugg, 1963). Purified spinach ferredoxin, ferredoxin-NADP reductase, and plastocyanin, which were routinely used in the assays for NADP photoreduction, as well as other chemicals used were the same as those reported previously (Vernon and Shaw, 1965). Chlorophyll was determined by the method of Vernon (1960). The esr spectra were determined with a Varian Model 4500 esr spectrometer operated at 2.5-mw microwave power (100-kcycles field modulation) and with the necessary accessory equipment (Treharne *et al.*, 1963). The light-induced absorbancy change at 430 nm was detected using the apparatus and techniques reported for an earlier investigation (Ke *et al.*, 1964). The light-induced bleaching of P700 was followed at 430 nm, which had the advantage of being free from fluorescence responses of the particle. There were no absorption changes owing to cytochromes, which would modify the 430-nm change. In some cases this apparatus was also used to measure NADP photoreduction and plastocyanin or cytochrome photooxidation on the same sample. Light intensity was measured with a Kettering-Yellow Springs Instrument Co. radiometer.

Results

NADP Photoreduction. Many of the properties of the NADP photoreduction system of the light P-D10 particle have been reported in a previous communication (Vernon *et al.*, 1966a). High rates of NADP photoreduction were obtained by supplying the reaction system with high amounts of purified plastocyanin. This protein had been solubilized and removed from the particle by the treatment with Triton. The highest rates observed were 1980 μmoles/mg of chlorophyll hr. These rates are considerably higher than rates of oxygen evolution or carbon dioxide fixation by intact cells or chloroplasts, which indicates that the electron-transfer system of photosystem 1 can function at higher rates when freed of the necessary coupling found in the chloroplast itself. Two other enzymes are also required to couple the chlorophyll of the P-D10 particle to NADP, *viz.*, ferredoxin and ferredoxin–NADP reductase. Figure 1a shows the absolute requirement this reaction has for added plastocyanin. Experiments showing the need for the other enzymes were reported previously (Vernon *et al.*, 1966a).

The P-D10 particle also has the ability to photoreduce compounds which have an oxidation potential lower than that of ferredoxin, as shown in Figure 1b. The particle will photoreduce DTNB with no additions, and the addition of methyl viologen depresses the reaction. The addition of a low-potential (−656 mv) viologen, however, causes a marked stimulation. In this reaction, which is carried out anaerobically, the viologen reduced photochemically is reoxidized by the added DTNB,

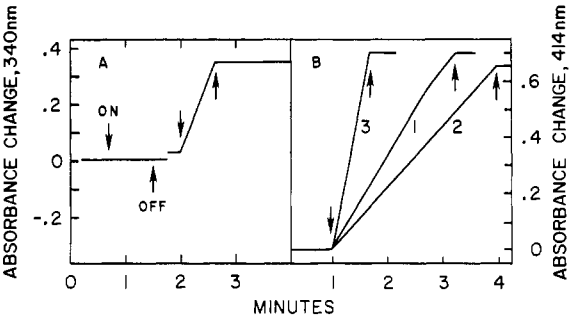


FIGURE 1: Plastocyanin requirement for NADP photoreduction and photoreduction of low-potential viologen dyes by P-D10 particle. (a) The experiments were performed anaerobically with a Beckman DB spectrophotometer. Initially the adapted Thunberg tube contained 0.05 M phosphate buffer (pH 7.0), 0.25 mM NADP, 5.3 mM sodium ascorbate, 67 μM DPIP, 2 units of purified spinach ferredoxin having a specific activity of 11 (Losada and Arnon, 1964), 6 units of purified ferredoxin–NADP reductase having a specific activity of 38 transhydrogenase units (Keister *et al.*, 1960), and P-D10 particles equivalent to 17 μg of chlorophyll in a volume of 3.0 ml. After the first illumination period indicated by the arrows, 8.4 nmoles of purified plastocyanin was added. A broad-band red filter (Corning 2403) combined with an infrared-absorbing filter (Corning 4600), which passed light beyond 640 nm with a light intensity of 3.2×10^5 ergs/cm^2 sec, was employed. (b) The experiments were performed anaerobically in a 3.0-ml volume containing (curve 1) 0.05 M Tris buffer (pH 7.8), 2.7 mM sodium ascorbate, 30 μM DPIP, 75 μM DTNB, and P-D10 particles containing 28 μg of chlorophyll. Where indicated there was also added (curve 2) 0.8 mM methyl viologen (MV), 1,1′-dimethyl-4,4′-bipyridylium dichloride, or (curve 3) 1,1′-trimethylene-2,2′-bipyridylium-4,4′-dimethyl dibromide.

whose reduction is followed spectrophotometrically. It is impossible to show an accumulation of reduced viologen in the absence of DTNB, probably because the reduced compound reacts back with the oxidized ascorbate in the system. The addition of the low-potential viologen also stimulates oxygen uptake by this particle, which indicates further that it is being reduced photochemically. The reduction of such low-potential compounds has been previously reported by Kok (1966) and Black (1965) for intact chloroplasts. (The viologen dyes used in this investigation were from the same preparations used by Black (1965) for his study.) The P-D10 particle will also photoreduce methyl red (with ascorbate-DPIP) and will photooxidize mammalian ferrocytochrome *c* aerobically in the presence of plastocyanin. Therefore, in all reactions followed to date the particle has the same characteristics as do intact chloroplasts, except that the reactions proceed at a faster rate. Therefore, a comparison of the photochemistry, esr signal,

