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THE PRESENCE OF P700 IN CHLOROPLAST FRAGMENTS PREPARED BY SHORT TIME INCUBATION WITH TRITON X-100

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SUMMARY

A procedure was recently described by R. Malkin (*Biochim. Biophys. Acta*, 253 (1971) 421) by which fragments having Photosystem II activity could be derived from chloroplasts. These fragments could photoreduce $NADP^+$ but were completely devoid of P700, thus giving considerable support to the new "Three-Light-Reaction" scheme in green plant photosynthesis. The results presented here show that the Triton chloroplast fragments still contain approximately 30 to 40 % initial P700 as assayed by EPR and difference spectroscopy. The failure to detect P700 in the earlier publication, either by chemical or photochemical assay, was because of the lack of plastocyanin in the reaction medium. This electron carrier is necessary for the reduction of P700 by ascorbate in the Triton fragments. The photoreduction of methyl viologen or $NADP^+$ by these fragments with ascorbate as electron donor in the presence of plastocyanin is shown to be a Photosystem I reaction. The maximal Photosystem I activity is about 35 % of that of the starting material. The fragments exhibit an increased EPR Signal II.

INTRODUCTION

The hypothesis of the "Two-Light-Reaction" or Z-scheme for green-plant photosynthesis has been continuously questioned since its presentation by Hill and Bendall[1]. The latest attempt to modify the original proposal was made by Knaff and Arnon[2] who introduced a concept of three-light reactions in chloroplasts: two System II short-wavelength (<700 nm) photoreactions that operate in series and a third System I long-wavelength (>700 nm) photoreaction that operates parallel to System II. The assignment of two short-wavelength photoreactions was based on several lines of evidence[3]: One of them was that preparations of chloroplast fragments, which were able to photoreduce $NADP^+$ with water as electron donor, were completely devoid of P700, the System I reaction-center pigment[4]. While the preparation of these particles–called DTS-III–was very laborious involving a combination of digitonin, Triton X-100, sonication, and sucrose density gradient treatments, R. Malkin[5] recently reported that chloroplast fragments, exhibiting features similar to the DTS-III particles, could be prepared by short-time incubation with Triton X-100 only.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol, DPC, 1,5-diphenylcarbazide.

It was expected that Malkin's preparations would be of value in the investigation of the EPR Signal II since EPR Signal I (oxidized P700[6]) would not be present. Yet the EPR studies revealed that a considerable amount of P700 was still detectable in these preparations. This communication reports these EPR experiments as well as other properties of the Triton fragments.

MATERIALS AND METHODS

Preparations of chloroplasts and fragments

Chloroplasts from spinach plants, bought at the local market, were isolated exactly as outlined by Malkin[5]. Before fragmentation, the chloroplast pellet was resuspended with the aid of a Dounce homogenizer (Kontes, with piston A) to obtain an even suspension for the EPR experiments. The fragmentation likewise followed the reported procedure and the final Triton chloroplast fragment pellet was resuspended in a minimum amount of grinding medium (0.4 M sucrose, 50 mM Tris (pH 7.8 at 20 °C), 10 mM NaCl) with the homogenizer as given above. As was published[5], this procedure decreases the chlorophyll *a*/chlorophyll *b* ratio by approximately 0.9.

Reagents

Tris, tricine, sodium ascorbate, and 2,6-dichlorophenolindophenol (DCIP) were obtained from Sigma Chemical Co.; methyl viologen from Mann Research Lab.; 1,5-diphenylcarbazide (DPC) and potassium ferricyanide from J. T. Baker Chemical Co.; and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) from E. I. DuPont de Nemours. The spinach plastocyanin was kindly prepared by Mr B. Nolan according to the method of Anderson and McCarty[8].

Instruments and activity measurements

The photooxidation of reduced DCIP was measured through the methyl viologen mediated oxygen uptake with a YSI Oxygen Monitor (Model 53). Photoreduction of DCIP was measured with the scattered transmission accessory of a Cary 14R spectrophotometer modified according to Sauer and Biggins[7] and equipped with a Dumont photomultiplier KM 2433 with S-20 response. Light *minus* dark difference spectra were recorded with the standard sample compartment of the Cary 14R accepting the same actinic light source as above and equipped with a Hamamatsu photomultiplier R446 with extended S-20 response. All measurements were made in semimicro cuvettes with 10 mm path length and width of 3 mm. Both side walls and the bottom plate of the reference cuvette were black. Only one side and the bottom of the sample cuvette were black, the other side served as the excitation window (Helma, "self-masking" cuvettes).

