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THE EFFECT OF ARTIFICIAL ELECTRON DONOR AND ACCEPTOR SYSTEMS ON LIGHT-INDUCED ABSORBANCE RESPONSES OF CYTOCHROMES *f* AND OTHER PIGMENTS IN INTACT CHLOROPLASTS

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SUMMARY

An investigation has been made into the effect of low concentrations of 2,6-dichlorophenolindophenol (DCIP) and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), in the presence of ascorbate, on light-induced absorbance changes in the α-band region of cytochrome *f* and *b*-type cytochromes. Strong photo-oxidation of cytochrome *f* occurred with DCIP, in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (2 μM), but not with TMPD. However, with TMPD a large light-induced absorbance increase occurred due to a broad band centred at 566 nm. With DCIP, the uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), in conjunction with the electron acceptor 1,1'-ethylene-2,2'-bipyridylium dibromide (diquat), inhibited the cytochrome *f* response and an absorbance increase in the 560–575 nm region occurred similar to that with TMPD. Neither FCCP nor diquat had any great effect on the TMPD system. The results support a pathway of electron transport between the two photosystems in which (a) DCIP–ascorbate interacts with an intermediate on the Photosystem II side of a coupling site; (b) TMPD–ascorbate interacts after this site, and (c) cytochrome *f* is located on the Photosystem I side of the site.

INTRODUCTION

Various electron donor systems have been used over the past decade in studies of photosynthetic electron flow and photophosphorylation[1–5]. It has been clearly established that in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) electron donation occurs at a site on the intermediate pathway between Photosystem I and Photosystem II[2,6,7]. However, the earlier view that these systems could be used as a tool for dissection of the intermediate pathway[7] has not been fully substantiated because of the complication that the dyes used in the donor systems act

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Diquat, 1,1'-ethylene-2,2'-bipyridylium dibromide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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also as co-factors for cyclic phosphorylation (see refs 4, 8, 9). Thus while the 2,6-dichlorophenolindophenol (DCIP) and ascorbate couple promotes electron flow through Photosystem I coupled to phosphorylation, the *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)–ascorbate couple mediates a largely uncoupled electron flow[7] and it has been argued that while DCIP acts both as an electron donor and catalyst for cyclic phosphorylation, TMPD, at low concentrations (see ref. 4), acts only as an electron donor[8,9].

Avron and Chance[10] reported that in swollen chloroplasts cytochrome *f* was not involved in the electron transport system induced by DCIP–ascorbate in the presence of DCMU. However, there is direct evidence that TMPD donates electrons to a site closer to Photosystem I than that served by DCIP[11]. It was therefore a logical development in a series of investigations of the absorbance changes in intact chloroplasts[12,13] to monitor the photo-oxidation of cytochrome *f* in the presence of DCIP–ascorbate and TMPD–ascorbate couples, since recent evidence has tended to confirm the location of cytochrome *f* on an intermediate pathway between the two systems[12,14] (other intermediate pathways, lacking cytochrome *f*, are not thereby excluded). This led to the discovery that the two couples resulted in quite different absorbance changes in the α-band region of cytochrome *f*. The situation was complicated by the appearance under these conditions of a broad band of positive or negative absorbances in the region 565–575 nm which tended to obscure the cytochrome *f* responses. Nevertheless, the results have shown a greater photo-oxidation of cytochrome *f* with DCIP compared with TMPD. These results, in conjunction with a study of P518 responses[13], are taken to indicate that DCIP donates electrons prior to a coupling site (*i.e.* on the Photosystem II side) in contrast to which TMPD donates electrons after the site, and that cytochrome *f* is located on the Photosystem I side of the site.

MATERIALS AND METHODS

The materials and methods used in this work have been described in detail elsewhere[12].