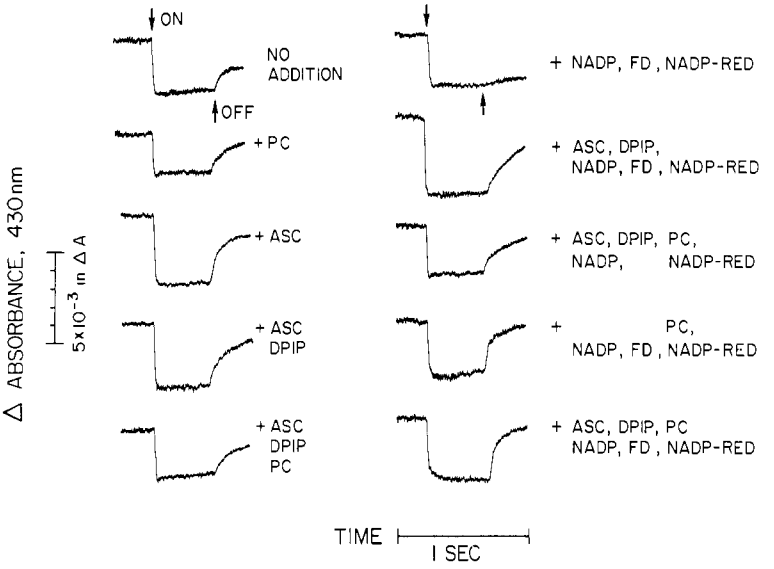


FIGURE 2: Light-induced absorbancy changes of the P-D10 particles under anaerobic conditions and in the presence of different donor and acceptor systems. The 3.0-ml reaction mixture in 0.05 M phosphate buffer (pH 7) contained chloroplast particles (P-D10) equivalent to 22 μg of chlorophyll, 8 μmoles of ascorbate, 0.1 μmole of DPIP, 8.4 nmoles of plastocyanin, and 0.75 μmole of NADP. The concentrations of ferredoxin and ferredoxin–NADP reductase were the same as for Figure 1a. The ratio of chlorophyll a: chlorphyll b in the particle was 7.2. The total time span for all absorbancy-change transients was 1 sec, and the illumination period was approximately 0.5 sec. The reactions were performed with the rapid-response flash spectrophotometer previously described (Ke *et al.*, 1964). The intensity of the actinic light (680-nm interference filter) was 6×10^4 ergs/cm^2 sec. A downward direction upon illumination indicates a decrease in absorbance. The initial horizontal portion represents the base line.

and the P700 response should relate to the situation found in the chloroplast.

The P700 Response. Figure 2 shows the light-induced absorbancy change at 430 nm observed for the P-D10 particle alone and with various additions. These data were obtained using a 0.5 sec illumination period, which gives more information than the previously employed short flashes. The P-D10 particle alone gives the P700 response, with a relatively slow decay time. Because the response is elicited at low light intensities, the effect of the measuring beam in the apparatus employed becomes important. The data for Figure 2 were obtained using as short an exposure as possible for the reaction mixture in the measuring beam prior to the onset of the actinic light. A period of about 5 sec was required to adjust the instrument so the actinic light could be turned on for each experiment. Longer periods in the measuring beam caused progressively more photooxidation of the P700, with resulting smaller signals when exposed to the actinic light.

Addition of plastocyanin to the system causes no significant change. Addition of ascorbate to the particle causes a larger light-induced signal plus a faster decay rate. The larger signal is due to the fact that the ascorbate keeps the P700 in a more reduced state prior to the actinic illumination, and the faster decay is related to its ability to chemically reduce oxidized P700. The addition of DPIP to the system containing ascorbate causes a somewhat slower decay of the signal, and further addition of plastocyanin results in a smaller signal and a slower decay than observed with ascorbate alone. The presence of the acceptor system of NADP, ferredoxin, and ferredoxin-NADP reductase greatly inhibits the decay of the signal.

When the reaction system has the additions required for the photoreduction of NADP, *i.e.*, ferredoxin, the reductase, and plastocyanin (with or without ascorbate and DPIP), there is a rapid turnover of the P700. Figure 2 shows the rapid decay of the P700 signal under such conditions, and Figure 3 shows the corresponding absorbance changes relating to the photoreduction of NADP. In the absence of ascorbate and DPIP, it is possible to follow the photooxidation of the added plastocyanin. Under these conditions the rate of NADP reduction and plastocyanin oxidation decreases with time, because of the limiting amount of plastocyanin added. The back reaction observed when the light is turned off is probably owing to reduction of plastocyanin by endogenous quinones contained on the particle (Vernon *et al.*, 1966b). In the presence of ascorbate and DPIP, the rate of NADP reduction continues at a linear rate for the entire illumination period, since under these conditions the oxidized plastocyanin is reduced again by the redox couple.

The data of Figure 2 are significant in several respects. They show that the special chlorophyll responsible for the P700 response will turn over during illumination in the absence of any added cofactor, but at a rather slow

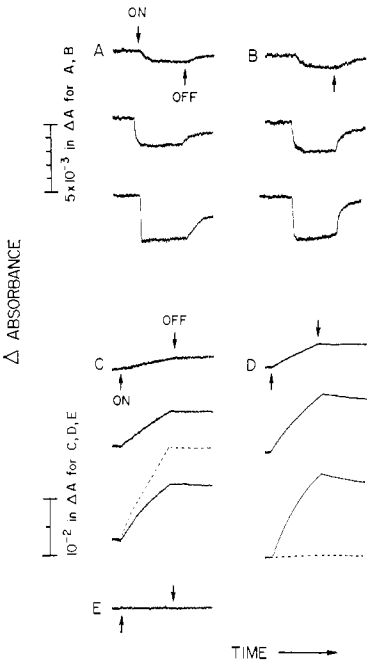


FIGURE 3: P700 photooxidation, NADP photoreduction, and plastocyanin photooxidation with the P-D10 particle.

