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ON TWO PHOTOREACTIONS IN SYSTEM II OF PLANT PHOTOSYNTHESIS

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SUMMARY

Light-induced absorbance changes of cytochrome b_{559} and C550 in chloroplasts indicate that noncyclic electron transport from water to ferredoxin (Fd)–$NADP^+$ is carried out solely by System II and includes not one but two photoreactions (IIa and IIb) that proceed effectively only in short-wavelength light. (C550 is a new chloroplast component identified by spectral evidence and distinct from cytochromes.) The evidence suggests that the two short-wavelength light reactions operate in series, being joined by a System II chain of electron carriers that includes (but is not limited to) C550, cytochrome b_{559}, and plastocyanin (PC).

$$H_2O \rightarrow IIb_{h\nu} \rightarrow C550 \rightarrow \text{cyt. } b_{559} \rightarrow PC \rightarrow IIa_{h\nu} \rightarrow Fd \rightarrow NADP^+$$

Photoreaction IIb involves an electron transfer from water to C550 that does not require plastocyanin and is the first known System II photoreaction resistant to inhibition by 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and *o*-phenanthroline. Cytochrome b_{559} is reduced by C550 in a reaction that is readily inhibited by DCMU or *o*-phenanthroline. Thus, the site of DCMU (and *o*-phenanthroline) inhibition of System II appears to lie between C550 and cytochrome b_{559}. Photoreaction IIa involves an electron transfer from cytochrome b_{559} and plastocyanin to ferredoxin–$NADP^+$.

INTRODUCTION

It is widely held that the light-induced reduction of $NADP^+$ by water requires the collaboration of Systems I and II of plant photosynthesis (see review[1]). However, recent observations provided a basis for attributing the light-induced reduction of $NADP^+$ solely to System II, which was subdivided into two photoreactions (IIb and IIa), operating in series and connected by a dark chain of electron carriers characteristic of System II. In the new scheme,

$$H_2O \rightarrow h\nu_{IIb} \rightarrow C550 \rightarrow \text{cyt. } b_{559} \rightarrow PC \rightarrow h\nu_{IIa} \rightarrow Fd \rightarrow NADP^+$$

we envisage that photoreaction IIb oxidizes water and reduces C550 (refs. 2, 3) and that photoreaction IIa oxidizes cytochrome b_{559} and plastocyanin (PC) and reduces ferredoxin (Fd)[2,4]. Reduced ferredoxin in turn reduces $NADP^+$ in a dark enzymic reaction[5]. (C550 is a new photoreactive chloroplast component distinct from cyto-

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

chromes[3].) According to this concept[2], System I operates in parallel to System II and is limited to cyclic electron flow (and phosphorylation) and its experimental variants, such as the light-induced reduction of $NADP^+$ by reduced dyes.

Earlier reports dealt with the photoreduction of C550 and the photooxidation of cytochrome b_{559} (refs. 2–4). This investigation was concerned with the oxidation of C550 and the reduction of cytochrome b_{559}, *i.e.* the electron transfer reactions that, according to the new scheme, link photoreactions IIa and IIb. Evidence was obtained that the photoreduction of cytochrome b_{559} by water *via* photoreaction IIb does not require plastocyanin, in contrast to the photooxidation of cytochrome b_{559} *via* photoreaction IIa which does require plastocyanin. Evidence will also be presented suggesting that the site of DCMU and *o*-phenanthroline inhibition of System II lies between C550 and cytochrome b_{559}.

METHODS

"Broken" spinach chloroplasts were prepared according to the method of WHATLEY AND ARNON[6] and Tris-treated chloroplasts, by a modification of the procedure of YAMASHITA AND BUTLER.[7] Sonicated chloroplasts were prepared by sonicating a chloroplast suspension with a Branson sonifier for 1 min at power setting 3 as described previously[2,8]. Chlorophyll was determined as described by ARNON[9].