Chloroplasts were isolated from pea leaves obtained from 10 to 14-day cultured pea plants. The preparations of intact chloroplasts involved the isolation of high-salt chloroplasts by a modification of the method of Harvey and Brown[15] employing a sucrose density gradient centrifugation and an isolation medium of 330 mM sorbitol, 5 mM $MgCl_2$, 1 mM $Na_4P_2O_7$, 1 mM ascorbic acid, 10 mM morpholinopropane sulphonate (MOPS) buffer (pH 6.5) and 0.1 % bovine serum albumin (Grade A). The final precipitate of chloroplasts was taken up in the reaction medium of 330 mM sorbitol, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (pH 7.5, buffered with KOH) and 0.1 % bovine serum albumin. These chloroplasts were of the Class I type with outer membranes intact.

Light-induced absorbance changes were recorded in a dual wavelength difference spectrophotometer with access to the chloroplast suspension from the side for illumination with actinic light. The 1 cm × 1 cm cuvette was kept at a constant temperature of 10 °C, in order to preserve the intact chloroplasts for longer periods; comparative tests at 25 °C showed no qualitative differences in the results. Actinic illumination of the wavelengths 680 nm and 720 nm was obtained using Baird Atomic interference filters with a 95 % transmission between ± 5 nm. The light intensity at the cuvette

was $4 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$s^{-1} at 680 nm and $2 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$s^{-1} at 720 nm. A combination of a glass filter (Corning CS 4-96) and a Kodak Wratten filter No. 59A screened the photomultiplier from actinic light.

Biochemicals were obtained from Calbiochem Co., Los Angeles. Carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), 1,1'-ethylene-2,2'-bipyridylium dibromide (diquat) and DCMU were gifts of P. G. Heytler, Experimental Research Station, E. I. Dupont Nemours Co. Ltd, Wilmington, Delaware. DCMU and FCCP were dissolved in ethanol and were used at a final concentration of less than 0.2 % ethanol.

RESULTS

Two wavelengths have been chosen to illustrate the effects of the electron donor and acceptor systems on light-induced absorbance changes in the α-band region of cytochrome *f*: (i) at 554 nm, the peak of the α-band of cytochrome *f*, and (ii) at 572 nm, where a broad light-induced absorbance decrease has previously been observed[12]. The response at 572 nm and related responses, described subsequently, are of great importance in the present work because they interfere, in certain situations, with cytochrome *f* changes at 554 nm. Far-red actinic light (720 nm) was used to stimulate Photosystem I largely[13], and red actinic light (680 nm) to stimulate both Photosystem I and Photosystem II; intensities of both beams were near to saturation for the cytochrome *f* response[12].

Fig. 1 shows the effects of ascorbate *plus* DCIP (20 μM) and ascorbate *plus* DCIP in the presence of DCMU (2 μM) on the light-induced changes. Although the

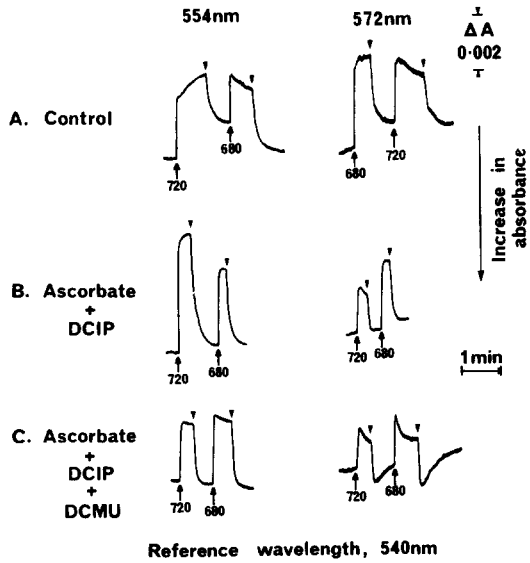

Fig. 1. The effect of ascorbate (2 mM potassium salt) *plus* DCIP (20 μM) and DCMU (2 μM) on the light-induced responses at 554 and 572 nm in intact pea chloroplasts measured by a dual wavelength difference spectrophotometer (reference wavelength, 540 nm). The chloroplasts were exposed to far-red light (720 nm, $2 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$s^{-1}) and red light (680 nm, $4 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$s^{-1}), indicated by the upwardly pointing arrows; "light-off" is indicated by the downwardly pointing arrowhead. Temperature 10 °C. Chlorophyll concentration (μg/ml): A, 65; B, 71; C, 68.

