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PHOTOOXIDATION OF CYTOCHROME *b*-559 IN LEAVES AND CHLOROPLASTS AT ROOM TEMPERATURE

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SUMMARY

1. Light-induced absorbance changes of cytochrome *b*-559 and cytochrome *f* in the α-band region were examined in leaves and in isolated chloroplasts.

2. Absorbance changes of cytochrome *b*-559 were not detected in untreated leaves or in most preparations of isolated chloroplasts. After treatment of leaves or chloroplasts with carbonyl cyanide *m*-chlorophenylhydrazone, high rates of photooxidation of cytochrome *b*-559 were obtained, both in far-red (>700 nm) and red actinic light. Cytochrome *f* was photooxidized in far-red light, but in red light it remained mainly in the reduced state. The initial rates of photooxidation of cytochrome *b*-559 in leaves or chloroplasts treated with carbonyl cyanide *m*-chlorophenylhydrazone were considerably decreased by 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea.

3. A slow photoreduction of cytochrome *b*-559 was observed in aged mutant pea chloroplasts in red light.

4. The results do not support the view that cytochrome *b*-559 is a component of the electron transport chain between the light reactions. It is suggested that cytochrome *b*-559 is located on a side path from Photosystem II, but with a possible additional link to Photosystem I.

INTRODUCTION

Chloroplasts of green plants and algae contain two *b*-type cytochromes, characterized by α-bands at 559 nm (cytochrome *b*-559) and 563 nm (cytochrome b_6) for the reduced pigments[1]. There is much uncertainty, however, regarding the role of the *b*-cytochromes in photosynthetic electron transport. Difficulties in the interpretation of light-induced absorbance changes have been due in some instances to confusion between cytochrome b_6 and cytochrome *b*-559. For example, the absorbance changes attributed to cytochrome b_6 in Euglena[2] were probably caused by cytochrome *b*-559. LEVINE AND GORMAN[3] investigated light-induced absorbance changes of cytochrome *b*-559 as well as cytochrome *c*-553 (cytochrome *f*) in chloroplast fragments of the wild type and mutant strains of *Chlamydomonas reinhardi*. Their results were explained by

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; TCIP, 2,3′,6-trichlorophenolindophenol.

the series formulation of photosynthetic electron transport[1] with both cytochromes as components of the pathway between the light reactions, and with cytochrome *b*-559 closer to Photosystem II. A slow photoreduction of cytochrome *b*-559 was observed in spinach chloroplasts by CRAMER AND BUTLER[4] in both red and far-red light. Since cytochrome *f* was oxidized by far-red light, it was concluded that cytochrome *b*-559 was nearer to Photosystem II than was cytochrome *f*, and that electron flow between cytochrome *b*-559 and cytochrome *f* was rate-limiting. Similar observations were made by BEN HAYYIM AND AVRON[5] for lettuce chloroplasts.

In contrast, HIND[6] and KNAFF AND ARNON[7] failed to observe light-induced redox changes in cytochrome *b*-559 in untreated spinach chloroplasts. HIND[6] reported, however, a photooxidation of cytochrome *b*-559 by Photosystem I light in chloroplasts in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). He suggested that CCCP, by acting as an uncoupler was increasing the rate of electron flow between cytochrome *b*-559 and Photosystem I. KNAFF AND ARNON[7] found that cytochrome *b*-559 was photooxidized at room temperature in spinach chloroplasts after treatment of the chloroplasts with Tris buffer, which inhibits electron flow between water and Photosystem II.

It seems likely that these differences in the redox behaviour of cytochrome *b*-559 on illumination of isolated chloroplasts are due largely to the condition of the chloroplasts. In an attempt to resolve the role of cytochrome *b*-559 in photosynthesis, we have examined the light-induced absorbance changes of cytochromes in leaves. Some work with isolated chloroplasts is also reported for comparison with the leaf studies. A chlorophyll-deficient pea mutant[8] was used in the leaf studies, but essentially the same observations were made with normal pea and with spinach.