Reactions	Additions to P-D10	Measuring Wavelength (nm)
A	None	430
B	NADP + Fd + NADP-Red + PC	430
C	NADP + Fd + NADP-Red + PC	340
C (dashed)	NADP + Fd + NADP-Red + PC + ASC + DPIP	340
D	NADP + Fd + NADP-Red + PC	590
D (dashed)	NADP + Fd + NADP-Red + PC + ASC + DPIP	590
E	NADP + NADP-Red + PC (no Fd)	340

The components of the reaction mixtures have the same concentrations as described in Figure 2. Absorbancy changes in experiments A–D were examined with 680-nm actinic light at three incident intensities: top, 6×10^3; middle, 3×10^4; and bottom tracing, 8×10^4 erg/cm^2 sec. The absorbancy changes in E and the dashed tracings in C and D were obtained with the highest incident intensity. Total time spans are 1 sec for A and B and 4 sec for C–E. Arrows indicate lights on and off. A downward direction represents a decrease in absorbance.

rate. The initial oxidation is rapid, but the decay is slow. Addition of ascorbate stimulates the decay, but surprisingly the addition of plastocyanin does not, either when added alone or with ascorbate and DPIP. The presence of the acceptor system (NADP, ferredoxin, and the reductase) does not affect the extent of the P700 reaction, but does prevent the decay in the dark, showing that this system can accept electrons from P700.

The fastest turnover of the P700 is observed under the conditions required for NADP photoreduction (the bottom two curves of the right-hand column of Figure 2). From the initial slopes of these two P700 decay curves, a decay rate of 680 μmoles of P700/hr mg of chlorophyll is calculated, which compares to a rate of 420 for the system with only ascorbate added. It is not known why plastocyanin is unable to support as fast a decay in the other experiments in which it is present. Its essential role in the photoreduction of NADP is shown in Figure 1, and its interaction with the P700 is seen by comparing the rates of P700 turnover in the complete system and in the corresponding system lacking only the plastocyanin.

The soluble cytochrome 552 isolated from *Euglena* will serve as an electron donor in the system under study and support NADP photoreduction, as shown in Figure 4. The rate of NADP photoreduction is about the same with either plastocyanin or cytochrome 552 as the donor molecule. When plastocyanin is also present, the particle will photooxidize both of these molecules, giving a slightly faster rate of NADP photoreduction and a slower rate of oxidation of the individual donors. Thus these two donors appear to act at the same site. Unpublished data obtained by S. Katoh in this laboratory (personal communication) show that with *Euglena* chloroplast particles both *Euglena* cytochrome 552 and plastocyanin are capable of donating electrons during the process of NADP photoreduction.

Ascorbate and PMS are an efficient electron-donating couple to photosystem 1 of chloroplasts (Ke, 1964), and PMS itself is a very active agent for catalyzing cyclic electron flow through this system. With the P-D10 particle, ascorbate and PMS give a unique response, allowing the P700 signal to decay rapidly back to the original level.

Experiments were performed with ascorbate–PMS at lowered temperatures, and the results are compared to data obtained with ascorbate–DPIP in Figure 5. This figure shows the different response at room temperature, and further shows that PMS continues to interact with P700 at temperatures as low as −196°, serving as an acceptor (some oxidized form would be present even with ascorbate in the system) and a donor in the reduced form. There is surprisingly little change in the reaction pattern over the entire temperature range investigated.

On the other hand, the ascorbate–DPIP system became inoperative slightly below 0°. For the PMS-supported reaction to proceed at −196° the dye molecule must be in close association with the P700, since appreciable movement of the molecules would not

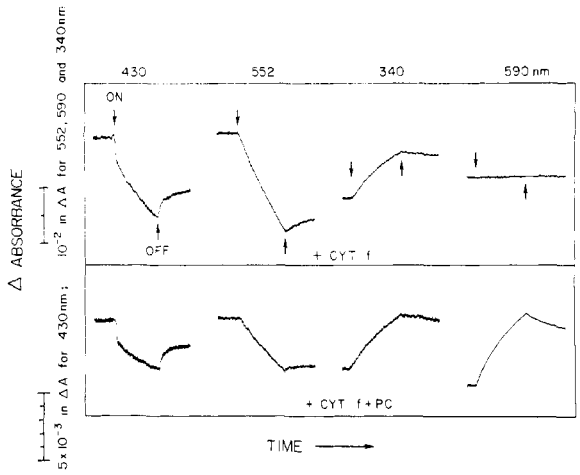


FIGURE 4: Photoreduction of NADP by the P-D10 particle coupled to the oxidation of *Euglena* cytochrome 552. The reaction mixtures have NADP, ferredoxin, and ferredoxin-NADP reductase (the acceptor system) at concentrations described in Figure 2. Where indicated, 8.4 nmoles of plastocyanin and/or 15.6 nmoles of *Euglena* cytochrome 552 were added to the system. The excitation intensity of the 680-nm light was 6×10^4 erg/cm^2 sec. The time spans are 1 sec for the 430-nm transients and 4 sec for the others. Arrows indicate light on and off. The wavelengths of the measuring light are indicated above the tracings (430 for P700, 552 for cytochrome *f*, 340 for NADP, and 590 nm for plastocyanin).

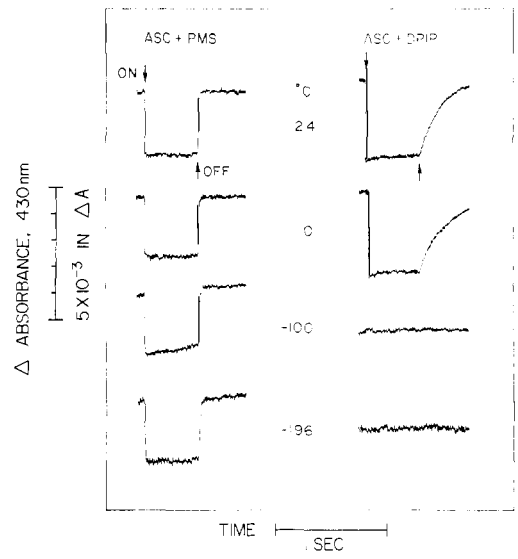


FIGURE 5: Effect of temperature on the P700 absorbancy changes of the P-D10 particle in the presence of ascorbate-PMS or ascorbate-DPIP. The low-temperature measurements were carried out in a specially constructed cold-finger cuvet with a path length of approximately 1 mm (detailed construction will be described in a subsequent publication). The reaction mixtures in 0.05 M phosphate and 0.25 M sucrose (pH 7) contain the following: chloroplast particles (P-D10) equivalent to 8 μg of chlorophyll/ml, 2 mM ascorbate, 5 μM PMS, and 30 μM DPIP. Excitation intensity of the 680-nm light was 6×10^4 erg/cm^2 sec. Time span was 1 sec, and the illumination period was approximately 0.5 sec. The indicated temperatures were actually measured on the frozen suspension with an immersed thermocouple.

take place. It appears the PMS may be complexed to the P700.