responses at the two wavelengths appear to be qualitatively similar the light–dark difference spectra (Fig. 2) revealed an extensive photo-oxidation of cytochrome *f*, as well as contributions from two other spectral components, previously reported for intact chloroplasts[12], (a) the negative response centred broadly between 565 and 575 nm (referred to as the P-572 response), and (b) a negative response centred at 550 nm. The spectra for the DCIP–ascorbate *plus* DCMU treatment were similar.

Since, in the presence of DCMU alone, cytochrome *f* cannot be reversibly photo-oxidised, the large reversible response in the presence of the donor couple and DCMU indicated that DCIP donated electrons prior to cytochrome *f* on an electron transport pathway between the two photosystems[12,14]. Furthermore, to account for the extensive reversible response of cytochrome *f* it was concluded that a resistance to electron flow existed between the site of interaction and cytochrome *f*; the observation elsewhere of a strong P518 response under identical conditions[13] provided evidence that this resistance was a site of coupling to phosphorylation (see also ref. 16).

In the absence of DCMU, the fully reversible responses indicated that the DCIP couple interacted at a similar site. However, the larger response to far-red light could mean either that Photosystem II activity increased the flow of electrons through the resistance to cytochrome *f* or that the flow of electrons from cytochrome *f* to Photosystem I was reduced. The latter explanation would entail a pathway between the photosystems by-passing cytochrome *f*.

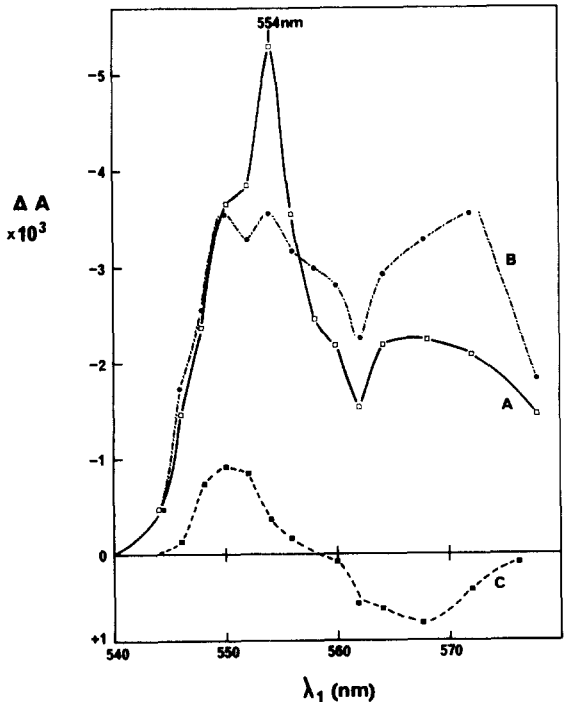

Fig. 2. Difference spectra for intact chloroplasts in the presence of DCIP (20 μM) and ascorbate (2 mM potassium salt); (A) the light–dark difference spectrum of the fast response to far-red (720 nm) light; (B) the light–dark difference spectrum of the final response to red (680 nm) light, and (C) the difference spectrum between the fast and final response to far-red light. Experiment same as in Fig. 1B. Experimental conditions as in Fig. 1. Chlorophyll concentration, 71 μg/ml.