MATERIALS AND METHODS

Spinach plants (*Spinacea oleracea* L.) were grown in nutrient solutions as described previously[9]. Pea plants (*Pisum sativum*, L. var. Greenfeast) were grown in a controlled temperature glasshouse of the C.S.I.R.O. phytotron. The mutant pea was derived from the commercial variety as a spontaneous mutation[8]. Total chlorophyll of the mutant leaves was about one-half that of normal pea leaves per mg dry weight and the ratio of chlorophyll *a*/chlorophyll *b* was 10–14. The average chlorophyll content of mutant leaves was 17.4 $\mu g/cm^2$.

Light-induced absorbance changes were measured with an Aminco-Chance dual-wavelength difference spectrophotometer (American Instrument Corporation, Silver Springs, Md. U.S.A.) fitted with a side-illumination attachment. Actinic light was provided by a 650 W tungsten iodine lamp, and passed through a 3 cm layer of water and interference filters (half band width 7–10 nm). Corning filters 2-64 and 4-77 were used in conjunction with the interference filter at 714 nm, and 2-64 and 7-59 with the 732 nm filter. Light intensities at the position of the sample were measured with a YSI-Kettering Model 65 Radiometer (Yellow Springs Instrument Company, Ohio, U.S.A.). The photomultiplier tube was protected by a Corning glass filter 4-96.

Rates of photooxidation of cytochrome *f* and cytochrome *b*-559 were calculated from the initial rates of absorbance decrease at 554 nm and 559 nm respectively, with 570 nm as reference wavelength. The absorbance of cytochrome *f* at 559 nm *vs.* 570 nm

was taken as one-third of its absorbance at 554 *vs.*570 nm, and the absorbance of cytochrome *b*-559 at 554 nm *vs.* 570 nm was taken as one-half of its absorbance at 559 nm *vs.* 570 nm. Molar extinction coefficients of $2.5 \cdot 10^4$ for cytochrome *f* and $2.0 \cdot 10^4$ for cytochrome *b*-559 were used.

A half leaf or piece of leaf, approximately 1 cm × 3 cm, was mounted between two pieces of thin perspex at an angle of 45° to the actinic light. The angle between the measuring beam and the leaf also was 45°. Treatment of the leaves with CCCP or 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) was carried out by gently pricking the surface of a leaf with a needle, prior to soaking the leaf in a 10 % ethanolic solution of 10^{-4} M CCCP or 10^{-5} M DCMU for 10–15 min. Leaves treated with 10 % ethanol resembled control leaves in their light-induced absorbance changes, except that the post-illumination reduction rate of cytochrome *f* was markedly increased.

Chloroplasts were isolated from spinach leaves or mutant pea leaves in 0.05 M phosphate buffer (pH 7.2) containing 0.3 M sucrose and 0.01 M KCl, as described previously[9]. For light-induced absorbance measurements, chloroplasts were suspended in the isolation medium in a cuvette with a 1-cm optical path. The chlorophyll concentration was 75 μg/ml for spinach chloroplasts and 50 μg/ml for mutant pea chloroplasts.

CCCP and DCMU were donated by Dr. P. G. Heytler and Dr. C. W. Todd of Du Pont and Co.

RESULTS

Light-induced absorbance changes in mutant leaves

The steady-state difference spectrum obtained on illumination of a mutant pea leaf with 703 nm actinic light is shown in Fig. 1. A minimum is observed at 554 nm, but there is no evidence for a shoulder at 559 nm. This means that cytochrome *f*, but not cytochrome *b*-559 is photooxidized when a leaf is illuminated with far-red light which is absorbed predominantly by Photosystem I. The small increase in absorbance at 563 nm is attributed to a slight degree of photoreduction of cytochrome b_6. Illumination of a leaf with 675 nm light also gave some photooxidation of cytochrome *f*, but the extent of the absorbance change was small compared to that observed in 703 nm light (Fig. 1). Treatment of a leaf with DCMU increased considerably the steady-state level of oxidation of cytochrome *f* in 675 nm light (Fig. 2). These results for cytochrome *f* are in agreement with previous studies which indicate that 675 nm light activates both photosystems and that DCMU inhibits electron flow between the two photoreactions. In none of the above experiments did we observe an absorbance change which could be attributed either to the photooxidation or photoreduction of cytochrome *b*-559.