Cytochrome Composition. In the previous communication (Vernon *et al.*, 1966a) the cytochrome composition was reported for P-D10 particles which had been treated with acetone to remove the chlorophyll. A broad band was shown in the difference spectrum (dithionite reduced minus ferricyanide oxidized) which peaked around 557 nm. The cytochromes were not resolved. Later experiments performed with unextracted particles clearly show the presence of cytochromes *f* and b_6 on the particle, as shown in Figure 6. The ascorbate-minus-untreated spectrum shows the presence of cytochrome *f*, which is at least partially oxidized. The dithionite-minus-untreated spectrum shows both cytochromes *f* and b_6, with the latter predominant. The total amounts of both cytochromes are shown in the lower curve. The peak of the cytochrome *f* occurs at 555–556 nm in the difference spectrum, while the cytochrome b_6 peak occurs at 564 nm. This is at a somewhat longer wavelength than that reported by Biggins and Park (1965) (560–562 nm) for chloroplast cytochromes detected in a protein preparation prepared from spinach chloroplasts through the action of the detergent sodium dodecyl sulfate. Figure 6 also shows the oxidation by ferricyanide of the P700 and some of the bulk chlorophyll which has an absorption peak at 675 nm. The 675-nm peak is not due to fluorescence of the particle. The accompanying paper (Ke and Vernon, 1967) shows that negligible fluorescence is shown by the P-D10 particle at this wavelength at room temperature. From the lower curve of Figure 6 the ratio of the cytochrome and chlorophyll components of the P-D10 particle has been calculated to be 0.5 P700:1 cytochrome *f*:1 cytochrome b_6:50 chlorophylls (the molar extinction coefficients of the various components are described elsewhere in the text; 2×10^4 was used for calculating cytochrome b_6).

Comparison of Esr and P700 Responses. The esr signal observed with the P-D10 particle is shown in Figure 7, along with the kinetics of its formation and decay in the presence of various donor and acceptor systems. The signal is not observed with nonilluminated particles, and in general its characteristics are those of "signal I" reported for algae and chloroplasts (Treharne *et al.*, 1963). For purposes of comparison the kinetics of the light-induced esr signal and P700 response were measured with the same particle preparation. Since the reaction mixture was exposed to a measuring beam for the P700 measurements, the esr measurements also included

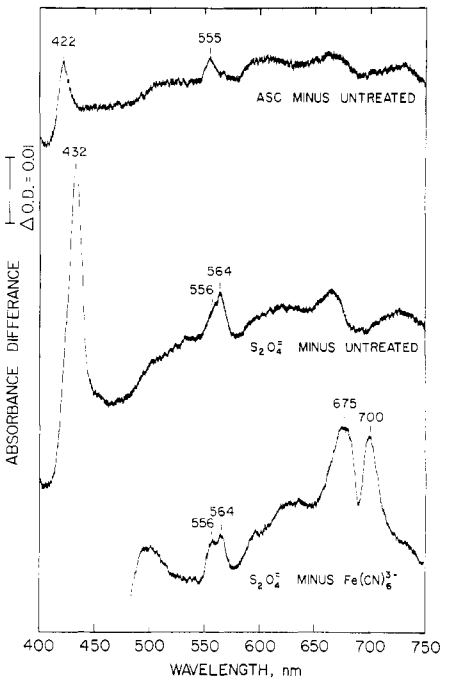


FIGURE 6: Cytochrome (*f* and b_6) and P700 composition of P-D10 particles. Difference spectra: top, ascorbate minus untreated; middle, dithionite treated minus untreated; bottom, dithionite treated minus ferricyanide treated. The particle suspension had a chlorophyll content of 21.6 μg/ml and an optical density of 1.38 at 680 nm. The difference spectra were measured on a Cary Model 14R spectrophotometer with a special slide wire of 0–0.05-ODU full scale. All reagents were added in the solid form in excess.

