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LIGHT-INDUCED RAPID ABSORPTION CHANGES DURING PHOTOSYNTHESIS

VII. SOME REACTIONS IN SONICATED CHLOROPLASTS*

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SUMMARY

1. Spinach chloroplasts subjected to sonication show light-induced absorption changes at 700 mμ characteristic of the photooxidation of the chlorophyll component P700. The appearance of P700 absorption changes probably resulted from the release of plastocyanin thus interrupting the electron flow between pigment systems 1 and 2. The general features of the absorption-change transients are similar to those observed previously with digitonin-treated chloroplasts. The addition of 2 mM ascorbate or 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea had practically no effect on either the magnitude or the dark decay of the transient absorption change.

2. Phenazine methosulfate (PMS) (in the presence or in the absence of ascorbate) reduction appeared to be coupled to P700 photooxidation, as shown by the corresponding transients at 430 and 388 mμ. The absorbance changes at these two wavelengths indicate that the amount of PMS photoreduced was equivalent to that of P700 photooxidized. Higher PMS concentrations accelerate the dark decay of the P700 signal. When PMS alone is present, anaerobiosis caused the dark decay to become more rapid than in the presence of ascorbate.

3. Unlike PMS, other redox agents such as 2,6-dichlorophenolindophenol, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine or diaminodurol in the presence of excess ascorbate, did not noticeably affect the kinetics of the dark decay at 430 or 703 mμ, suggesting that these reduced species are not efficiently coupled to photooxidized P700.

4. The onset and decay rates of the P700 transient in the presence of PMS and excess ascorbate was insensitive to temperature between 25° and 0°. However, when the chloroplast sample was frozen at temperatures ranging from —5° to —196°, all reactions ceased. When the frozen (—196°) sample was brought back to the room temperature, the reaction was restored completely. Fresh broken chloroplasts behave similarly. Digitonin-treated chloroplasts persisted down to about —25° but with diminishing magnitude and slower decay.

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; P700, the special chlorophyll component of chlorophyll showing absorption changes near 700 mμ; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate.

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INTRODUCTION

Chloroplasts subjected to sonication are unable to photoreduce $NADP^+$ either by the Hill-reaction or by the ascorbate–DCIP system[1]. The loss of $NADP^+$-photoreduction activity parallels the release of plastocyanin from the chloroplasts as a result of sonication and the activity is restored by adding a catalytic amount of plastocyanin to the sonicated chloroplasts[2]. These results indicate clearly that plastocyanin is one component of the electron-transfer chain in the photosynthetic apparatus.

The light-induced absorption changes at 700 mμ associated with the photooxidation of the chlorophyll component P700 in the long-wavelength pigment system (PS1) can be clearly observed when the short-wavelength pigment system (PS2) of the chloroplasts is inactivated[3,4]. It was of interest to see if similar light-induced absorption changes occur when the interaction between PS2 and PS1 is interrupted by sonication. The results presented herein characterize further the properties of sonicated chloroplasts measured by the flash-induced absorption-change technique. In addition, the effects on these absorption changes when a portion (only PS1) of the overall light-reaction system is coupled to an external redox agent are described.

EXPERIMENTAL

Broken spinach chloroplasts were prepared by swelling in water. They were suspended in 0.05 M phosphate buffer (pH 7.8) containing 0.4 M sucrose and 0.01 M NaCl. Sonication was carried out in a refrigerated Raytheon oscillator operated at 10 kcycles/sec for 10 min. The chloroplast samples usually contained approx. 15 μg chlorophyll per ml.

The flashing-light spectrophotometer and its use in conjunction with a CAT computer to improve the signal-to-noise ratio were described previously[5]. The spectrophotometer was operated in a single-beam mode by using a well regulated power supply (model 6273A, Harrison Laboratories, Berkeley Heights, N.J.) for the measuring light source. The low-temperature experiments were conducted either in a regular 1-cm cuvette (for 25° to 1°) or in a cuvette formed by microscope slides separated by 1 mm with a rubber gasket (for 0° to −196°). The cuvette was held in a metal holder which was connected to a liquid-N_2 reservoir and the entire assembly was placed in an optical dewar[6]. Other experimental conditions were the same as described previously[7].