If a mutant pea leaf was treated with CCCP prior to illumination with 703 nm actinic light, the light *minus* dark difference spectrum shown in Fig. 3 was obtained. The minimum is at 554 nm, but there is also a distinct shoulder at 559 nm, showing that under these conditions there is some photooxidation of cytochrome *b*-559. Illumination of the CCCP-treated leaf with 667 nm actinic light gave the difference spectrum shown in Fig. 4. The minimum now is at 559 nm with a shoulder at 554 nm, suggesting that under these conditons, the steady-state level of cytochrome *b*-559 is more oxidized than that of cytochrome *f*.

Initial rates of photooxidation of cytochrome *f* and cytochrome *b*-559 for control

mutant pea leaves and for CCCP-treated leaves are shown in Table I. For a control leaf, the rate of photooxidation of cytochrome *f* in 703 nm actinic light was 6-fold higher than the rate in 667 nm actinic light. The rate of photooxidation of cytochrome *b*-559 was negligible in either light. After treatment of the leaf with CCCP, high rates

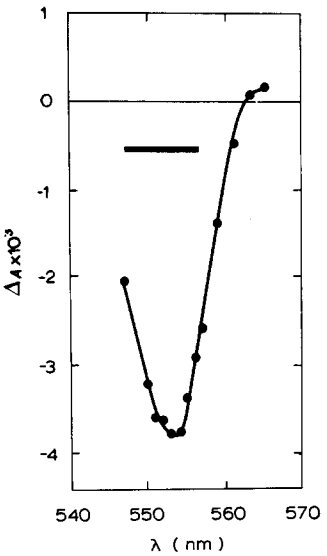
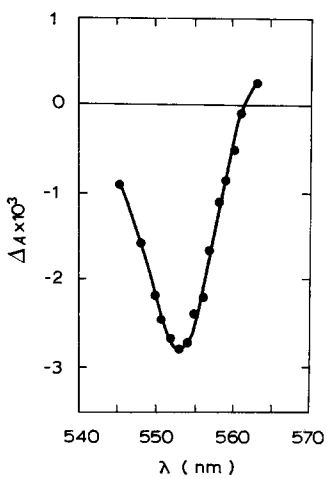


Fig. 1. Light *minus* dark difference spectrum obtained on illumination of a mutant pea leaf with 703 nm actinic light of intensity $1.5 \cdot 10^4$ erg·cm^{-2}·sec^{-1}. Reference wavelength, 570 nm. The bar indicates the extent of the absorbance change at 554 nm produced by 675 nm actinic light.



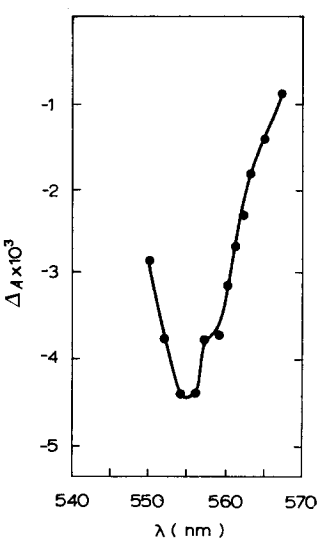


Fig. 2. Light *minus* dark difference spectrum of a mutant pea leaf following treatment with 10^{-5} M DCMU. Reference wavelength, 570 nm. Actinic light: 675 nm of intensity $1.5 \cdot 10^4$ erg·cm^{-2}·sec^{-1}.