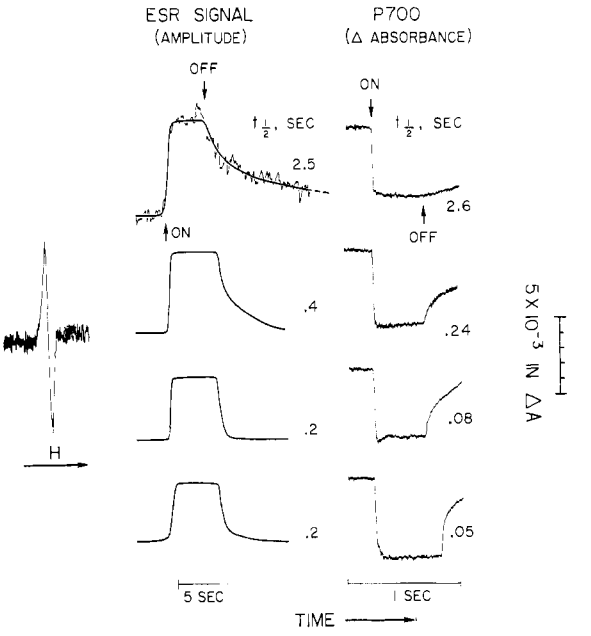


FIGURE 7: Light-induced esr spectrum of the P-D10 particle (left) and kinetics of light-induced esr and P700 responses (right) in the presence of various donor molecules. For the experiments involving esr signal the chlorophyll concentrations were 0.52 mg/ml in the flat cuvet, which gave an absorbancy at 678 nm of 0.85. The spectrometer was set for an amplification of 1000 with a field modulation of 4.8 gauss. When present ascorbate was 8 mM; DPIP (0.1 mM), NADP (0.7 mM), and ferredoxin, plastocyanin, and ferredoxin-NADP reductase as given for Figure 2. For the P700 measurements the chlorophyll concentration was 8 μg/ml in the regular 1-cm cuvet, giving an absorbance of 0.5 at 678 nm. The concentrations of the other additions listed were the same as given in Figure 2. The time scale is indicated, and arrows show where the light was turned on and off. On the first kinetic tracing of the esr signal the noise is shown along with the best smooth line drawn through the tracing. For the other experiments only the drawn smooth curve is shown. The times ($t_{1/2}$) shown for each experiment are the times in seconds for the signal to decay to one-half its original value. The actinic light was of 680 nm (interference filter), at an intensity of 3.2×10^4 erg/cm² sec for the esr measurements and 6×10^4 erg/cm² sec for the P700 measurements. The intensity of the measuring beam for the P700 measurements was approximately 200 erg/cm² sec, and correspondingly a 200-erg/cm² sec background illumination (680 nm) was employed for the esr measurements. The reaction systems contained the following: top pair, P-D10 particle in 0.05 M phosphate buffer, pH 7.0; second pair, plus ascorbate; third pair, plus ascorbate, DPIP, and plastocyanin; bottom pair, plus ascorbate, DPIP, plastocyanin, ferredoxin, reductase, and NADP. The response time of the esr spectrometer was 10^{-4} sec, and the time required for full-scale deflection by the recorder was 1 sec. The deflections recorded were slightly less than one-half of the full scale.

a 200 erg/cm² sec background of 680-nm light, to which was added 32,000 erg/cm² sec where indicated. The background light continued after the actinic light was turned off. The times for the responses to decay to one-half the original value in the dark are also included. The data show that the particle alone had a relatively long decay time in the dark, which was shortened an order of magnitude by the addition of ascorbate. Further addition of DPIP and plastocyanin caused a still more rapid decay. (Separate experiments show the faster response in this case is due primarily to the plastocyanin.) The fastest decay for the P700 signal was observed in the presence of all the components necessary for NADP photoreduction (including in this case ascorbate and DPIP). The decay rates observed for the esr signal were about the same for the latter two systems, but this was due to the slower response time of the instrumentation used to measure the esr signal. There is no reason to believe that the half-times for the esr decay would not be as fast as the P700 signal response if it were possible to measure the decays accurately. For the particle alone and in the presence of ascorbate, the agreement between the two measurements is good, and qualitative agreement was observed upon the addition of the other com-

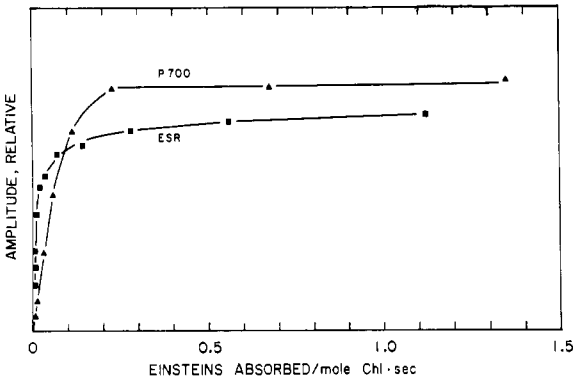


FIGURE 8: Response of the P-D10 esr signal amplitude and P700 absorbance change to increasing light intensity. The conditions were the same as given for Figure 7 regarding physical arrangements for the experiments. The ordinate is a measure of the extent of each reaction under steady illumination, being the amplitude of the esr signal in arbitrary units and the absorbancy change at 430 nm for the P700 measurement. The abscissa measures the quanta absorbed per chlorophyll in each system, which is proportional to light intensity.

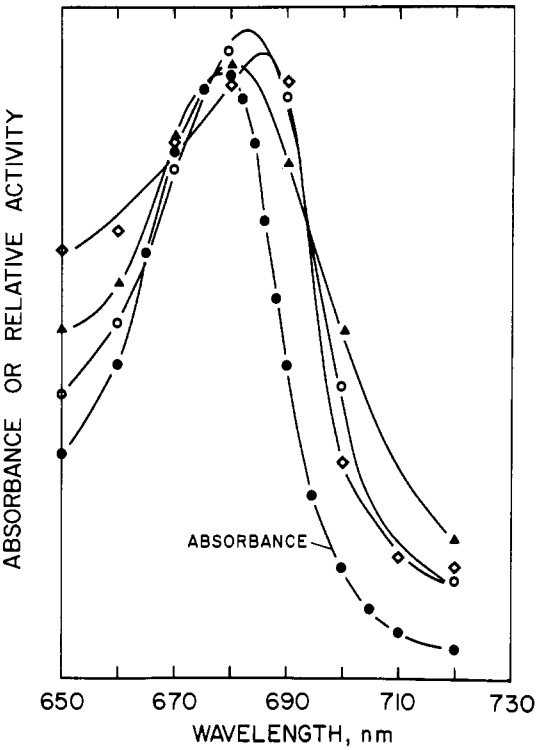


FIGURE 9: Action spectra for the light-induced esr (△) and P700 responses (◇) of the P-D10 particle and for NADP photoreduction (○). The experimental conditions for the esr experiments were those of Figure 7, employing only 250 μg of chlorophyll/ml of P-D10 particles suspended in 0.05 M phosphate buffer (pH 7.0). The actinic light was adjusted at each wavelength to give an intensity of 14 ergs/cm² sec. The ordinate represents the amplitude of the esr signal in arbitrary units. The amplitude at the peak was about 80% of the saturation value. The P700 response and NADP photoreduction were measured on the same sample, as outlined in Figure 3 with the reaction mixture having the composition given in Figure 2 for the complete reaction system. In the present case the chlorophyll content was 8 μg/ml, and the actinic light was adjusted to give an intensity of 5×10^4 ergs/cm² sec at each wavelength. The absorption spectrum was taken with a Cary 14 spectrophotometer, and gave an absorbance of 0.5 at its peak. The maximal rate of NADP photoreduction in this reaction was 914 μmoles/mg of chlorophyll hr.

ponents. The relatively slow instrument response for the esr signal also precluded any measurement of the rise time of the esr signal.