Plastocyanin was prepared according to a modification of the method of KATOH *et al.*[10]. Ferredoxin–$NADP^+$ reductase[11] and ferredoxin[12] were prepared as described previously.

Absorbance changes were measured with a dual-wavelength spectrophotometer (Phoenix Precision Instrument Co.) as described previously[2–4]. The half-band width of the measuring beams was 2.0 mμ when 540 mμ was used as the reference wavelength and 2.3 mμ for measurements made with 570 mμ as the reference wavelength.

Monochromatic illumination was introduced through a hole in the side of the spectrophotometer as described previously[2–4,13]. Incident illumination intensity was measured with a YSI Kettering Model 65 radiometer.

The oxidation–reduction potentials of the cytochromes were determined *in situ* by measuring the extent of their oxidation in chloroplasts incubated in solutions containing different proportions of potassium ferricyanide and ferrocyanide. The potentials of the chloroplast suspensions were measured with a Radiometer Model 26 pH meter and a PK 149 platinum electrode. The extent of oxidation of each cytochrome was determined as a ratio of two absorbance changes, one when the cytochrome underwent a complete transition from a fully reduced to a fully oxidized state (ferricyanide *minus* ascorbate) and the other when the cytochrome underwent a transition from a partly oxidized state (in a particular ferro–ferricyanide solution) to a fully oxidized state (excess ferricyanide). In some cases the procedure was reversed and the ratio was obtained by transition to a fully reduced state, using excess ascorbate. The respective absorbance changes were measured at 561 mμ *minus* 570 mμ for spinach chloroplast cytochrome b_{559}, 550 mμ *minus* 540 mμ for spinach chloroplast cytochrome *f*, and 558 mμ *minus* 570 mμ for *Euglena gracilis* cytochrome b_{558}. To reduce the instrument noise level in the potential determinations, the Optics Technology 600-mμ short pass cut-off filter and the Corning 4-96 filter, normally used to protect the phototube from the actinic light, were removed, after it had been

determined that the presence or absence of the filters made no difference in the absorbance changes.

RESULTS AND DISCUSSION

Photooxidation of cytochrome b_{559} at physiological temperatures

We previously reported[2,4] that cytochrome b_{559} photooxidation can be consistently observed only when its photoreduction by electrons from water is blocked, either by performing the experiment at liquid N_2 temperature or by Tris-treating the chloroplasts at room temperature—a treatment that eliminates the oxidation of water but does not otherwise interfere with System II activity[7,8,14]. It therefore seemed possible that cytochrome b_{559} photooxidation could also be observed at a physiological temperature without the Tris-treatment if the experiments were performed at a pH where the photooxidation of water does not take place[15].

Fig. 1 shows the photooxidation of cytochrome b_{559} at 20° and pH 10.0. In agreement with our earlier results[2,4], System II light (664 mμ) was again much more effective than System I light (715 mμ) in photooxidizing cytochrome b_{559}. However, other investigators have reported that the photooxidation of cytochrome b_{559} is a System I reaction[16–18]. In our experiments, the photooxidation of cytochrome b_{559} by