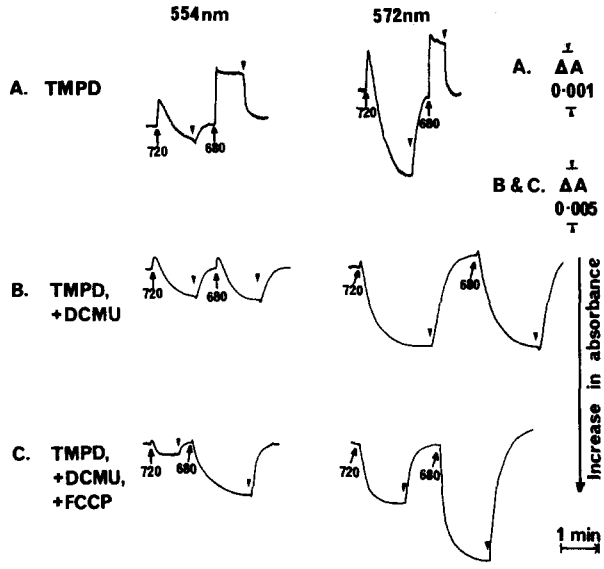

Fig. 3. The effect of TMPD (30 μM), DCMU (2 μM), FCCP (2 μM) and diquat (10 μM) in various combinations on the light-induced responses at 554 and 572 nm in intact pea chloroplasts (ascorbate as the potassium salt was present at a concentration of 2 mM in all treatments). Experimental conditions as in Fig. 1. Chlorophyll concentrations (μg/ml): A and B, 48; C, 55.

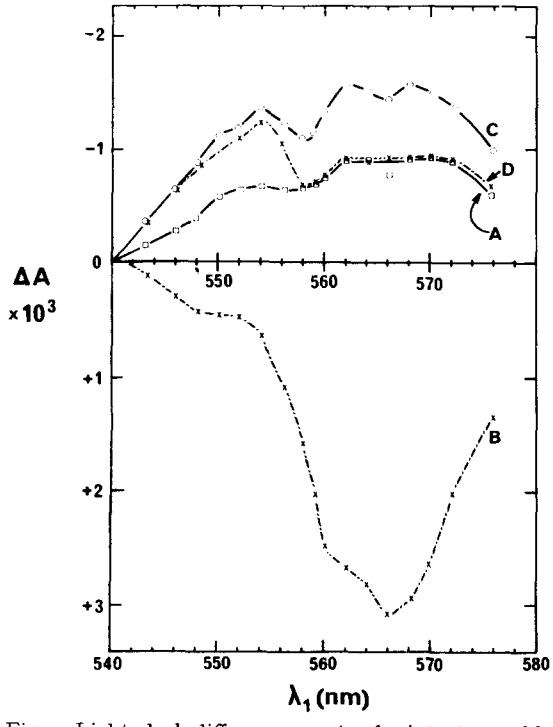

Fig. 4. Light–dark difference spectra for intact pea chloroplasts in the presence of TMPD (30 μM) *plus* ascorbate (2 mM, potassium salt): A, fast far-red (720 nm) light response; B, final far-red light response; C, fast red (680 nm) light response; D, final red light response. Experiment same as in Fig. 3A. Experimental conditions as in Fig. 1. Chlorophyll concentration: 48 μg/ml. Reference wavelength, 540 nm.

Since TMPD–ascorbate has been shown to donate electrons at a site closer to Photosystem I than cytochrome *f*, without coupled phosphorylation[7,11,17] the effects of this donor couple were tested in the present system. The effects of 30 μM TMPD and 2 mM ascorbate are shown in Figs 3 and 4. In far-red light the broad negative band seen in the presence of DCIP–ascorbate was replaced by a broad positive band whose peak was shifted to approx. 566 nm (nevertheless, changes at 572 nm were still monitored for comparative purposes and were very similar to those at the peak wavelength). As shown in Figs 3 and 5 the addition of DCMU to the TMPD system resulted in the broad positive band in response to red light as well as far-red light, which was now centred at 562 nm.