It should be noted that the decay characteristics of the P-D10 preparation used for the data of Figure 7 are somewhat different from those shown in Figure 2. For Figure 7 the addition of plastocyanin in the absence of the complete acceptor system (ferredoxin, the reductase, and NADP) resulted in a faster decay rate for the P700 signal. However, the fastest decay rate was always observed for the complete system. The discrepancy reflects differences in the particles as prepared, and in the present case the stimulating effect of plastocyanin upon all decay rates allows for a closer comparison between the P700 and esr signal.

The effect of increasing light intensity upon the esr signal and the P700 response of the P-D10 particle is shown in Figure 8. Since these experiments were performed with a different apparatus under different conditions, a common abscissa of einsteins absorbed per mole of chlorophyll per second is utilized, which is proportional to light intensity but corrects for the different concentrations of chlorophyll used in each experiment. Although the chlorophyll concentration in the flat cuvet used for esr measurements was greater than that used for the P700 determinations, the small thickness (light path) of the esr cuvet gave an optical density comparable to that of the sample used in the P700 measurement. The esr signal and the P700 response to light intensity are quite similar. For both of these measurements the amplitude of the response is plotted, since it is impossible to calculate initial rates of the esr signal even on a relative basis, because of the slow time response of the apparatus. The saturation characteristics of the P700 amplitude with increasing light intensity are quite different from those of the initial rate of P700 oxidation, because of the slow rate of the back reaction with the particle.

Action Spectra for the Esr Signal, P700 Response, and NADP Photoreduction. The action spectra of the three activities of the P-D10 particle are shown in Figure 9. For measurement of the rates of formation of the P700 signal and the photoreduction reaction the per cent absorption of light by the chlorophyll and the light intensities employed were not much different. The experi-

mental conditions for the esr measurement (which was of signal amplitude, not the rate of formation) required that higher chlorophyll concentrations and lower light intensities be used. However, the per cent absorption of light by chlorophyll in the esr cuvet was not much different from that of the other experimental systems because of the flat cuvet used in the esr spectrometer.

The data of Figure 9 show that all three activities have essentially similar maxima in the action spectra, which are located around 682 nm. The slight difference for the P700 signal response is not considered significant. This contrasts to a maximum in the absorption spectrum at about 678 nm. These data clearly indicate that the longer wavelength form of chlorophyll a is more effective in catalyzing all three reactions, and in terms of the initial light absorption act and subsequent transfer of excitation energy, all photoreactions seem to be due to the same chlorophyll system. When Triton X-100 is added to the reaction mixture, the maximum for the action spectrum is not shifted from that shown in Figure 9, although the absorption maximum of the particle is shifted to 673 nm. This indicates the presence of chlorophyll on the particle which is inactive in the photoreduction of NADP, and which is accessible to the added Triton.

The quantum requirements for NADP photoreduction, as well as for cytochrome *c* (mammalian) photooxidation at various wavelengths, were determined by Dr. Martin Schwartz on the same P-D10 particle which was used for the experiments reported in Figures 2, 9, and 11. Figure 10 shows the data obtained, utilizing the double-beam spectrophotometer described previously (Hoch and Martin, 1963) in conjunction with an integrating sphere. This apparatus allows the determination of the absorption spectrum and rate of the photoreaction on the same preparation under the same experimental conditions. The data obtained for cytochrome *c* photooxidation show that this is a 1-quantum process at longer wavelengths. The slightly higher values observed for NADP photoreduction are not greatly different from a value of 1 quantum/equiv of NADP reduced, and in view of the lability of the NADP photoreduction system (it is more sensitive to heating, etc.) it is reasonable to assign a maximum quantum efficiency of 1 equiv of NADP reduced/quantum of incident light for this reaction also. From other experiments it is known that viologen dyes react more readily with the photosystem of P-D10, since low concentrations of the dyes completely inhibit NADP photoreduction. The lower quantum requirement for the viologen-mediated oxidation of cytochrome *c* reflects this more efficient coupling to viologen dyes. From both spectra of Figure 10, the presence of some inactive chlorophyll on the particle is indicated, which could be some contaminating chlorophyll from the photosystem 2 particle which was mostly removed by the treatment with Triton X-100. Since addition of Triton to the reaction mixture causes a change in the absorption maximum but not in the maximum of the action spectrum, this also indicates the presence of some inert chlorophyll on the particle.

Comparison of P700 Response, NADP Photoreduction, and Plastocyanin Photooxidation. Figure 11 shows the

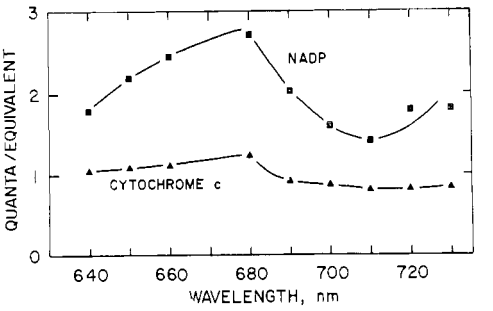


FIGURE 10: Quantum requirement for NADP photoreduction and ferrocytochrome *c* photooxidation by P-D10 particles. The reaction mixture for NADP photoreduction had the composition given for Figure 2 for the complete system. In the present case the chlorophyll concentration was 5.0 μg/ml. The reaction mixture for ferrocytochrome *c* photooxidation contained 25 μM ferrocytochrome *c* reduced with hydrogen and palladium, 6.0 μM plastocyanin, 50 μM methyl viologen, 0.15 M phosphate buffer (pH 6.8), and P-D10 particles equivalent to 5.0 μg of chlorophyll/ml. Other experimental conditions and information concerning the double-beam spectrophotometer and integrating sphere used to measure absorbance changes are given elsewhere (Schwartz, 1967). The lowest quantum requirements were observed for 710 nm, which gave values of 0.82 quantum/equiv for cytochrome *c* oxidation and 1.4 quanta/equiv for NADP photoreduction. The intensities of the actinic light were adjusted to give approximately 5 nEinsteins absorbed/min at each wavelength, which was on the linear portion of the light-intensity curve. The absorbance of the particle suspension was 0.274 at 680 nm.