EPR experiments were carried out in a Varian X-band spectrometer (V 4502) equipped with a double-beam light source as described by Margozzi *et al.*[9]. Light intensity measurements were made with a YSI Radiometer (Model 65).

Reaction mixtures

(a) The reaction mixture (1 ml) for the water→DCIP reaction contained the following in μmoles/ml: Tricine (pH 7.5), 50; $MgCl_2$, 4.5; DCIP, 0.027; NH_4Cl, 8; chlorophyll 13 μg/ml.

(b) For the 1,5-diphenylcarbazide (DPC)→DCIP reaction the reaction mixture (a) *plus* 0.5 μmoles/ml DPC was used.

(c) The reaction mixture (3 ml) for the ascorbate + DCIP→methyl viologen reaction contained in μmoles/ml: Tricine (pH 7.5), 50; $MgCl_2$, 7.5; methyl viologen, 0.2; KCN, 0.2; DCMU, 0.01; DCIP, 0.027; NH_4Cl, 8; chlorophyll, 50 μg/ml; sodium ascorbate, 15; plastocyanin, saturating amount.

For the EPR experiments, chloroplasts or Triton chloroplast fragments were suspended in the sucrose–Tris–NaCl medium. Chlorophyll concentrations varied between 2 and 4 mg/ml.

Chlorophyll concentrations were determined according to Ziegler and Egle[10] using the following equations:

$$\text{Chlorophyll } a \ (\mu g/ml) = 11.78 \ (A_{664\,nm}) - 2.29 \ (A_{647\,nm})$$

$$\text{Chlorophyll } b \ (\mu g/ml) = 20.05 \ (A_{647\,nm}) - 4.77 \ (A_{664\,nm})$$

$$\text{Chlorophyll } (a + b) \ (\mu g/ml) = 7.01 \ (A_{664\,nm}) + 17.76 \ (A_{647\,nm})$$

where $A\lambda$ = absorbance of the chlorophyll solution in 80% acetone at wavelength λ.

RESULTS

Several criteria were used to assure that the chloroplast fragments obtained were similar to those prepared by Malkin[5]. Their photochemical activity was tested, using as a Photosystem II reaction the photoreduction of DCIP with DPC as an electron donor and, as a Photosystem I reaction, the methyl viologen mediated oxygen reduction with reduced DCIP as electron donor. As seen in Fig. 1A the Photosystem II activity is comparable with the reported values but is only about 60% of the activity of fresh, uncoupled chloroplasts. Similarly the poor Photosystem I activity with the ascorbate/DCIP electron donor couple is in agreement with the results of Malkin.