Fig. 3. Light *minus* dark difference spectrum of a mutant pea leaf following treatment with 10^{-4} M CCCP. Actinic light: 703 nm of intensity $1.5 \cdot 10^4$ erg·cm^{-2}·sec^{-1}. Reference wavelength, 570 nm.

of photooxidation of cytochrome *b*-559 were obtained both in 703 nm light and in 667 nm light. The rates of photooxidation of cytochrome *f* were not altered in this experiment to any significant extent by treating the leaf with CCCP, although in other experiments the rates were sometimes lowered. Thus, in the presence of CCCP, cytochrome *b*-559 is rapidly oxidized by 667 nm light, whereas cytochrome *f* remains mainly reduced. Since 667 nm light is absorbed by both Photosystems, it is not clear whether the oxidation of cytochrome *b*-559 is driven by light absorbed by Photosystem I or by Photosystem II.

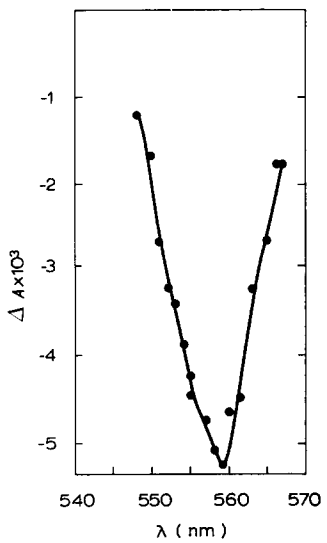


Fig. 4. Light *minus* dark difference spectrum of a mutant pea leaf, following treatment with 10^{-4} M CCCP. Actinic light: 667 nm of intensity $0.93 \cdot 10^4$ erg·cm^{-2}·sec^{-1}. Reference wavelength, 570 nm.

TABLE I

INITIAL RATES OF LIGHT-INDUCED CYTOCHROME OXIDATION IN MUTANT PEA LEAF

One-half of a mutant pea leaf was treated with 10^{-4} M CCCP for 15 min, the other half served as control. The intensities of actinic light were $1.5 \cdot 10^4$ erg·cm^{-2}·sec^{-1} for 703 nm and $0.93 \cdot 10^4$ erg·cm^{-2}·sec^{-1} for 667 mn.

Actinic light (nm)	*Cytochrome oxidation (nmoles·min^{-1}·cm^{-2}, leaf area)*			
	Control leaf		*CCCP-treated leaf*	
	Cyt f	*Cyt b-559*	*Cyt f*	*Cyt b-559*
667	0.7	0	0.3	7.1
703	4.0	0	4.0	4.4

In an attempt to answer this question, a CCCP-treated leaf was illuminated successively with actinic light at wavelengths of 667, 688, 714 and 732 nm. Sufficient time was allowed between illuminations to enable the leaf to return to its dark steady-state condition. Initial rates of oxidation of cytochrome *f* and cytochrome *b*-559 are shown in Table II. The rates of photooxidation of cytochrome *b*-559 in 732 nm, and

714 nm actinic light are higher than the corresponding rates of cytochrome *f*. The effectiveness of different wavelengths of actinic light suggests that the oxidation of cytochrome *b*-559 is driven by light absorbed by Photosystem I. It is difficult to decide whether cytochrome *b*-559 is also photooxidized by light absorbed by Photosystem II. The higher rate of oxidation of cytochrome *b*-559 in 667 nm actinic light compared with 688 nm light may simply reflect the higher absorbance of Photosystem I at 667 nm than at 688 nm. We conclude, however, that cytochrome *b*-559 is not photoreduced at a significant rate by light absorbed by Photosystem II, otherwise we could not explain the high rate of photooxidation of cytochrome *b*-559 in 667 nm actinic light. In contrast, the relatively low rate of oxidation of cytochrome *f* in 667 nm light reflects the well-known antagonism of light absorbed by Photosystems I and II with Photosystem I oxidizing cytochrome *f* and Photosystem II reducing it.