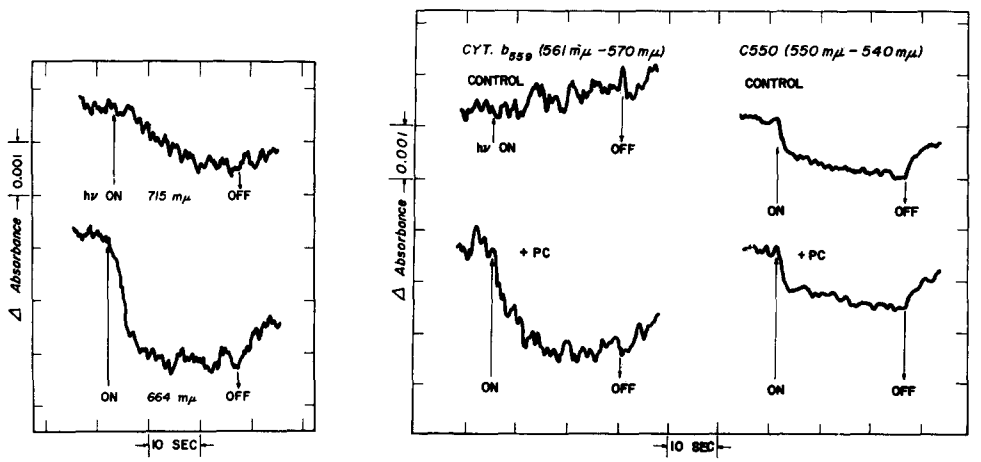


Fig. 1. Photooxidation of cytochrome b_{559} at 20° and pH 10.0 by 664- and 715-mμ light (561 mμ *minus* 570 mμ). The reaction mixture contained (per 1.0 ml) P_{1S} spinach chloroplasts (equivalent to 75 μg chlorophyll) and the following in μmoles: glycine buffer (pH 10.0), 33.3; K_2HPO_4, 5; $MgCl_2$, 2; sodium ascorbate, 1; ferredoxin, 0.01; and $NADP^+$, 1. Gas phase, N_2. The 664-mμ actinic light had an intensity of $1.5 \cdot 10^4$ ergs/cm² per sec and the 715-mμ actinic light had an intensity of $0.9 \cdot 10^4$ ergs/cm² per sec.

Fig. 2. Effect of plastocyanin (PC) on components of System II. The reaction mixture for the cytochrome b_{559} photooxidation measurement contained (per 1.0 ml) sonicated, Tris-treated spinach chloroplasts (equivalent to 75 μg chlorophyll) and the following in μmoles: 2-(*N*-morpholino)ethane sulfonic acid buffer (pH 6.2), 33.3; K_2HPO_4, 5; $MgCl_2$, 2; sodium ascorbate, 1; ferredoxin, 0.01; $NADP^+$, 1; ferredoxin–$NADP^+$ reductase equivalent to an absorbance at 456 mμ of 0.0095; and, where indicated, plastocyanin (PC), 0.01. The reaction mixture for the C550 photoreduction measurement contained (per 1.0 ml) sonicated spinach chloroplasts (equivalent to 75 μg chlorphyll) and the following in μmoles: 2-(*N*-morpholino)ethane sulfonic acid buffer (pH 6.2), 33.3; K_2HPO_4, 5; $MgCl_2$, 2; potassium ferricyanide, 5; and, where indicated, plastocyanin (PC), 0.01. Gas phase, N_2. 664-mμ illumination as in Fig. 1.

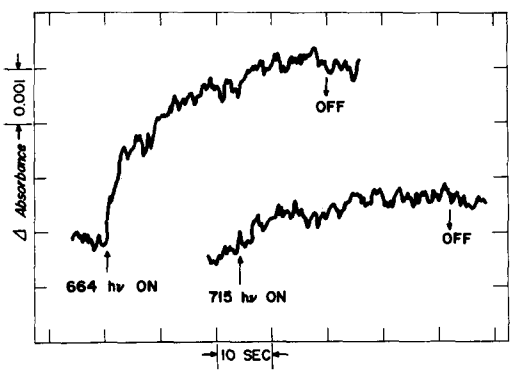
System II light was stimulated by the presence of ferredoxin and $NADP^+$, a fact suggesting a close connection between the photooxidation of cytochrome b_{559} by System II and the reduction of ferredoxin and $NADP^+$.

Plastocyanin requirement

We suggested earlier, on thermodynamic grounds, that the photoreaction (IIa) responsible for photooxidation of cytochrome b_{559} must be different from the photoreaction (IIb) responsible for the photooxidation of water and the concomitant photoreduction of C550 (ref. 2). Further evidence for the existence of two different System II photoreactions comes from their contrasting requirement for plastocyanin. Photooxidation of cytochrome b_{559} (refs. 2, 8) requires plastocyanin, but the photoreduction of C550 does not. Fig. 2 shows that the addition of plastocyanin was essential for cytochrome b_{559} photooxidation to chloroplasts (control) depleted of plastocyanin by sonication[2,8]. By contrast, the photoreduction of C550 proceeded equally well in the plastocyanin-depleted chloroplasts (control) with or without the addition of plastocyanin.