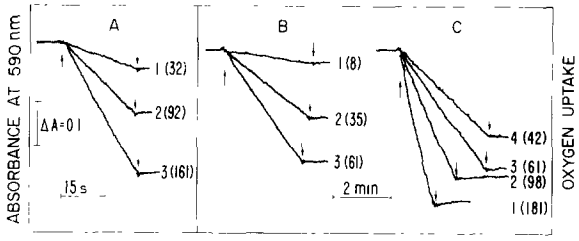

Fig. 1. Photochemical activities of Triton chloroplast fragments. The reaction mixture is given in the text. Upward arrows indicate light on, downward arrows light off. Numbers in parentheses indicate activity as μmoles acceptor reduced per mg chlorophyll per h. (A) Photosystem II activity Actinic light at 675 nm; halfbandwidth, 30 nm; incident intensity, $2.5 \cdot 10^5$ ergs·cm^{-2}·s^{-1}. Trace 1: [H_2O→DCIP] reaction with Triton fragments, Trace 2: [DPC→DCIP] reaction with Triton fragments, Trace 3. [water→DCIP] reaction with chloroplasts (control) (B) Plastocyanin requirement for the [ascorbate + DCIP→methyl viologen] reaction with Triton fragments Actinic light at 703 nm; halfbandwidth, 10 nm; incident intensity, $4 \cdot 10^4$ ergs·cm^{-2}·s^{-1}. Absolute concentration of plastocyanin was unknown Trace 1: no plastocyanin added, Trace 2: 10μl plastocyanin added, Trace 3: 20μl or 25 μl plastocyanin added. (C) Photosystem I activity. With chloroplasts: Trace 1: [ascorbate + DCIP→methyl viologen] reaction. Illumination conditions as for Fig. 1B. Trace 2: [ascorbate + DCIP→methyl viologen] reaction. Actinic light at 660 nm; halfbandwidth, 10 nm; incident intensity, $4 \cdot 10^4$ ergs·cm^{-2}·s^{-1}. With Triton fragments: Trace 3: [ascorbate + DCIP + plastocyanin→methyl viologen] reaction. Illumination conditions as for Fig. 1B. Trace 4: [ascorbate + DCIP + plastocyanin→methyl viologen] reaction. Illumination conditions as for Trace 2.

Only the addition of plastocyanin, as was reported for the photoreduction of NADP by Malkin, resulted in a substantial activity increase (Fig. 1B) and gave about 35% of the rate obtainable with whole chloroplasts. This reaction is clearly a Photosystem I reaction since far-red (703 nm) light was more effective than was red (660 nm) light (compare Traces 1 and 2 for chloroplasts and 3 and 4 for Triton fragments in Fig. 1C).

Another criterion which indicated that the correct chloroplast fragments were obtained was the apparent absence of P700 in an oxidized *minus* reduced difference spectrum. As can be seen in Fig. 2, no change in the absorption spectrum was recorded when ascorbate was added to the reference cuvette and ferricyanide to the sample cuvette. However, if in addition the sample cuvette was illuminated with blue light (the photomultiplier was shielded by two 645 nm cut-off filters), while the reference cuvette was kept in darkness, a small absorbance decrease was recorded around 700 nm. Obviously either ascorbate or ferricyanide, or both, were unable to act upon the P700 moiety. Since it was known from the photochemical activity measurements that plastocyanin was necessary for the photoreduction of methyl viologen or $NADP^+$, it was also added, together with ascorbate, to the reference cell. The result was a spectrum similar to that seen in the light *minus* dark difference spectrum (Trace 3, Fig. 2).

Because it is not possible to record a true difference spectrum with the Cary 14, due to limited sensitivity, and possible distortion by fluorescence, a light *minus* dark difference spectrum was obtained on an instrument, especially suitable for this kind of measurements (Fork and Murata[11]). (I am very indebted to Dr D. Fork for performing this experiment.) As is obvious from Fig. 3, these Triton chloroplast fragments certainly did contain P700. Using a millimolar extinction coefficient of 64 for P700 at 700 nm (ref. 12) it was calculated that these fragments contained about 1 P700 molecule per 900 chlorophyll *a* molecules or approximately 33% of the P700 concentration of untreated chloroplasts[13]. When compared with the Photosystem I

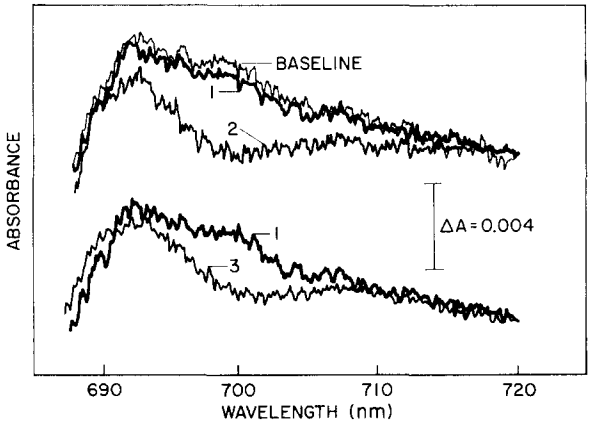

Fig. 2. P700 content of Triton chloroplast fragments. The reaction mixture (1 ml) contained Triton chloroplast fragments (equivalent to 100 μg chlorophyll/ml) in Tricine buffer (0.05 M, pH 7.5). Trace 1: 10 μmoles of ascorbate were added to the reference cuvette and 10 μmoles ferricyanide to the sample cuvette Trace 2: the sample cuvette was illuminated in addition with blue light (431 nm; halfbandwidth, 44 nm); incident intensity, $4 \cdot 10^4$ ergs·cm^{-2}·s^{-1}. Trace 3: saturating amounts (40 μl) of plastocyanin of unknown concentration were added to the reference cuvette and 40 μl buffer to the sample cuvette. Other conditions as for Trace 1.