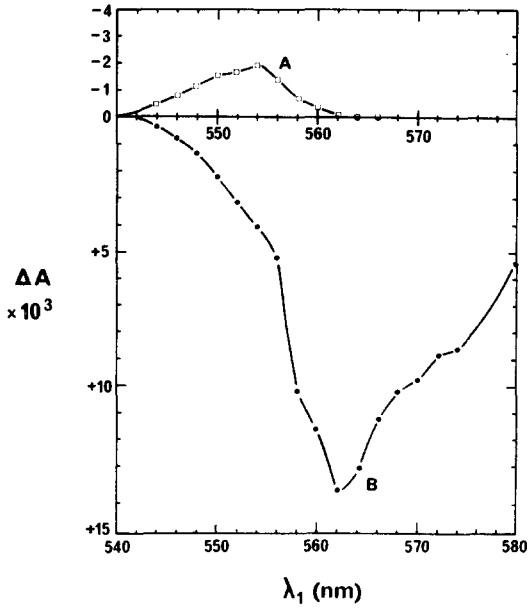

Fig. 5. Light–dark difference spectra for intact chloroplasts in the presence of TMPD (30 μM) ascorbate (2 μM, potassium salt) and DCMU (2 μM): A, fast red (680 nm) light response; B, final red light response. Experiment same as in Fig. 3B (*i.e.* far-red (720 nm) light response and red light response very similar). Experimental conditions as in Fig. 1. Chlorophyll concentration: 55 μg/ml. Reference wavelength, 540 nm.

The absence of any steady-state photo-oxidation of cytochrome *f* with the TMPD system in the presence of DCMU supports the hypothesis that TMPD interacts closer to cytochrome *f* than DCIP and the lack of a P-518 response under these conditions[13] supports an interaction closer to Photosystem I than the postulated coupling site. In the absence of DCMU it is possible that cytochrome *f* is a component of the large negative response in red light (Fig. 3A), although there was little indication of this from the difference spectra (Fig. 4). An accompanying P-518 response[13], indicated the occurrence of energy conservation under these circumstances, and it is possible that TMPD interacted at two sites in the absence of DCMU (*cf.* ref. 18).

The explanation that DCIP interacts with an intermediate prior to a coupling site (Fig. 9) was tested further by the use of the uncoupler of phosphorylation, FCCP,

which should remove any resistance to electron flow caused by coupling to phosphorylation. However, the use of FCCP changed the cytochrome *f* and *b* responses of intact chloroplasts to those characteristic for swollen Class II chloroplasts (A. W. D. Larkum and W. D. Bonner, unpublished). The same effect is brought about by a number of inhibitors, by certain cations and by maltreatment[12]. It seemed to be an effect, not specific to FCCP, on electron transport through Photosystem I since the oxidation rate of cytochrome *f* in far-red light was very slow. With FCCP-treated chloroplasts restoration of the original rates could be effected by the addition of the artificial electron acceptor, diquat which would catalyse under these conditions a pseudocyclic flow of electrons to oxygen with the formation of hydrogen peroxide[19].

The effects of diquat (10 μM) *plus* FCCP (2 μM) are shown in Fig. 6A. The responses were distinctive because of contributions of cytochrome *b*-559 and cytochrome b_6, in the region of 559–563 nm, which will be described separately (A. W. D. Larkum and W. D. Bonner, unpublished); there were also the familiar contributions from cytochrome *f*, P-572 and the component at 550 nm.