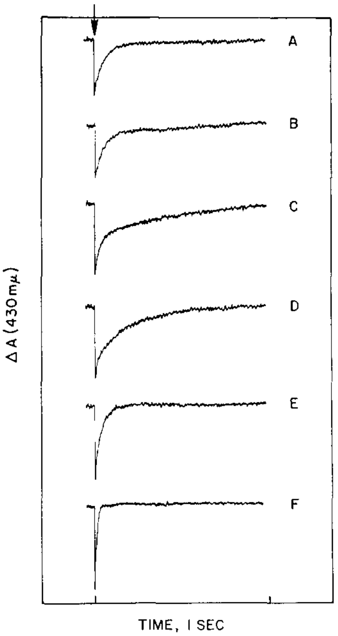
Most chemicals were obtained from commercial sources and were used without further purification. Diaminodurol was a gift from Dr. A. Trebst to Dr. L. P. Vernon. Where indicated, glucose and glucose oxidase were used to create anaerobic conditions.

RESULTS

Light-induced absorption-change transients and difference spectra

Sonicated chloroplasts in the absence of exogenously added redox agents show transient absorption changes characteristic of P700 photooxidation when illuminated with a 20-μsec light flash[3]. The half-life of the dark decay of the transient is approx. 35 ± 5 msec. The general features of the absorption-change transients of sonicated chloroplasts are similar to those observed previously with digitonin-treated chloroplasts. In contrast, the dark decay is slower with the digitonin preparations[3].

The addition of 2 mM ascorbate or 10 μM DCMU to the sonicated chloroplasts had practically no effect on either the magnitude of the transient absorption change or the kinetics of the dark decay. Upon addition of PMS to sonicated chloroplasts in the presence of ascorbate (2 mM), the magnitude of the absorption change increased and the decay kinetics were modified. At 0.33 μM PMS, the magnitude of the absorption change was increased by more than 50 % and the dark decay became biphasic; the half-life of the slower portion was about 200–300 msec, while that of the rapid portion was only about 20 msec. Upon addition of more PMS, the dark decay became simple exponential and rapid. A typical series of measurements at 430 mμ with sonicated chloroplasts, in the presence of ascorbate and PMS, is shown in Fig. 1.



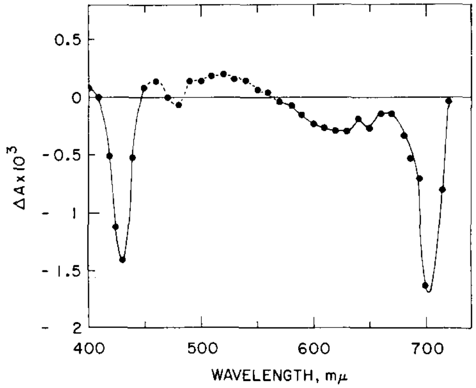


Fig. 1. Absorption-change transients at 430 mμ induced by a 20-μsec light flash (arrow). (A) sonicated chloroplasts alone; (B) sonicated chloroplasts *plus* 2 mM ascorbate; (C) to (F) *plus* ascorbate and 0.33, 1, 10 and 33 μM PMS.

Fig. 2. Light-*minus*-dark difference spectrum of sonicated chloroplasts in the presence of 2 mM ascorbate and 20 μM PMS.

A corresponding series of absorption changes was observed at 703 mμ.

Unlike PMS, other redox agents such as DCIP, TMPD or diaminodurol, in the presence of excess ascorbate, did not noticeably affect the kinetics of the dark decay of the 430- or 703-mμ transients. In most cases, only the magnitude of the absorption change increased at high dye concentrations.

The light-*minus*-dark difference absorption spectrum of sonicated chloroplasts was obtained by flash illuminating the suspension in the presence or absence of a redox couple. The light-*minus*-dark difference absorption spectrum, measured in the presence of ascorbate and PMS, is shown in Fig. 2. The profile of the difference spectrum is similar to that of P700 photooxidation observed previously with both digitonin-treated chloroplasts[3,4] and a partially purified pigment complex from spinach[8].

It should be noted that sonicated chloroplasts exhibit slight absorption changes at 515 and 475 mμ (Fig. 2, dashed curve). However, these changes have different decay kinetics and can thus be differentiated from the P700 reactions.