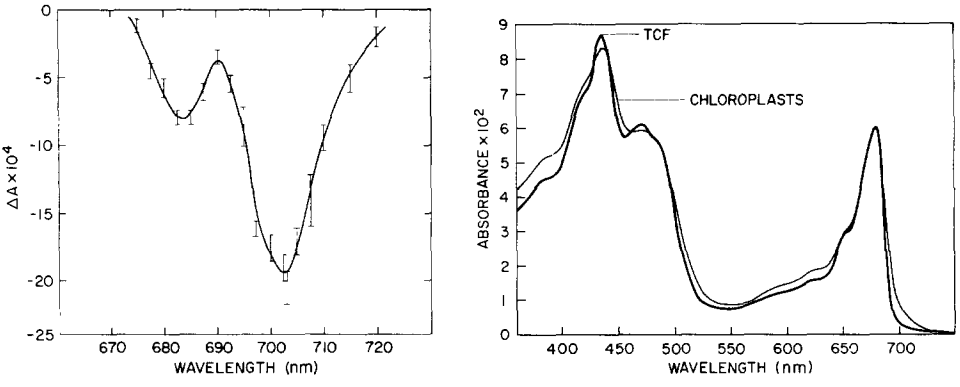

Fig. 3. Light-induced difference spectrum for absorption changes produced in the red region upon illumination with blue-green light of Triton chloroplast fragments The halfbandwidth of the measuring beam was 2.5 nm. The intensity of the actinic light was $1.4 \cdot 10^6$ ergs·cm^{-2}·s^{-1}. The final concentration of the substances in the reaction mixture were: methyl viologen, 10 μM; sodium ascorbate, 1 mM; plastocyanin, 0.26 μM; Tricine buffer, 50 mM (pH 7.5). Chlorophyll *a* concentration, 27 μg/ml.

Fig. 4. Absorption spectrum of Triton chloroplast fragments (TCF) and chloroplasts as measured with the scattered transmission accessory of the Cary 14R The chloroplast concentration was equal to 1.24 μg chlorophyll/ml in 0.05 M Tricine buffer (pH 7.5). The TCF concentration was adjusted to give the same absorption at the peak at 678 nm (approx. 1.4 μg chlorophyll/ml). The resolution at 700 nm was 0.37 nm; path length, 1 cm.

activity in the DCIP + plastocyanin→methyl viologen reaction (see above) it can be concluded that all the remaining P700 molecules are active.

In order to compare rates of photochemical activity at various wavelengths it was necessary to know the absorption at these wavelengths. For this reason the absorption spectrum of the Triton chloroplast fragments was measured with the scattered transmission accessory of the Cary 14R. For dilute samples this method gave very acceptable results with little distortion (Fig. 4). The spectra indicate that the Triton fragments have less than half of the absorption at 700 nm than the starting material.

EPR experiments

A typical EPR spectrum of freshly prepared whole chloroplasts is shown in Fig. 5. Even in the light the spectrum consists mainly of Signal II as evidenced by the small changes between the spectrum recorded in light and that recorded in darkness (for nomenclature details on EPR signals see a recent review by Weaver[6]). However, Signal I was recorded easily after addition of DCMU. The most obvious explanation for these results is that in intact chloroplasts in the light the steady state concentration of oxidized P700 (the compound giving rise to Signal I) was very low. After interruption of the electron flow by DCMU, thereby preventing the reduction of $P700^+$, its concentration increased and became recordable.

The EPR spectra obtained with Triton fragments are similar to those observed with whole chloroplasts, but some differences should be noted. As mentioned by Kohl[14] the amplitude at Point A is entirely due to Signal II, whether recorded in the light or in darkness. Since in both experiments the total chlorophyll concentration was equal, it can be calculated from the amplitude at Point A that the Triton fragments contained about 35 % more of Signal II than the starting material. Further-

more, for chloroplasts there was almost no difference at Point A between the traces recorded in light or in the dark because of the very slow decay of Signal II. In Triton chloroplast fragment preparations this signal decayed faster, resulting in a lower amplitude at A for the dark trace. More details on Signal II will be reported elsewhere.