Reduction of cytochrome b_{559}

Further support for the validity of the proposed scheme for System II came from experiments on the reduction of cytochrome b_{559} by System II. The scheme predicts that, since plastocyanin is on the oxidizing side of cytochrome b_{559} (compare ref. 19) the reduction of this cytochrome could occur in chloroplasts depleted of plastocyanin by sonication. Fig. 3 shows that this was indeed observed and that the photoreduction of cytochrome b_{559} was induced much more effectively by System II light (664 mμ) than by System I light (715 mμ), in agreement with work in other laboratories[16–19].



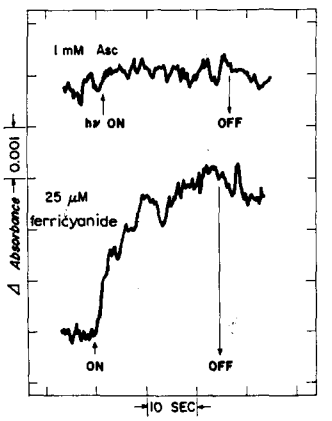


Fig. 3. Photoreduction of cytochrome b_{559} in sonicated chloroplasts by 664- and 715-mμ light (560 mμ *minus* 570 mμ). The reaction mixture contained (per 1.0 ml) sonicated spinach chloroplasts (equivalent to 75 μg chlorophyll) and the following in μmoles: 2-(*N*-morpholino)ethane sulfonic acid buffer (pH 6.2), 33.3; K_2HPO_4, 5; $MgCl_2$, 2; and potassium ferricyanide, 0.025. Gas phase, N_2. Illumination as in Fig. 1.

Fig. 4. Photoreduction of cytochrome b_{559} in sonicated chloroplasts in the presence and absence of ascorbate (560 mμ *minus* 570 mμ). Experimental conditions were as described in Fig. 3, except that, where indicated, 1 μmole (per 1.0 ml) sodium ascorbate (Asc) replaced potassium ferricyanide. 664-mμ illumination as in Fig. 1.

Because chloroplasts contain two *b*-type cytochromes (cytochrome b_6 (refs. 16, 20–22) with an α-band at 563 mμ and cytochrome b_{559} (refs. 16, 17, 21–23) with an α-band at 559 mμ) with similar absorption spectra, it was necessary to determine if the light-induced increase in absorbance was caused by the reduction of cytochrome b_6, cytochrome b_{559}, or both. That the observed increase in absorbance was indeed a measure of reduction of cytochrome b_{559} and not of cytochrome b_6 is corroborated by the ascorbate treatment shown in Fig. 4. Cytochrome b_{559}, but not cytochrome b_6, is completely reduced by ascorbate in the dark[16,21,22]. Thus, a pretreatment of chloroplasts with ascorbate would be expected to leave cytochrome b_{559} in a completely reduced state and would abolish the light-induced reduction, an expectation that was experimentally verified (Fig. 4).

Further substantiation that the light-induced increase in absorbance resulted mainly from the photoreduction of cytochrome b_{559} was obtained by measuring the spectrum in the region from 559 to 570 mμ. (Interference from absorbance changes due to C550 (ref. 3) and cytochrome *f* (refs. 3, 21, 22, 24) prevented determination of the complete spectrum.) The spectrum had a maximum at 560 mμ, as would be expected for cytochrome b_{559} (refs. 4, 16, 17) A shoulder at 563 mμ indicated that a small amount of cytochrome b_6 photoreduction did occur[16,20–22].