Correlation between the absorption changes of P700 and PMS

While PMS in the presence of excess ascorbate markedly affects the absorption-change transients associated with P700 photooxidation, PMS alone also profoundly modifies the decay. At 0.1 μM PMS, the transient was little affected. At PMS concentrations greater than 0.5 μM, the transient immediately showed a biphasic decay. Fig. 3 shows typical biphasic transients in sonicated chloroplasts at 430 mμ in the presence of 0.1 μM and 5 μM PMS, respectively. The biphasic transient at the higher PMS concentration is presented on two different time scales. Above 5 μM PMS, the decay became simple exponential and rapid but slower than in the presence of excess ascorbate (*cf.* Fig. 1).

The direct reaction between photooxidized P700 and PMS is further evidenced by a concomitant change in the absorption of PMS at 388 mμ. Transient absorption changes at 430 and 388 mμ for sonicated chloroplasts containing 5 μM PMS are shown

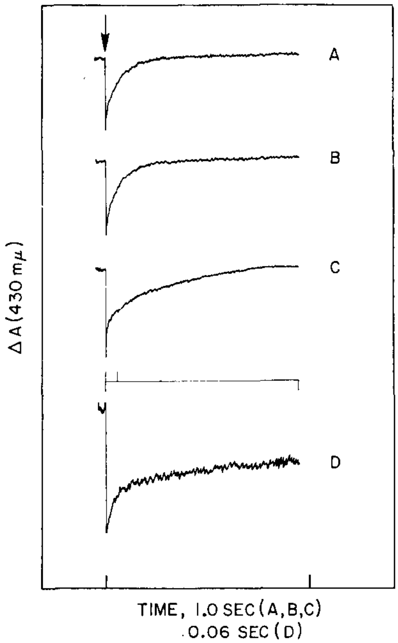


Fig. 3. Light-induced absorption-change transients at 430 mμ. (A) sonicated chloroplasts *plus* 0.1 μM PMS; (B and C) sonicated chloroplasts *plus* 0.5 and 5 μM PMS, respectively; (D) shows only the initial 60-msec portion of (C).

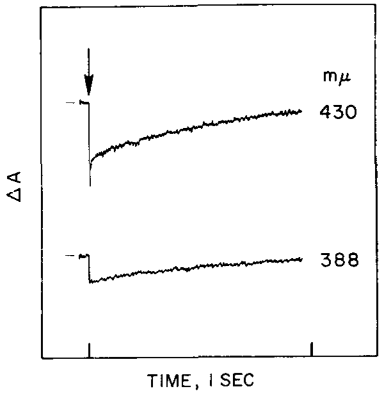
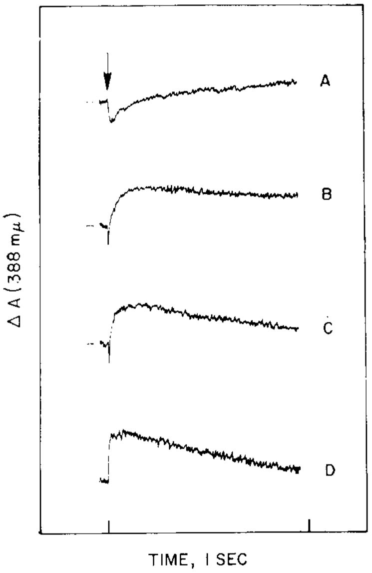


Fig. 4. Light-induced absorption-change transients at 430 and 388 mμ in sonicated chloroplasts containing 5 μM PMS. The magnitude of the 388-mμ transient is magnified by a factor of 5/3.

in Fig. 4. At 430 mμ, the transient shows the typical biphasic decay (*cf.* Fig. 3). At 388 mμ, the initial decrease in absorption indicates a rapid reduction of PMS, presumably coupled to P700 photooxidation. The kinetics of the dark decay at 388 mμ correspond well with those of that portion of the change at 430 mμ (slow decay) which represents the re-reduction of oxidized P700. Unlike the 430-mμ transient, the 388-mμ transient does not show the rapid negative spike.

The magnitudes of the slow decay portion of the 430-mμ absorption change and the total absorption change at 388 mμ are $8 \cdot 10^{-4}$ and $2 \cdot 10^{-4}$ absorbance unit, respectively. The molar extinction coefficient for PMS at 388 mμ is 26300 (ref. 9). Assuming that the molar extinction coefficient of P700 is the same as that of chlorophyll *a* (approx. 10^{-5}, ref. 8), the amount of PMS reduced photochemically is nearly equivalent to the amount of P700 photooxidized.