Likewise, there is already to some extent a signal recordable at Point B, without the addition of DCMU; but here too DCMU has a stimulatory effect. This signal has

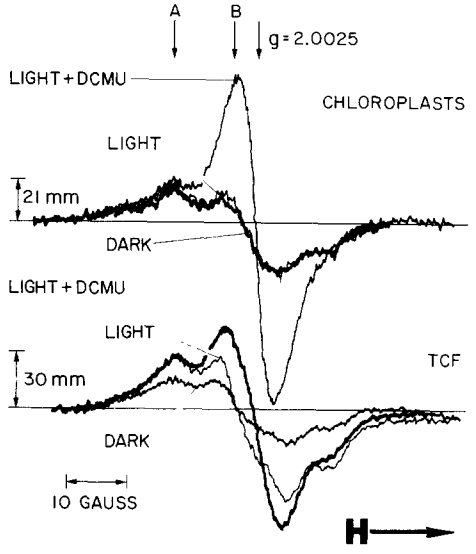

Fig. 5. EPR spectra of chloroplasts and Triton chloroplast fragments (TCF). Chloroplasts or Triton fragments were suspended in the sucrose–Tris–NaCl medium (equivalent to 3 mg chlorophyll/ml). Where indicated 0.5 mM DCMU was added. Actinic light at 675 nm; halfbandwidth, 30 nm; incident intensity, $5 \cdot 10^5$ ergs·cm^{-2}·s^{-1}. For the trace recorded in darkness the light was shut off at the beginning of the trace. Instrument settings: microwave power, 36 mW; modulation amplitude, 2 7 G; time constant, 0.3 s; scan rate, 20 G/min

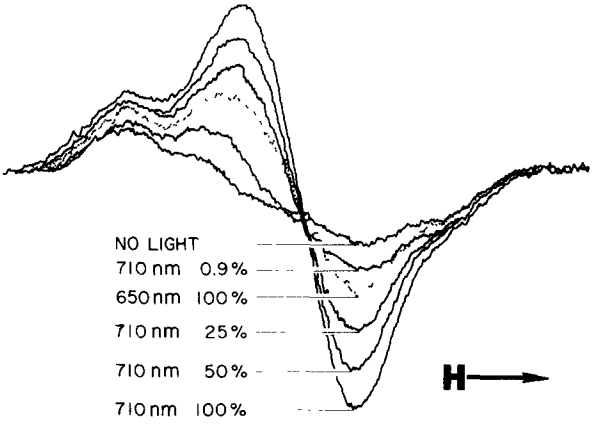

Fig. 6. Relationship between EPR spectrum and far-red light intensity TCF in sucrose–Tris–NaCl medium and 0.5 mM DCMU (equivalent to 3 mg chlorophyll/ml). 100% light (710 or 650 nm) corresponds to $1.4 \cdot 10^4$ ergs·cm^{-2}·s^{-1}. Halfbandwidth, 10 nm. Instrument settings: microwave power, 40 mW; modulation amplitude, 4 4 G; time constant, 0.3 s; scan rate, 40 G/min.

the same peak to peak width ($\Delta H_{pp} = 7.7$ G) and the same g-value (2.0025) as the Signal I in whole chloroplasts; furthermore, far-red (710 nm) light is more effective than red (650 nm) light in activating the signal (Fig. 6). Signal I was also detected at 77 °K. By subtracting the contribution of Signal II to the amplitude at Point B it could be calculated that the amplitude of Signal I and therefore the spin concentration in the Triton fragments is approximately 40 % of that in whole chloroplasts.