TABLE II

EFFECT OF WAVELENGTH OF ACTINIC LIGHT ON INITIAL RATES OF CYTOCHROME OXIDATION IN CCCP-TREATED LEAF

The mutant pea leaf was treated with 10^{-4} M CCCP for 10 min.

Leaf No.	*Actinic light (nm)*	*Intensity ($erg \cdot cm^{-2} \cdot sec^{-1} \times 10^{-3}$)*	*Cytochrome oxidation ($nmoles \cdot min^{-1} \cdot cm^{-2}$, leaf area)*	
			Cyt f	*Cyt b-559*
1	667	2.1	0.8	7.8
	688	2.1	2.0	5.9
	714	2.1	1.4	1.4
	732	6.0	0.6	1.1
	667	0.9	0.3	4.3
	688	0.9	1.7	2.9
2	667	2.1	0.5	7.7
	688	2.1	5.0	4.8
	714	2.1	1.3	2.0
	732	6.0	0.7	1.0
	688	0.4	0.6	1.0
	688	0.9	2.5	2.5

TABLE III

EXTENTS OF LIGHT-INDUCED ABSORBANCE CHANGES

A mutant pea leaf was treated with 10^{-4} M CCCP for 15 min. The extents of the light-induced absorbance changes were measured at 554 nm and 559 nm. Reference wavelength was 570 nm. Amounts of oxidized cytochrome *f* and cytochrome *b*-559 at the steady-state were calculated as described in METHODS. Intensities of actinic light, $3.0 \cdot 10^3$ $erg \cdot cm^{-2} \cdot sec.^{-1}$.

Actinic light (nm)	*Absorbance change $\times 10^3$*		*Amount of oxidized cytochrome ($nmole \cdot cm^{-2}$, leaf area)*	
	554 nm	*559 nm*	*Cyt f*	*Cyt b-559*
667	2.85	4.1	0.039	0.19
675	2.9	3.75	0.050	0.17
688	4.2	4.1	0.074	0.16
700	4.9	4.2	0.14	0.15
714	2.75	2.75	0.066	0.11

Illumination of a CCCP-treated leaf with high intensity red light (Corning filter 2-64, $\lambda > 660$ nm, intensity 10^6 erg·cm^{-2}·sec^{-1}, causes photooxidation both of cytochrome *f* and cytochrome *b*-559. On turning off the light, cytochrome *f* is rapidly reduced, but the reduction of cytochrome *b*-559 is considerably slower. In the experiments with low intensity monochromatic light reported in Table II, the decay rates of cytochrome *f* were always faster (about 5 ×) than those of cytochrome *b*-559.

The extents of the light-induced absorbance changes at 554 and 559 nm in the steady state are shown in Table III, together with the calculated amounts of oxidized cytochrome *f* and cytochrome *b*-559. The amount of cytochrome oxidized by 700 nm actinic light accounts for all of the cytochrome *f* and about half of the cytochrome *b*-559 in the mutant leaf. We estimate that in 667 nm actinic light at least two-thirds, and in some experiments 90 %, of the cytochrome *b*-559 in the leaf was photooxidized, while about three-quarters of the cytochrome *f* remained in the reduced state. The extents of the absorbance changes support our conclusion from the rate measurements that, in the presence of CCCP, cytochrome *b*-559 of the mutant is photooxidized in 667 nm light and cytochrome *f* remains largely reduced.

Table IV reports the effect of DCMU on the initial rates of cytochrome oxidation in a CCCP-treated leaf. DCMU has little effect on the rate of oxidation of cytochrome *f* by 700 nm actinic light, but it causes a high rate of oxidation in 675 nm light. (A similar effect is observed when control leaves are treated with DCMU.) Rates of oxidation of cytochrome *b*-559 are decreased considerably by DCMU.

If a mutant pea leaf is treated first with DCMU and then with CCCP, some photooxidation of cytochrome *b*-559 is observed both in 667 nm and 703 nm actinic light.