Some of the more important combinations of diquat, FCCP and DCMU with the DCIP–ascorbate couple are shown in Figs 6B–6D. The negative absorbance changes in the 565–575-nm region, characteristic of the DCIP–ascorbate couple alone, were no longer observed, being replaced by the positive changes more characteristic of the TMPD–ascorbate system. However, there was a steady-state absorbance decrease at 554 nm in some treatments (Figs 6B and 6C). From the light–dark difference spectra (Fig. 7) it was found that these absorbance decreases had a peak at 552 nm indicating contributions from both the 550 nm component and cytochrome *f* photooxidation. In the presence of the four agents together, *viz.* diquat, FCCP, DCMU

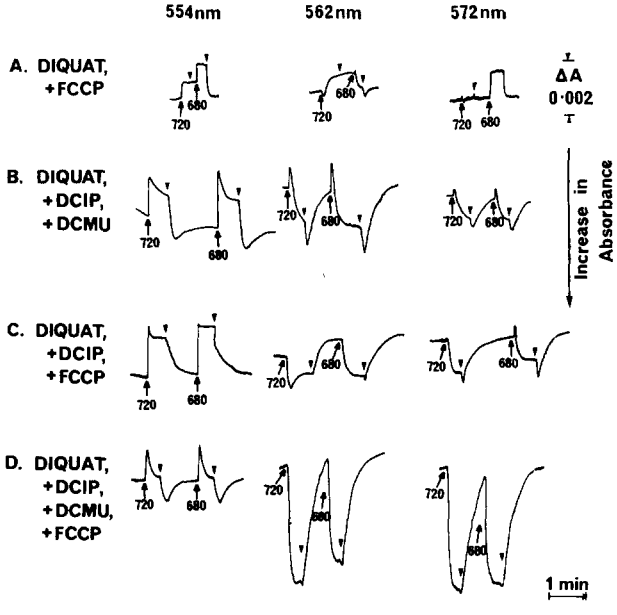

Fig. 6. The effect of diquat (10 μM), FCCP (2 μM), DCIP (30 μM, together with 2 mM potassium ascorbate) and DCMU (2 μM) on the light-induced responses at 554, 562 and 572 nm in intact pea chloroplasts. Experimental conditions as in Fig. 1. Chlorophyll concentration: 57 μg/ml.

and the DCIP couple (Fig. 6D), the responses approximated to those found in the TMPD–DCMU system. As shown in Figs 6D and 7, under those conditions, the positive component between 560 nm and 575 nm was enhanced and the cytochrome *f* and 550 nm components became small and transitory.

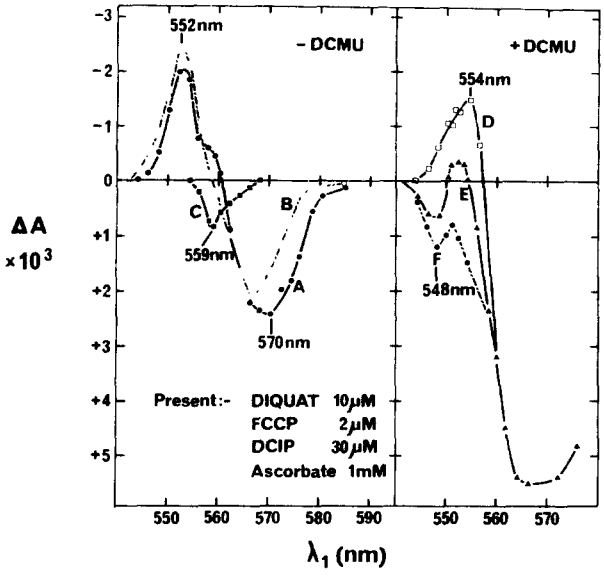

Fig. 7. Light–dark difference spectra for intact pea chloroplasts treated with diquat (10 μM), FCCP (2 μM), DCIP (30 μM) and potassium ascorbate (1 mM) in the absence and presence of DCMU (2 μM): A, final far-red (720 nm) light response; B, final red (680 nm) light response; C, difference spectrum between the fast and final far-red light responses; D, fast far-red light response; E, final far-red light response; F, total slow response to far-red light, *i.e.* measuring the absorbance change from the height of the fast response instead of the dark base-line. Experimental conditions as in Fig. 1. Chlorophyll concentration: 57 μg/ml.