DISCUSSION

According to the "Three-Light-Reaction" theory for green plant photosynthesis put forward by Arnon and coworkers[3,4] it should be possible to subdivide chloroplasts into fragments completely devoid of P700, yet having the ability to photoreduce $NADP^+$ with water as electron donor. According to the conventional Z-scheme the loss of P700 should be coupled with the inability to photoreduce NADP. However, Arnon *et al.*[4] and later Malkin[5] were able to obtain preparations which could reduce $NADP^+$ when no P700 was detectable. In both systems the presence of P700 was tested by a standard assay[15] for this pigment. Repeating this assay confirmed that no P700 could be detected in chloroplast fragments prepared according to Malkin[5]. However, EPR experiments revealed that the narrow ($\Delta H_{pp} = 7.7$ G) light induced signal with a g-value of 2.0025, usually ascribed to be $P700^+$ in chloroplasts, is also present in the Triton chloroplast fragments. Certainly, one would also expect the reaction center chlorophyll of light reaction II_a in the new scheme to produce a similar radical and hence EPR signal. This signal should not be inducable with far-red light, but the results presented here (Fig. 6) clearly indicate a higher activity of 710 nm compared to 650 nm light. Thus, there is no evidence for a new EPR signal which would represent light reaction II_a.

Puzzling for the moment is the effect of DCMU on the narrow signal. Since there is no plastocyanin and, according to Malkin[5], no cytochrome f present, in addition to the very low ability to oxidize water, one would expect that no linear electron flow is possible. Yet the concentration of $P700^+$ is maximal only in the presence of DCMU. One explanation would be that there is still some linear electron flow retained in the Triton fragments, resulting in a partial reduction of $P700^+$. However, this would mean that cytochrome f was not totally lost during the preparation. Or it is possible that the treatment with Triton X-100 opened up an artificial cyclic electron pathway around P700 which is not operating in normal chloroplasts and is sensitive to DCMU, as low temperature or Tris treatment introduce an artificial cyclic electron flow around Photosystem II[16]. Neither explanation is completely satisfactory and this effect needs further clarification.

While the EPR spectrum does not absolutely identify $P700^+$, the appearance of its distinctive absorption spectrum (Fig. 3) leaves no doubt that this pigment is present in the Triton fragments. The EPR as well as the spectroscopic data indicate that approximately 60 to 70 % of the normal P700 concentration of chloroplasts is lost during the fragmentation process. This amount corresponds very well with the maximal photochemical Photosystem I activity achievable with these fragments.

As noted by Malkin[5] the Triton chloroplast fragments are distinguished from other Triton X-100[17] or Digitonin[18] or French press[19] preparations by the inability of DCIP to influence electron transfer from ascorbate to $NADP^+$, either in the absence

of presence of plastocyanin. Triton fragments also differ from these preparations in respect to the inability of ascorbate to keep P700 mainly in its reduced state. Only in the presence of plastocyanin is the reduction fast enough (see Fig. 1B) to compete with the oxidation by ambient room light or by the measuring beam. Since relatively high chlorophyll concentrations were used (100 μg/ml) the slits were fairly wide and the measuring beams thus of moderate intensity (approx. 800 ergs·cm^{-2}·s^{-1}). The measuring light keeps the pigment in its oxidized state and the addition of ascorbate alone does not change this equilibrium in the reference cuvette. However, one would expect that the addition of ferricyanide to the sample cuvette would shift the equilibrium further to the side of P700$^+$. Since this is not the case it seems necessary to conclude that ferricyanide cannot act upon the pigment either. Only if strong actinic light is given, in addition to the oxidant, is the balance between the two cells disturbed.

These differences between the Triton chloroplast fragments and the other preparations might be due to their different origin in the chloroplast. It was shown by Sane *et al.*[19] that Photosystem I particles prepared with the French pressure cell or with Digitonin originate from the stroma lamellae. The authors anticipate the same to be true for System I fractions prepared by Triton X-100. In contrast to this, the Triton fragments which are Photosystem II fragments originate mainly from the grana region, as judged by their sedimentation behaviour and their appearance under the light microscope. The detergent treatment might have changed the structure around P700 and rendered it less accessable to ascorbate or ferricyanide.

A similar explanation can be given for the failure to detect the photooxidation of P700 at 77 °K (ref. 5). It is certainly not an artifact introduced by low temperature, since at this temperature the formation of P700$^+$ can be recorded with the EPR instrument. Most probably P700 was already oxidized when the sample was frozen, since only ascorbate and no additional plastocyanin was added, and therefore no subsequent further oxidation was seen.