TABLE IV

INITIAL RATES OF LIGHT-INDUCED CYTOCHROME OXIDATION IN MUTANT PEA LEAF TREATED WITH CCCP AND DCMU

One half of a mutant pea leaf was treated with 10^{-4} M CCCP for 15 min, the other half was treated with 10^{-4} M CCCP followed by 10^{-5} M DCMU. Intensities of actinic light: $1.5 \cdot 10^4$ erg·cm^{-2}·sec^{-1} at 703 nm, $1.2 \cdot 10^4$ erg·cm^{-2}·sec^{-1} at 700 nm, $0.9 \cdot 10^4$ erg·cm^{-2}·sec^{-1} at 688 nm, $1.0 \cdot 10^4$ erg·cm^{-2}·sec^{-1} at 675 nm and $0.9 \cdot 10^4$ erg·cm^{-2}·sec^{-1} at 667 nm.

Leaf No.	*Actinic light (nm)*	*Cytochrome oxidation (nmoles·min^{-1}·cm^{-2}, leaf area)*					
		CCCP alone		*CCCP + DCMU (after 15 min)*		*CCCP + DCMU (after 30 min)*	
		Cyt f	*Cyt b-559*	*Cyt f*	*Cyt b-559*	*Cyt f*	*Cyt b-559*
1	675	0	3.5	4.0	0.7	–	–
	700	5.2	3.0	5.9	0.5	–	–
2	688	2.3	4.9	4.6	2.5	3.9	0.9
	703	5.4	2.9	5.2	2.0	4.4	0

Absorbance changes in heat-treated leaf

Fig. 5 shows the light *minus* dark difference spectrum obtained on illumination of a mutant pea leaf after heat treatment for 2 min. It is apparent from the shape of the curve that cytochrome *b*-559 as well as cytochrome *f* is photooxidized by 675 nm actinic light. The period of heating was found to be critical and it was difficult

to always reproduce the spectrum shown in Fig. 5 with different leaves. With too short a heat treatment the difference spectrum was similar to a control leaf, and too long a treatment abolished the absorbance changes due to both cytochromes.

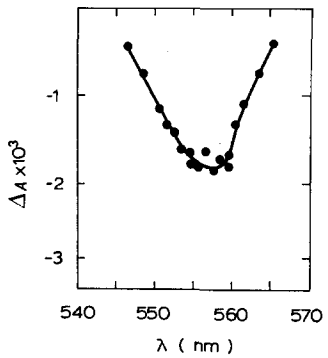


Fig. 5. Light *minus* dark difference spectrum obtained on illumination of a heat-treated mutant pea leaf with 675 nm actinic light of intensity $1.5 \cdot 10^4$ erg·cm^{-2}·sec^{-1}. Reference wavelength, 570 nm. The leaf was heated for 2 min. 47°.

Light-induced absorbance changes with spinach chloroplasts

Spinach leaves gave similar results to those reported for the pea mutant leaf, except that the extents of the absorbance changes were somewhat smaller. For example, the light-induced oxidation of cytochrome *b*-559 could not be detected in spinach leaves in either 667 nm or 703 nm actinic light, but after treatment of a leaf with 10^{-4} M CCCP for 15 min cytochrome *b*-559 was oxidized at a rate of 2.6 nmoles·min^{-1}·cm^{-2} (leaf area) in 667 nm light ($0.93 \cdot 10^4$ erg·cm^{-2}·sec^{-1}) and 2.2 nmoles min^{-1}·cm^{-2} (leaf area) in 703 nm light ($1.5 \cdot 10^4$ erg·cm^{-2}·sec^{-1}).