rates of photoreduction of NADP (340 nm), photooxidation of plastocyanin (590 nm), and the rate of P700 photooxidation calculated from both the initial absorbancy change at 430 nm and also the initial rate of decay of the 430-nm signal when the light is turned off. The latter would represent the rate of turnover of the P700. The data shown in Figure 11, which were calculated from curves similar to those shown in Figure 2 (with intermediate light intensities), show that the photoreactions of NADP and plastocyanin have similar rates. Since the P700 change represents a one-electron change (Kok, 1961) the data in Figure 11 indicate that the rate of P700 oxidation is only slightly more than one-half that of NADP photoreduction. It must be emphasized, however, that there are numerous assumptions involved in calculating the rate of P700 photooxidation, and the true extinction coefficient of P700 is unknown. In view of this uncertainty, the concept that P700 is involved as the photocatalyst in NADP photoreduction is compatible with the data obtained. Upon aging, the rates observed for NADP photoreduction, plastocyanin photooxidation, and P700 turnover calculated from the initial decay all decreased by about 50% while the initial rate of P700 change was not affected. This again would re-

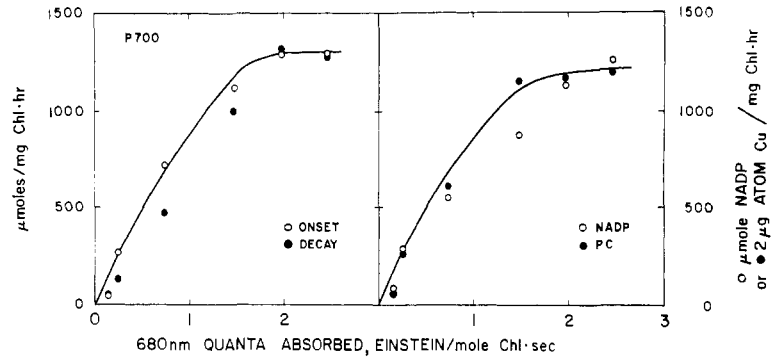


FIGURE 11: Rate of P-700 photooxidation, NADP photoreduction, and plastocyanin photooxidation with P-D10 particles as a function of 680-nm excitation intensity. The 3.0-ml reaction mixture in 0.05 M phosphate buffer (pH 7) contained P-D10, NADP, Fd, NADP-Red, and PC at concentrations identical with those used in Figure 2. Ascorbate and DPIP were not present since the rate of plastocyanin oxidation can only be determined in their absence. The extinction coefficient used for P700 was 5.7×10^4 l./mole cm at 430 nm, and for plastocyanin the value of 9.8×10^3 l./mole cm at 590 nm was employed. The P700 change is on a molar basis, which would be a 1-equiv change, while the NADP and plastocyanin changes are for 2 equiv.

late the P700 turnover to NADP photoreduction. At the highest intensity employed the rate of the P700 response is approaching the limiting rate of the instrumentation used, and there is more uncertainty concerning the apparent saturation of this reaction (particularly the onset of the signal). Because of the lower specific absorptivities of both NADP and plastocyanin, the same limitation does not apply, and the saturation at high light intensities is real.

Kok *et al.* (1965) and Jones and Kok (1966a,b) have shown that chloroplasts are strongly inhibited by exposure to either visible or ultraviolet light. Ultraviolet irradiation affected photosystem 2 rather specifically whereas visible light affected both photosystems. We have inhibited NADP reduction activity of the P-D10 particle by irradiation with visible light and at the same time followed the P700 and esr activities. Table I shows that only NADP photoreduction activity was inhibited. The light intensity employed in the present case was considerably lower than that used by Jones and Kok (1966b), who reported simultaneous destruction of both NADP photoreduction activity and P700 response. In their case, however, the P700 response was less affected

TABLE I: Inactivation by Preillumination with Visible Light.[a]

Irradiation Time (min)	Per Cent Activity		
	NADP Reduction	P700 Amplitude	Esr Amplitude
0	100	100	100
5	75	100	98
10	—	100	98
20	70	100	—
40	25	100	103

[a] For the assays the experimental conditions given for Figures 1, 2, or 4 were used. For NADP photoreduction the system contained 11 μg of chlorophyll/ml and the actinic light intensity was 1.4×10^4 ergs/cm² sec. For the esr experiments the mixture contained 0.165 mg of chlorophyll/ml and the actinic light intensity was 2.8×10^4 ergs/cm² sec. For the P700 measurements experiments were run with or without the ascorbate–DPIP couple, with identical results. The reaction mixture contained 8 μg of chlorophyll/ml and the 680-nm actinic light was 6×10^4 ergs/cm² sec. The P-D10 particles were illuminated at 20° prior to the assays in 50 mM phosphate buffer (pH 6.7) containing 0.25 M sucrose at a concentration of 0.165 mg of chlorophyll/ml. The intensity of white light (tungsten lamp) for this preillumination was 1.5×10^6 ergs/cm² sec.