The new scheme envisages that the photoreduction of cytochrome b_{559}, as distinguished from its photooxidation, is dependent on light reaction IIb that involves an electron flow from water *via* C550. Evidence for this interpretation was found in experiments with Tris-treated chloroplasts (Fig. 5). The Tris treatment,

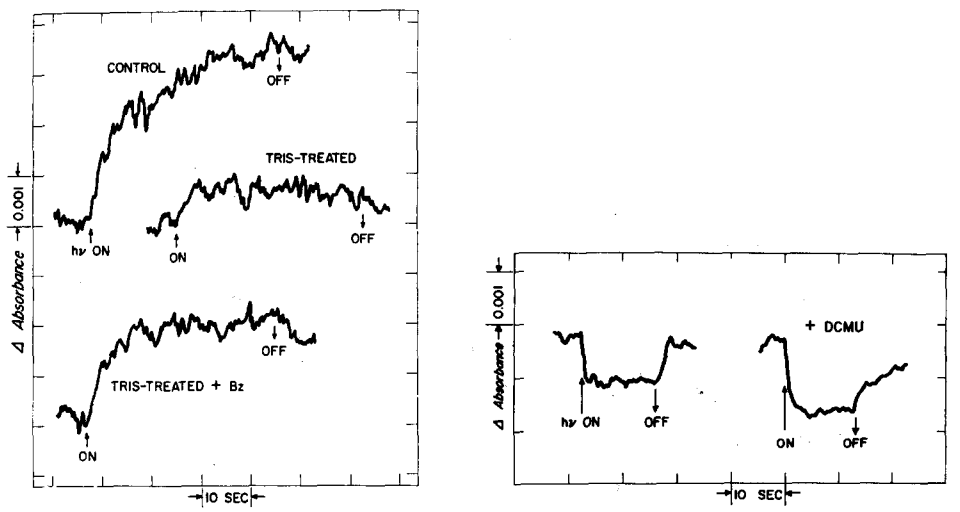


Fig. 5. Effect of Tris treatment and a System II electron donor on cytochrome b_{559} photoreduction (560 mμ *minus* 570 mμ). Experimental conditions were as described in Fig. 3 except that, where indicated, Tris-treated sonicated chloroplasts replaced sonicated (control) chloroplasts and 0.067 μmole (per 1.0 ml) benzidine (Bz) was present. 664-mμ illumination as in Fig. 1.

Fig. 6. Effect of DCMU on C550 photoreduction in Tris-treated chloroplasts (550 mμ *minus* 540 mμ). The reaction mixture contained (per 1.0 ml) Tris-treated chloroplasts (equivalent to 75 μg chlorophyll) and the following in μmoles: Tricine buffer (pH 8.2), 33.3; K_2HPO_4, 5; $MgCl_2$, 2; potassium ferricyanide, 0.5; and, where indicated, DCMU, $2 \cdot 10^{-4}$. Gas phase, N_2. 664-mμ illumination as in Fig. 1.

which inhibits the electron flow from water, has also effectively inhibited the photoreduction of cytochrome b_{559}. The addition to Tris-treated chloroplasts of a substitute electron donor, benzidine[25], has restored the photoreduction of cytochrome b_{559} (Fig. 5). Here again, System II light (664 mμ) was much more effective than System I light (715 mμ).

DCMU inhibition

Other evidence in support of the new scheme was obtained from experiments with 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and *o*-phenanthroline, two well-known inhibitors of System II (ref. 26). In earlier experiments with untreated chloroplasts[2,3], these two inhibitors inhibited the oxidation but not the reduction of C550. Fig. 6 shows that in Tris-treated chloroplasts DCMU again inhibited only the oxidation of C550; reduction was actually stimulated. Similar results were obtained with *o*-phenanthroline. Thus, the photoreduction of C550 became the first known reaction of System II that is not inhibited by DCMU or *o*-phenanthroline.