For comparison with our leaf studies, we also investigated light-induced absorbance changes in spinach chloroplasts. Contrary to the reports of CRAMER AND BUTLER[4] and BEN HAYYIM AND AVRON[5] but consistent with the observations of HIND[6] and KNAFF AND ARNON[7] we did not normally observe light-induced absorbance changes due to cytochrome *b*-559 in untreated spinach chloroplasts (Table V). Photo-

TABLE V

LIGHT-INDUCED OXIDATION OF CYTOCHROMES IN ISOLATED SPINACH CHLOROPLASTS

Chloroplasts were suspended in the isolation medium. Chlorophyll concentration, 75 μg/ml; CCCP, 10^{-5} M. Intensities of actinic light, $3.0 \cdot 10^3$ erg·cm^{-2}·sec^{-1}.

Actinic light (nm)	*Cytochrome oxidation*							
	Initial rate (nmoles·min^{-1}·cm^{-3})				*Extent (nmoles·cm^{-3})*			
	No addition		*+ CCCP*		*No addition*		*+ CCCP*	
	Cyt f	*Cyt b-559*	*Cyt f*	*Cyt b-559*	*Cyt f*	*Cyt b-559*	*Cyt f*	*Cyt b-559*
655	0	0	1.6	7.4	0	0	0.054	0.20
688	3.3	0	4.2	7.1	0.082	0	0.11	0.25
714	3.6	0	3.6	1.0	0.12	0	0.12	0.19
732	1.6	0	2.1	0.3	0.15	0	0.15	0.11

oxidation of cytochrome *f* occurred in 688, 714 and 732 nm actinic light, but not in 655 nm light. In the presence of 10^{-5} M CCCP, high initial rates of photooxidation of cytochrome *b*-559 were observed in 655 nm and 688 nm light, with lower rates in 714 nm and 732 nm light. In contrast, the initial rate of photooxidation of cytochrome *f* was much lower in 655 nm actinic light than in 688 nm or 714 nm light. The rate of oxidation of cytochrome *f* was 7-fold higher than that of cytochrome *b*-559 in 732 nm actinic light and about 4-fold higher in 714 nm light. This is in contrast to the rates shown in Table II for the mutant pea leaf where the initial rates of oxidation of cytochrome *b*-559 in Photosystem I light were slightly higher than the corresponding rates for cytochrome *f*. However, the variation in the initial rate of photooxidation of cytochrome *b*-559 with wavelength of actinic light was similar to that observed for the mutant pea leaf. The conclusion that the oxidation of cytochrome *b*-559 in the leaf is driven by Photosystem I and possibly also by Photosystem II seems to apply also to the isolated spinach chloroplasts. The extent of the photooxidation of cytochrome *b*-559 in 732 nm light is about 50 % of that observed in 655 nm light, and in 714 nm light, the extent is only slightly less than the value in 655 nm light. The amount of cytochrome oxidized in 655 nm light in the presence of CCCP accounts for about half of the cytochrome *b*-559 of the chloroplast and about one-fourth of the cytochrome *f*. About three-fourths of the cytochrome *f* is photooxidized in 732 nm light, either in the presence or absence of CCCP. In an occasional preparation of spinach chloroplasts, a slow photooxidation of cytochrome *b*-559 was observed in far-red light in the absence of CCCP, but the extent of the photooxidation did not account for more than 20 % of the cytochrome *b*-559 of the chloroplast.

Addition of 10^{-5} M DCMU to spinach chloroplasts caused photooxidation of cytochrome *f* in 655 nm light as well as in far-red light, but there were still no absorbance changes which we could attribute to cytochrome *b*-559 (Table VI). On treatment of chloroplasts with CCCP + DCMU, the effectiveness of actinic light for the oxidation of cytochrome *f* was similar to the wavelength effect in DCMU alone (Table VII). The initial rates of oxidation of cytochrome *b*-559 were lower for choroplasts treated with CCCP + DCMU, than for chloroplasts in CCCP, particularly in 655 nm and 688 nm actinic light (*cf.* Table V). But in spite of these lower rates of oxidation, the extents of cytochrome *b*-559 oxidation were not diminished to a significant extent by the addition of DCMU to the CCCP-treated chloroplasts.