TABLE II: Inactivation of P-D10 Particles by Heating.[a]

Heating Temp (°C)	Per Cent Activity		
	NADP Reduction	P700 Amplitude	Esr Amplitude
25	100	100	100
30	85	91	100
36	70	100	100
42	30	100	101
50	22	—	—
52	0	59	63
56	0	11	17

[a] The experimental conditions for the assays were those given in Table I, with a 10-min preheating being substituted for the preillumination.

by the intense illumination, and could be restored to about one-half its original value by the addition of ascorbate and PMS along with detergent. Qualitatively the results of our experiments are in agreement, except that under our conditions of less intense illumination, neither the P700 nor the esr response are affected at all.

The effects of heating on the activities of the P-D10 particle are shown in Table II. Heating at 52° for 10 min completely inactivated the particle for NADP photoreduction, yet allowed retention of considerable P700 and esr activity. These data along with those of Table I show that the photoreduction system is clearly the most sensitive of the three in terms of inactivation, and that the P700 and esr responses are similar.

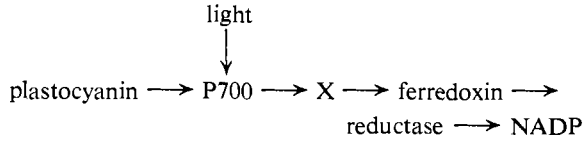
Discussion

The data obtained in this investigation show that the special long-wavelength form of chlorophyll known as P700 has the properties expected of the photocatalyst of photosystem 1. It is readily demonstrated with the particle, and it shows the greatest rate of turnover under conditions where NADP is being photoreduced. Plastocyanin is the electron donor for NADP photoreduction and its presence is required for the rapid rates of P700 turnover, as shown by the fast decay when the light is turned off. Allowing for the uncertainty of the absorption coefficient for P700, which leads to uncertainty in calculated rates of reaction of the P700, the observed rates for P700 photooxidation and NADP photoreduction (and plastocyanin oxidation) are close enough to be reasonable. It is significant that in their quantitative examination of the number of spins produced (from the esr spectrum) and P700 molecules oxidized by light, Beinert and Kok (1963) also observed that the number of P700 molecules transformed were less than the number of spins produced by illumination, and again by a factor of about 2. The same difficulty concerning the number of P700 molecules reacting would also be encountered in those experiments. By combining the data from the present investigation with those of Beinert and Kok, one can conclude that the rate of production of spins by illumination is approximately equal to the rate of photoreduction of 1 equiv of NADP.

The quantum requirements for NADP photoreduction and for cytochrome *c* photooxidation show that the P-D10 particles contain an efficient photosystem 1 with a little contaminating inactive chlorophyll, whose absorption in the region of 660–690 nm causes a slight increase in quantum requirement in this region. Thus, the particle as isolated does represent photosystem 1 as it exists in the intact chloroplast, and the high quantum efficiencies attest to the gentle nature of the splitting caused by Trition X-100.

The similarity in the response of the P700 signal and the light-induced esr signal indicate that both are manifestations of the same moiety, a partially oxidized chlorophyll molecule. Both the P700 and esr signals show about the same stability to heating and light (Tables I and II), and the data of Figure 8 show that both have a similar response to light intensity. Furthermore, the half-decay times of the P700 and esr signals are similar. In considering photosynthetic tissue of various types, from intact chloroplasts to derived particles and *Scenedesmus* mutants which lack either photosystem 1 or photosystem 2 (Weaver and Bishop, 1963), there is a complete correlation between the presence of the esr signal studied here (the sharp, fast decaying signal) and the demonstration of a P700 signal. For these reasons it is logical to assign the esr signal to a P700 molecule which has lost one electron to become half-oxidized. In this form it would be expected to give an esr signal of the type observed. The salient feature of both the P700 and esr signals is the fast onset of the signals upon illumination and the slow decay in the dark with the particle alone. This allows weak light to produce both signals, which saturate at low-intensity values. This indicates that the primary photochemical step of P700 is one leading to oxidation of P700, and this initial step proceeds readily in the absence of added cofactors. This further indicates that the primary acceptor for the P700 must be present initially in the P-D10 particle. The nature of the primary acceptor is not known, but is unlikely to be ferredoxin since the P700 signal is observed in the absence of added ferredoxin, and the P-D10 particle is essentially devoid of this iron protein.

The experiments reported here are entirely consistent with the presently held concept that plastocyanin reacts directly with the oxidized form of the reaction center, P700, allowing the transfer of electrons to ferredoxin *via* photosystem 1. Furthermore, the similarity of rates of plastocyanin photooxidation and NADP photoreduction shows they are linked in a stoichiometric fashion. Although the P-D10 particle contains both cytochromes *f* and b_6, these are not sufficient to allow electron flow from ascorbate and DPIP to NADP. Added *Euglena* cytochrome *f* does support NADP reduction, but in terms of solubility properties the *Euglena* cytochrome *f* resembles spinach plastocyanin more than it does spinach cytochrome *f*. The data support the transfer sequence outlined below:



These experiments do not give much information upon the nature of the primary photochemical reaction, because it was not possible to influence the P700 response by adding either the donor molecule (plastocyanin) or the acceptor systems independently. The fact that ferredoxin itself, with or without the ferredoxin–NADP reductase and NADP, does not significantly affect the P700 response upon illumination indicates it is not involved directly in the initial photochemical act. Furthermore, the ease with which ferredoxin is removed from the chloroplast indicates it is not in the tight complex one would expect if it reacted directly with the P700. Because of these facts, it seems logical to include in the reaction scheme an unknown compound, X, which in-

teracts directly with P700 to produce the P700 bleaching always observed in active P-D10 particles prepared from spinach chloroplasts.

The experiments with PMS shown in Figure 5 indicate that this molecule is capable of reacting directly with P700 in a primary photoact, since the P700 change is still observed in the presence of PMS at −196°. This would explain the fact that PMS-catalyzed photophosphorylation does not saturate in the usual manner with increasing light intensity and also the fact that PMS at very low concentrations prevents NADP photoreduction in reconstituted systems. It has been our experience that PMS at 10^{-8} M completely inhibits NADP photoreduction by the P-D10 particle, indicating it is reacting at the same site as does ferredoxin.

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