Thus, despite the complexity of the electron donor and acceptor systems employed and despite the complexity of the spectral components, it is still apparent that in the complete system, FCCP does have the effect of decreasing the extent of the steady-state photo-oxidation of cytochrome *f*, in accord with the hypothesis that DCIP donates electrons before a coupling site on the intermediate pathway prior to cytochrome *f*, as shown in Fig. 8.

The acute effects of FCCP on the DCIP–ascorbate systems are in strong contrast to those found for the TMPD–ascorbate *plus* DCMU system (Fig. 3C) where the only effect was a decrease in the small and transient photo-oxidation of cytochrome *f*. The further addition of diquat to the latter system was found to have no effect, the responses being identical to those shown in Fig. 3C. Such a lack of effect would be expected (a) if TMPD–ascorbate donated electrons to a site after a coupling site and (b) if, in the presence of FCCP, electron flow were not inhibited. In intact chloroplasts, as mentioned previously, FCCP normally inhibits electron flow through cytochrome *f* (A. W. D. Larkum and W. D. Bonner, unpublished). However, the action of TMPD in stimulating a pseudocyclic electron transport[4,19] can account for a sustained rate of electron flow in the absence of diquat and would further account for the similarity

between the response of the positive component between 566–575 nm in the DCIP–ascorbate–DCMU–diquat treatment (Fig. 6B) and the TMPD–ascorbate–DCMU treatment (Fig. 3B).

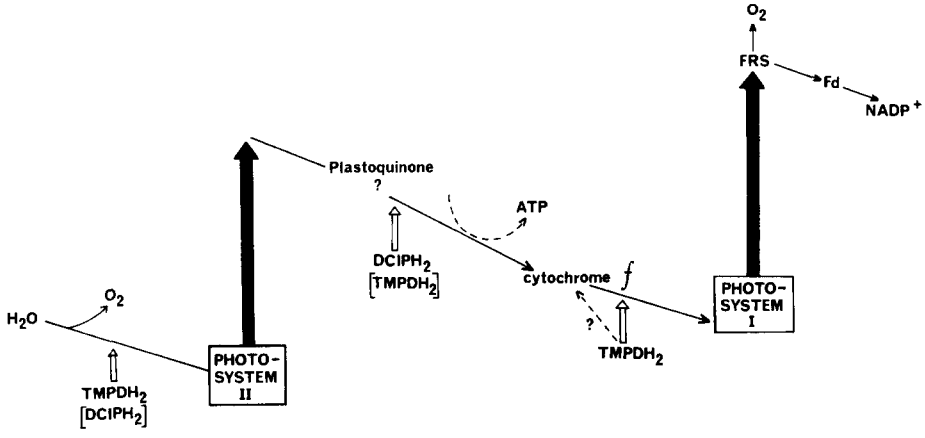

Fig. 8. Proposed scheme for the donation of electrons to the photosynthetic electron transport pathway by the couples, DCIP–ascorbate and TMPD–ascorbate. Square brackets indicate electron donation at high concentrations (above 1 mM DCIP or TMPD). FRS: ferredoxin reducing substance; Fd: ferredoxin.

DISCUSSION

The present results provide evidence for the interaction of the donor couples, DCIP–ascorbate and TMPD–ascorbate, on either side of a coupling site which lies before cytochrome *f* on the intermediate electron transport pathway, as shown in Fig. 8. Schwartz[4] showed that at high concentrations of TMPD coupled phosphorylation does occur so it is probable, as suggested by Schwartz, that at high concentrations TMPD interacts at a similar site to DCIP. The scheme shown in Fig. 8 provides the simplest explanation for the effects of the donor couples and FCCP on the photo-oxidation of cytochrome *f* and it is not at all clear how the present results could be interpreted in terms of a simultaneous action of DCIP as an electron donor and as a co-factor for cyclic photophosphorylation[8,9]. Previous work on cytochrome *f* photo-oxidation and P518 responses in various types of chloroplasts is also in agreement with the present hypothesis[12,13].