Since the new scheme envisages that the reduction of cytochrome b_{559} follows the oxidation of C550, DCMU and *o*-phenanthroline would be expected to inhibit the photoreduction of cytochrome b_{559}. We reported earlier that DCMU (and *o*-phenanthroline) inhibit the photooxidation of cytochrome b_{559} (ref. 2). Further investigation has now shown that, as observed earlier by other investigators[16–18], DCMU also inhibits the photoreduction of cytochrome b_{559}. Fig. 7 demonstrates that DCMU inhibited, to the same extent, the photoreduction of cytochrome b_{559} in untreated chloroplasts where water was the electron donor and in Tris-treated chloroplasts where benzidine served as the artificial substitute electron donor. Similar results were obtained with *o*-phenanthroline. These results indicate that

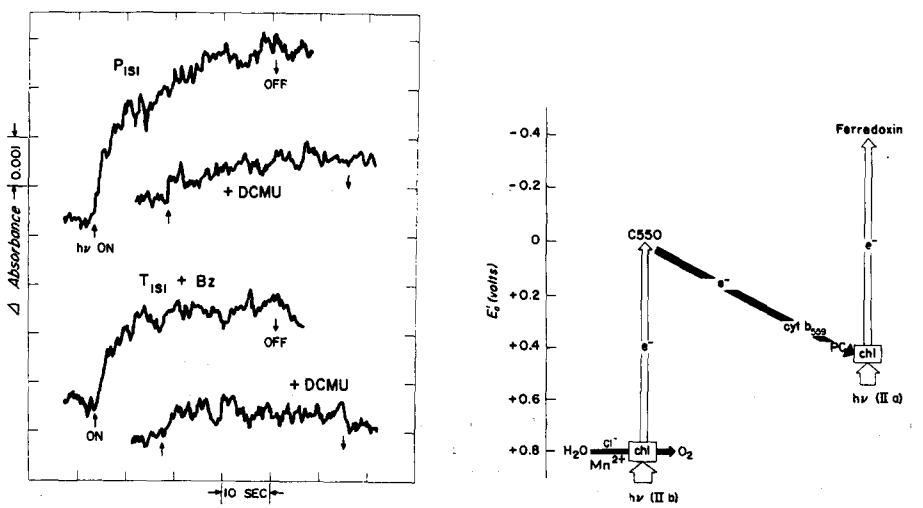


Fig. 7. Effect of DCMU on cytochrome b_{559} photoreduction (560 mμ *minus* 570 mμ). Experimental conditions were as described in Fig. 5, except that, where indicated, 0.001 μmole (per 1.0 ml) DCMU was present. Sonicated chloroplasts (P_{ISI}), sonicated Tris-treated chloroplasts (T_{ISI}) and benzidine (Bz) were present as indicated.

Fig. 8. Scheme for two photoreactions in System II.

DCMU (and *o*-phenanthroline) inhibit System II activity in chloroplasts by inhibiting electron transfer through cytochrome b_{559}.

To recapitulate, the concept put forward earlier[13,15,22,26] that noncyclic electron transport from water to ferredoxin–$NADP^+$ involves only System II, then known to include only one photoreaction, has now been further elaborated, in the light of new evidence, to include two short-wavelength photoreactions (IIb and IIa) that operate in series and are joined by an electron transport chain that includes (but is not limited to) C550, cytochrome b_{559}, and plastocyanin (Fig. 8). Noncyclic photophosphorylation is envisaged as being coupled to the electron transport chain between photoreactions IIb and IIa.

THERMODYNAMIC CONSIDERATIONS

The placement of cytochrome b_{559} in the new scheme was accompanied by a measurement of its midpoint oxidation–reduction potential as + 0.33 V at pH 8.2 (ref. 2). This value, which was in good agreement with that reported by BENDALL[27], was recently questioned by FAN AND CRAMER[28] who obtained a value of + 0.04 V for the midpoint potential of spinach cytochrome b_{559} at pH 8.0. We have therefore reinvestigated the matter and obtained from eight determinations, using potassium ferricyanide as the oxidant, a value for E_0 (pH 8.2) of + 0.325 ± 0.015 V. The corresponding value for n, the number of electrons transferred, was 0.9 ± 0.1. Fig. 9 shows a representative Nernst plot obtained in a potential determination using ferricyanide as the oxidant.