A similar correlation between the absorption changes at 430 mμ and 388 mμ was noted in the presence of excess ascorbate. The absorption changes at 388 mμ for sonicated chloroplasts containing PMS *plus* excess ascorbate are shown in Fig. 5. At low PMS concentration, an initial photoreduction of PMS occurred, presumably coupled to P700 photooxidation (Fig. 5, curve A). The dark decay kinetics at 388 mμ parallel those of the dark re-reduction of oxidized P700 shown in Fig. 1. At intermediate PMS concentrations, some rapid photoreduction of PMS was still observed (Fig. 5, curves B and C). At 50 μM PMS, the dark decay of the 430-mμ signal was very rapid (*cf.* Fig. 1, curve F). In contrast, no initial negative absorption change



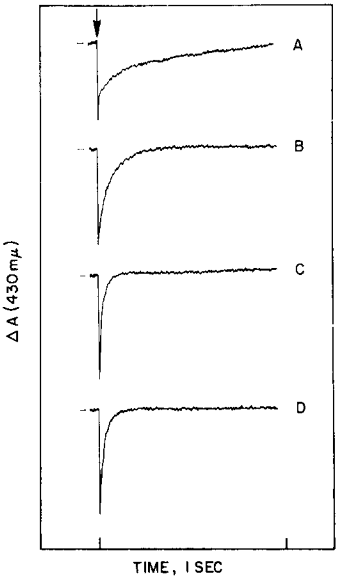


Fig. 5. Absorption-change transients at 388 mμ in the sonicated chloroplasts containing 2 mM ascorbate and varying amounts of PMS: (A) 0.5 μM; (B) 5 μM; (C) 10 μM and (D) 50 μM.

Fig. 6. Effect of anaerobicity on the absorption-change transients at 430 mμ in sonicated chloroplasts containing an external redox agent. (A) 5 μM PMS; (B) 5 μM PMS and 2 mM ascorbate; (C) 5 μM PMS *plus* glucose–glucose oxidase couple and 5 μM DCMU; (D) 5 μM PMS, 2 mM ascorbate, and glucose–glucose oxidase couple.

indicative of PMS reduction could be observed (Fig. 5, curve D). The very slow decay in all cases probably represents the chemical reduction of PMS by ascorbate.

Effect of anaerobiosis on the coupled reactions

Since reduced PMS is autooxidizable[10], the exclusion of O_2 should have an effect on the coupled reaction with oxidized P700, especially in the absence of ascorbate. When the chloroplast suspension containing PMS and ascorbate was made anaerobic with glucose and glucose oxidase, the absorption-change transient was affected very slightly. However, when only PMS was present, anaerobiosis caused the dark decay to become more rapid than even in the presence of ascorbate. These results are summarized in Fig. 6.

Effect of temperature on P700 changes

The photooxidation of P700 is generally considered to be a primary photochemical reaction and, thus, should be temperature independent. In contrast, the dark reaction, which requires the collision with a reductant, should be temperature dependent. RUMBERG AND WITT[4] reported evidence in support of both hypotheses. However, results from this work do not confirm either aspect.

When the temperature was decreased from 25° to 0°, either in the absence or in the presence of an exogenously added redox couple, the initial decrease in absorption at 430 mμ was unchanged. In the absence of the redox couple, the dark decay was retarded slightly. However, in the presence of PMS and ascorbate, the dark decay was practically temperature insensitive.

Representative absorption-change transients measured at 24°, 0° and from −5° to −196°, respectively, induced by a 20-μsec flash are shown in Fig. 7 (left side).

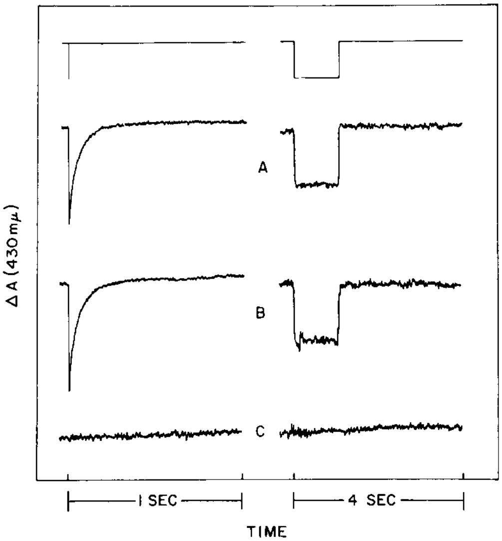


Fig. 7. Light-induced absorption-change transients at 430 mμ in sonicated chloroplasts containing 2 mM ascorbate and 5 μM PMS at several temperatures. The excitation flashes are represented by the profiles at the top (left: 20 μsec; right: approx. 1 sec). (A) room temperature (24°); (B) approx. 0°; (C) −5° to −196°.