There is, however, an obvious difference of interpretation between this work and that of Avron and Chance[10]. The latter authors concluded that the DCIP–ascorbate couple donated electrons to Photosystem I independently of cytochrome *f*. This conclusion was based on the evidence that in swollen chloroplasts in the presence of DCIP–ascorbate, $NADP^+$ caused photo-oxidation of cytochrome *f* only in the absence of DCMU. A somewhat similar effect has been shown in the present work in the FCCP-treated DCIP system where steady-state photo-oxidation of cytochrome *f* appears to be much greater in the absence of DCMU (Fig. 7). However, the precise involvement of cytochrome *f* in these "uncoupled" systems was obscured by the presence of the spectral component at 550 nm. Clearly, this is an area for further investigation but it would be unreasonable to dismiss the involvement of cytochrome *f*

in the DCIP–ascorbate system on these grounds, since the evidence for the involvement of cytochrome *f* in the "coupled" chloroplasts is so strong, even in the presence of DCMU.

One of the most interesting findings of the present work was the extensive but slow absorbance increase centred broadly in the region 562 and 575 nm, which was found in the presence of the electron donor couples under many circumstances. Since this occurred with the TMPD system and the DCIP system in the presence of FCCP, the results suggest that the rate of electron transport is an important factor in the slow absorbance response. This is supported by the work of Izawa *et al.*[17] who found that similar electron donor and acceptor systems, under conditions which have been shown here to give rise to the slow absorbance change, produced remarkably high rates of electron flow. From such a possible correlation and bearing in mind its slow nature, the response might seem to arise either by a swelling effect or by an electrochromic effect[13], due, perhaps, to a net movement of ions. The reversible nature of the response makes the electrochromic effect more likely than swelling. Similar slow absorbance changes, but of opposite sign, have been observed in the 518-nm region under identical conditions[13]. It is therefore possible that the response is due to an electrochromic effect of P-518 itself and indicates charge transfer reactions across the coupling membrane in the opposite direction to normal.

A further development of such an hypothesis would be to link the P-518 and P-572 responses, since although of opposite sign, they react in a very similar way to many inhibitors and uncouplers. It is well known that the light-induced carotenoid absorbance change in photosynthetic bacteria, at about 525 nm, has associated positive and negative peaks in other regions of the spectrum[20,21]. Similar shifts in algae and higher plants have been ascribed to carotenoids but the spectra so far presented to not extend to the 570 nm region[22] (spectra for swollen chloroplasts[23] do not show any peak in this region but neither is the P-572 response found in such chloroplasts, A. W. D. Larkum and W. D. Bonner, unpublished).

Finally, some comment is necessary on the contribution of other spectral components. Except for the treatment of FCCP *plus* diquat there has been no clear evidence for the involvement of *b*-type cytochromes. Only in the TMPD–ascorbate–DCMU treatment was any other spectral peak found in the cytochrome *b* region, in this instance, at 562 nm. The absorption band was very broad and could therefore have components of both the 566–572-nm band and a *b*-type cytochrome. Since cytochrome *b*-559 would be in the reduced state under these conditions and the contribution from cytochrome b_6 is normally so small (ref. 24 and A. W. D. Larkum and W. D. Bonner, unpublished), it seems more likely that the 566–572-nm band was shifted. This might account for the poor definition of the light-dark difference spectra with TMPD.

The peak at 550 nm which was most marked in the presence of diquat, could be due to a derivative of cytochrome *f*, resulting perhaps from reactions involving hydrogen peroxide[25]. A number of cytochromes with α-bands between 548 and 555 nm have been isolated from green cells[26], some of the *c*-type, some of the *f*-type and others which may be derivatives of an *f*-type[27]. However, it is tempting to draw a comparison between this 550 nm component and that discovered by Knaff and Arnon[28] which is photo-oxidised and whose identity is unknown.

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