With ascorbate as the reductant, the midpoint potential measured was routinely somewhat higher (0.015 V) than that obtained with ferricyanide as the oxidant.

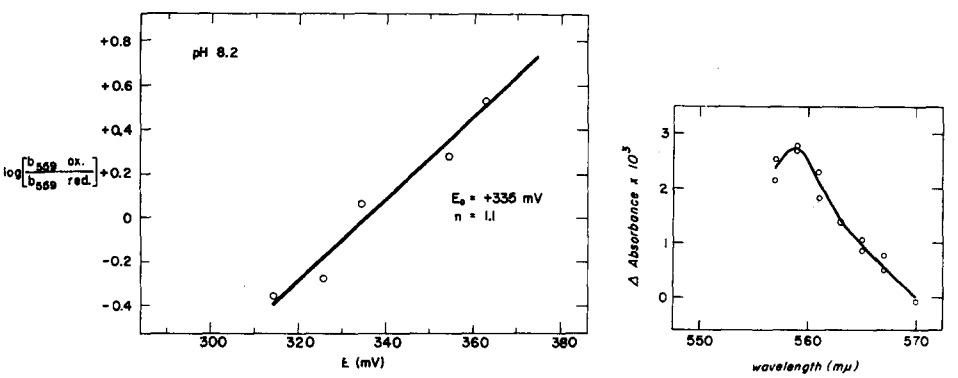


Fig. 9. Oxidation–reduction potential of cytochrome b_{559}. The reaction mixture contained (per 1.0 ml) P_{1S} spinach chloroplasts (equivalent to 75 μg chlorophyll) and the following in μmoles: Tricine buffer (pH 8.2), 67.6; K_2HPO_4, 5; $MgCl_2$, 2; and potassium ferricyanide–potassium ferrocyanide, 0.5. The absorbance change (561 mμ *minus* 570 mμ) on addition of 25 μmoles (in 0.05 ml) potassium ferricyanide to 5.0 ml of the reaction mixture was measured as a function of the potential. Gas phase, N_2.

Fig. 10. Reduction of cytochrome b_{559} by ferrocyanide (570-mμ reference). The reaction mixture contained (per 1.0 ml) P_{1S} spinach chloroplasts (equivalent to 75 μg chlorophyll) and the following in μmoles: Tricine buffer (pH 8.2) 67.6; and potassium ferricyanide, 0.05. To 5.0 ml of the reaction mixture (E = + 0.425 V) were added 25 μmoles (in 0.05 ml) of potassium ferrocyanide which lowered the potential to + 0.293 V. Gas phase, air.

The same potential values were obtained either after 5 min of vigorous flushing with N_2 or in air.

The spectra of the absorbance changes produced on addition of either ferricyanide or ascorbate had maxima at 559 mμ, indicating that the changes were indeed those of cytochrome b_{559} (refs. 21, 22, 27, 28).

Using the same technique, the potential of spinach chloroplast cytochrome *f* was found to be + 0.390 V and that of *Euglena gracilis* chloroplast cytochrome b_{559}, + 0.305 V, both values being in good agreement with those obtained in other laboratories[27-29].

Further substantiation of the potential measured for cytochrome b_{559} was sought from treatment with ferrocyanide. If the potential of cytochrome b_{559} were more positive than + 0.30 V, the protein should be reducible by a weak reductant such as ferrocyanide[29], but not if the potential were + 0.04 V (ref. 28). Fig. 10 shows that the spectrum of the absorbance changes obtained on addition of ferrocyanide corresponds to that of reduced cytochrome b_{559}.