In the presence of PMS and ascorbate, the kinetics of the transient absorption change were unaltered when the temperature was varied more than 20°.

When the sample was frozen (—2° to —5°), all reactions ceased. Since the photooxidation of P700 at —150° reported by RUMBERG AND WITT[4] was induced by longer flashes (approx. 1 sec), we measured also the 430-mμ absorption changes with flashes of comparably long duration. Again both the onset and decay of the P700 reaction coupled to reduced PMS were unaffected by temperature between 25° and 0°. Below 0°, no reaction was observed. The long-flash experiments are shown on the right side of Fig. 7. In one experiment, the cuvette containing the chloroplasts and reduced PMS was quickly brought to —196° by immersing the cuvette directly in liquid N_2. The result was the same as when the sample was cooled gradually to —196°. It should be noted that, in all cases, when the frozen sample (—196°) was brought back to room temperature, the reaction was restored completely with no change in the kinetics.

Similar experiments were performed with both digitonin-treated chloroplasts and broken fresh chloroplasts in the presence of ascorbate and PMS. With broken fresh chloroplasts, conditions as comparable as possible to those described by RUMBERG AND WITT[4] were used. In both cases, no change in decay time was observed at temperatures down to 0°. Below 0°, the decay became retarded. For instance, in digitonin-treated chloroplasts at —10°, the half-life was five times longer than at room temperature. The chloroplasts suspension became frozen between —5° and —10°, but the reaction persisted down to —25°, although with diminishing magnitude and slower return.

DISCUSSION

The discovery and possible significance of P700 as an intermediate in photosynthesis has recently been reviewed by KOK[12]. He states that "the kinetic behavior of P700 as observed in difference spectroscopy can only be explained by accepting that two antagonistic light reactions with different sensitizers operate on it, in addition to at least one dark reaction". PS1 catalyzes the oxidation of reduced P700; the converse reaction is catalyzed by PS2 and may possibly be rate limiting. Thus, the light-induced absorption changes characteristic of P700 photooxidation are observed only when the two photosystems become unbalanced as when photosystem 2 is inactivated, *e.g.*, by digitonin treatment[3,4], by aging[11], by an inhibitor such as DCMU (ref. 11), *etc.* As shown herein and by KOK[12], sonicated chloroplasts exhibit a reversible photo-bleaching of P700 without any additions. This may be explained by the release of plastocyanin from chloroplasts during sonication thereby interrupting electron flow through the dark thermochemical electron-transport chain connecting the two photosystems. The activities associated with the two individual photosystems are retained in sonicated chloroplasts; however, the pathway of electron flow from PS2 to PS1 (P700) has been interrupted. Thus, PS2 of sonicated chloroplasts cannot supply electrons for the photoreduction of oxidized P700 but PS1 does catalyze the photooxidation of reduced P700.

According to KOK AND HOCH[13], PS1, sensitized by chlorophyll *a*, yields oxidized P700 and the simultaneous formation of a reductant (XH). The dark reduction of oxidized P700 presumably results from the interaction of XH and oxidized P700.

Although less likely, the dark reduction of P700 could involve an unknown endogenous reductant present in sonicated chloroplasts.

The reversible absorption changes of P700 exhibited by sonicated chloroplasts can be markedly enhanced by PMS. KOK[12] proposed that the PMS in aqueous solution was reversibly reduced by light. The dark reduction of oxidized P700 can be coupled to reduced PMS, as shown by the profound effect of the latter on the decay kinetics of the P700 absorption-change transient.

When PMS alone is present, its photoreduction appears to be coupled directly to P700 photooxidation (Fig. 4). The photoreduced PMS can then back react with the oxidized P700 in the dark. At low PMS concentrations, a complex biphasic transient was always observed. The extent of the absorption change at 388 mμ indicates that PMS is involved in the slow portion of the transient. This is further supported by the fact that the magnitude of the absorption change in the slow portion of the P700 transient and of the total 388-mμ transient are nearly equivalent. The nature of the rapid transient spike remains unknown.