Clearly all these results indicate that the failure to detect P700 by chemical or photochemical methods is not sufficient evidence to claim that P700 is not present. Because the evidence for the lack of the Photosystem I reaction center pigment in the socalled DTS-III fragments was based on the same tests, and since the authors reported that plastocyanin was necessary to demonstrate NADP$^+$ reduction, although it was not included in the chemical assay[4], it seems very likely that P700 was nevertheless present in those preparations.

As evidence that the NADP$^+$ reduction was a Photosystem II reaction Malkin used the higher rates obtained with 664 nm light compared to 715 nm light. Such a comparison is not valid, since the Triton fragments absorb at 664 nm about 40 times as much as at 715 nm (Fig. 4). When the rate is divided by the absorption to obtain a crude quantum efficiency, it is clear that the far-red light is approximately 2 to 3 times more efficient.

CONCLUDING REMARKS

The concept of three photoreactions arranged in two parallel photosystems was based mainly upon three lines of evidence: the lack of enhancement effect; the photooxidation and photoreduction of cytochrome b_{559} both by Photosystem II light; and the preparation of chloroplast fragments with features as mentioned above[3]. Mean-

while it was reported[16] that the photooxidation of cytochrome b_{559} by Photosystem II light was an artifact introduced by low temperatures and perhaps by Tris treatment and that the inability to measure an enhancement effect was either due to inadequate concentrations of divalent cations (Mg^{2+} or Mn^{2+}) present in the reaction mixture[20] or the lack of a necessary yet unknown cofactor normally present in the cell[21]. From the results presented here it would also seem that the latter evidence is no longer valid.

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REFERENCES

1 R Hill and F. Bendall, *Nature*, 186 (1960) 136.
2 D. B. Knaff and D. I. Arnon, *Proc. Natl. Acad. Sci. U.S.*, 64 (1969) 715.
3 D. I. Arnon, D. B. Knaff, B. D. McSwain, R. K. Chain and H. Y. Tsujimoto, *Photochem. Photobiol.*, 14 (1971) 397
4 D. I Arnon, R. K. Chain, B. D. McSwain, H. Y. Tsujimoto and D. B. Knaff, *Proc. Natl. Acad. Sci. U.S.*, 67 (1970) 1404.
5 R. Malkin, *Biochim. Biophys. Acta*, 253 (1971) 421.
6 E. C. Weaver, *Annu. Rev. Plant Physiol.*, 19 (1968) 283.
7 K. Sauer and J. Biggins, *Biochim. Biophys. Acta*, 102 (1965) 55.
8 M. M. Anderson and R. E. McCarty, *Biochim. Biophys. Acta*, 189 (1969) 193.
9 A. P. Margozzi, M. Henderson and E. C. Weaver, *Photochem. Photobiol.*, 9 (1969) 549.
10 R. Ziegler and K. Egle, *Beitr. Biol. Pflanz.*, 41 (1965) 11.
11 D C. Fork and N Murata, *Photochem. Photobiol.*, 13 (1971) 33.
12 T. Hiyama and B. Ke, *Biochim. Biophys. Acta.*, 267 (1972) 160.
13 N. K. Boardman, in A San Pietro, *Methods in Enzymology*, Vol 23, Acadmic Press, New York, 1971, p 268
14 D Kohl, in H. M. Swartz, J. R. Bolton and D. C. Borg, *Biological Applications of ESR*, John Wiley, New York, in the press.
15 T. V. Marsho and B Kok, in A. San Pietro, *Methods in Enzymology*, Vol. 23, Academic Press, New York, 1971, p. 515.
16 K Erixon and W. L. Butler, *Photochem. Photobiol.*, 14 (1971) 427.
17 L. P. Vernon, B. Ke and E R. Shaw, *Biochemistry*, 6 (1967) 2210.
18 J. M. Anderson and N. K Boardman, *Biochim. Biophys. Acta*, 112 (1966) 403.
19 P. V. Sane, D J. Goodchild and R. B. Park, *Biochim. Biophys. Acta*, 216 (1970) 162.
20 A. S. Sun and K. Sauer, *Biochim. Biophys. Acta*, 256 (1972) 409.
21 P. V. Sane and R. B. Park, *Biochem. Biophys. Res Commun*, 44 (1971) 491.