ADDENDUM

The reduction of C550 is no longer demonstrable only by illuminating chloroplasts. We have recently observed a decrease in absorbance at 550 mμ, unrelated to cytochromes, by treating chloroplasts with sodium dithionite. Similar results were reported earlier by ERIXSON AND BUTLER[30].

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REFERENCES

1 N. K. BOARDMAN, *Ann. Rev. Plant Physiol.*, 21 (1970) 115.
2 D. B. KNAFF AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 64 (1969) 715.
3 D. B. KNAFF AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 963.
4 D. B. KNAFF AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 956.
5 M. SHIN AND D. I. ARNON, *J. Biol. Chem.*, 240 (1965) 1405.
6 F. R. WHATLEY AND D. I. ARNON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 6, Academic Press, New York, 1963, p. 308.
7 T. YAMASHITA AND W. L. BUTLER, *Plant Physiol.*, 43 (1968) 1978.
8 D. B. KNAFF AND D. I. ARNON, *Biochim. Biophys. Acta*, 223 (1970) 201.
9 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
10 S. KATOH, I. SHIRATORI AND A. TAKAMIYA, *J. Biochem. Tokyo*, 51 (1962) 32.
11 M. SHIN, K. TAGAWA AND D. I. ARNON, *Biochem. Z.*, 338 (1963) 84.
12 M. LOSADA AND D. I. ARNON, in H. F. LINSKENS, B. D. SANWAL AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Vol. 7, Springer, Berlin, 1964, p. 569.
13 B. D. MC SWAIN AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 989.
14 T. YAMASHITA AND T. HORIO, *Plant Cell Physiol.*, 9 (1968) 268.
15 H. Y. TSUJIMOTO, B. D. MC SWAIN, R. K. CHAIN AND D. I. ARNON, in H. METZNER, *Progress in Photosynthesis Research*, Vol. 3, Laupp, Tübingen, 1969, p. 1241.
16 W. A. CRAMER AND W. L. BUTLER, *Biochim. Biophys. Acta*, 143 (1967) 332.
17 G. BEN-HAYYIM AND M. AVRON, *European J. Biochem.*, 14 (1970) 205.
18 R. P. LEVINE AND D. S. GORMAN, *Plant Physiol.*, 41 (1966) 1293.
19 D. S. GORMAN AND R. P. LEVINE, *Plant Physiol.*, 41 (1966) 1648.
20 R. HILL, *Nature*, 174 (1954) 501.
21 N. K. BOARDMAN AND J. ANDERSON, *Biochim. Biophys. Acta*, 143 (1967) 187.

22 D. I. ARNON, H. Y. TSUJIMOTO, B. D. MC SWAIN AND R. K. CHAIN, in K. SHIBATA, A. TAKAMIYA, A. T. JAGENDORF AND R. C. FULLER, *Comparative Biochemistry and Biophysics of Photosynthesis*, University of Tokyo Press, Tokyo and University Park Press, State College, Pa., 1968, p. 113.
23 H. LUNDEGARDH, *Physiol. Plantarum*, 15 (1962) 390.
24 M. AVRON AND B. CHANCE, *Brookhaven Symp. Biol.* 19 (1966) 149.
25 T. YAMASHITA AND W. L. BUTLER, *Plant Physiol.*, 44 (1969) 435.
26 D. I. ARNON, H. Y. TSUJIMOTO AND B. D. MC SWAIN, *Nature*, 214 (1967) 562.
27 D. S. BENDALL, *Biochem. J.*, 109 (1968) 46P.
28 H. N. FAN AND W. A. CRAMER, *Biochim. Biophys. Acta*, 216 (1970) 200.
29 I. IKEGAMI, S. KATOH AND A. TAKAMIYA, *Biochim. Biophys. Acta*, 162 (1968) 604.
30 K. ERIXSON AND W. L. BUTLER, *51st Ann. Meeting Pacific Div. Am. Assoc. Advancement Sci., Berkeley, Calif., 1970.*