A similar correlation between the P700 and PMS absorption changes was found for illuminated chloroplasts containing both PMS and ascorbate. At low PMS concentrations, an initial photoreduction of PMS coupled to P700 photooxidation still occurs. Presumably, part of the PMS remains in the oxidized state even in the presence of ascorbate, thus acting as an electron acceptor for P700 photooxidation. At higher PMS concentrations, there is more reduced and more oxidized PMS relative to the amount of P700. Even under these conditions, some PMS photoreduction took place. Since the back reaction was fast at the highest PMS concentration used, it is possible that the rapid negative change may have been masked. In all cases shown in Fig. 5, the kinetics of the absorption increase at 388 mμ appear to correspond to its back oxidation in the dark (*cf.* Fig. 1).

The dependence of the kinetics of the dark decay of oxidized P700 on the concentration of PMS was essentially similar in the presence or absence of ascorbate. When the concentration of PMS was high, the dark decay was simple and rapid even in the absence of ascorbate. When O_2 was removed, the rate of reduction of P700 was very rapid. Under these conditions, the rate was faster than that in the presence of ascorbate and O_2 and was unaffected by the further addition of ascorbate. Since the ascorbate should have reduced most of the PMS present initially, it appears that the concentration of reduced PMS was not the decisive factor in determining the dark decay kinetics of P700. An alternative explanation is that the PMS was converted to the semiquinone form of the dye by illuminated chloroplasts rather than to the fully reduced form. Thus, whereas the reduction of oxidized P700 by reduced PMS might be slow, the reaction of oxidized P700 with the reactive semiquinone form of PMS might proceed very rapidly. ZAUGG[9] showed that although the semiquinone form of PMS was oxidized rapidly by O_2, a significant amount of the semiquinone could exist at the pH employed in the present studies under anaerobic conditions. Excess ascorbate or anaerobiosis would, therefore, favor the reaction of the semiquinone form of PMS with oxidized P700.

It appears that sonication also enhances the photoactivity of PSI as evidenced by the very high rate of $NADP^+$ reduction with ascorbate, DCIP and plastocyanin[2]. If the only effect of sonication was the removal of plastocyanin, one would expect that sonicated chloroplasts *plus* plastocyanin would be equivalent to untreated chloroplasts. In terms of the P700 absorption changes, this is not the case. Untreated chloroplasts exhibit little P700 absorption changes whereas sonicated chloroplasts do even in the presence of plastocyanin. It should be noted that addition of plastocyanin to sonicated chloroplasts resulted in an increase in the kinetics of the dark decay. This observation supports the postulation that plastocyanin functions in the electron-transport pathway from PS2 to P700. It also suggests that sonication increases the reactivity of PS1 so that the photooxidation of P700 is very fast and never the rate-limiting step. Thus, an increase in the rate of electron flow from PS2 to P700 in the presence of plastocyanin will not cause any significant change in the absorption changes of P700.

As found previously for digitonin-treated chloroplasts, the dark decay in sonicated chloroplasts also does not appear to be coupled directly to reduced DCIP, TMPD or diaminodurol. The magnitude of the P700 absorption change was always enhanced when any of these reduced dyes was present. Presumably, through an indirect and slow action, they maintain a high steady-state concentration of reduced P700 prior to illumination. These results agree with the observation[2] that neither ascorbate nor ascorbate and DCIP serve as efficient electron donor for $NADP^+$ photoreduction in sonicated chloroplasts in the absence of plastocyanin.

The effect of low temperature on the P700 absorption-change transient observed here was quite contrary to that reported previously[4]. The major differences are: (1) From room temperature to slightly below 0°, but above the freezing temperature, the decay kinetics remained constant. This means that the activation energy for the reaction between photooxidized P700 and reduced PMS is practically zero. Previously, RUMBERG AND WITT[4] reported an activation energy of 3.8 kcal/mole for this reaction. (2) As soon as the reaction mixture became frozen, usually around $-5°$ to $-10°$, the P700 transient disappeared. It was reported previously that P700 could be photooxidized at $-150°$, and the reaction was irreversible even in the presence of reduced PMS. The apparent differences found here cannot yet be explained. It is possible that P700 may have been irreversibly photooxidized by the measuring beam at the low temperature. However, this remains to be demonstrated. It should also be noted that P700 photooxidation reappeared upon thawing the reaction mixture. The temperature independence of the dark reaction over a temperature span of more than 20° indicates that P700 and reduced PMS are tightly coupled.

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