TABLE VI

LIGHT-INDUCED OXIDATION OF CYTOCHROMES IN SPINACH CHLOROPLASTS TREATED WITH DCMU

Chloroplasts were suspended in isolation medium. Chlorophyll concentration, 75 μg/ml; DCMU, 10^{-5} M. Intensities of actinic light, $3.0 \cdot 10^3$ erg·cm^{-2}·sec^{-1}.

Actinic Light (nm)	*Cytochrome oxidation*			
	Initial rate (nmoles·min^{-1}·cm^{-3})		*Extent (nmole·cm^{-3})*	
	Cyt f	*Cyt b-559*	*Cyt f*	*Cyt b-559*
655	4.3	0	0.14	0
663	4.2	0	0.11	0
688	4.9	0	0.10	0
714	3.4	0	0.12	0
732	2.3	0	0.13	0

TABLE VII

LIGHT-INDUCED OXIDATION OF CYTOCHROMES IN SPINACH CHLOROPLASTS TREATED WITH CCCP + DCMU

Chloroplasts were suspended in isolation medium. Chlorophyll concentration, 75 μg/ml; DCMU 10^{-5} M; CCCP, 10^{-5} M. Intensities of actinic light, $3.0 \cdot 10^3$ erg·cm^{-2}·sec^{-1}.

Actinic light (nm)	*Cytochrome oxidation*			
	Initial rate (nmoles·min^{-1}·cm^{-3})		*Extent (nmole·cm^{-3})*	
	Cyt f	*Cyt b-559*	*Cyt f*	*Cyt b-559*
655	2.3	1.4	0.13	0.22
688	2.3	1.4		
714	1.1	0.4	0.16	0.13
732	0.6	0.2		

Photoreduction of cytochrome b-559 in aged mutant pea chloroplasts

Light-induced absorbance changes observed with freshly prepared mutant pea chloroplasts were similar to those obtained with spinach chloroplasts. Cytochrome *f* was photooxidized in Photosytem I light, but absorbance changes of cytochrome *b*-559 were absent unless CCCP was added to the chloroplasts, and then a photooxidation was observed.

If the mutant pea chloroplasts were aged at 0° for a day a light-induced increase in absorbance at 559 nm occurred in 650 nm actinic light (Fig. 6). On turning off the actinic light, the decrease in absorbance was very slow, and it was increased somewhat with 714 nm or 732 nm light. A light *minus* dark action spectrum for the absorbance increase in 650 nm actinic light shows a peak at 559 nm, indicative of a photoreduction of cytochrome *b*-559 (Fig. 7).

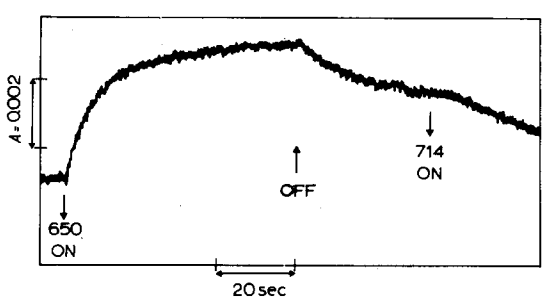


Fig. 6. Light-induced absorbance changes at 559 nm in mutant pea chloroplasts after ageing for 1 day at 0°. Reference wavelength, 570 nm. Intensities of actinic light: $0.95 \cdot 10^4$ erg·cm^{-2}·sec^{-1} at 650 nm; $0.62 \cdot 10^4$ erg·cm^{-2}·sec^{-1} at 714 nm. Chlorophyll concentration, 50 μg/ml.

DISCUSSION

In the present work, light-induced absorbance changes of cytochrome *b*-559 were not detected in untreated leaves or in most preparations of isolated chloroplasts. In our preparations of spinach chloroplasts or freshly prepared mutant pea chloro-

plasts, cytochrome *b*-559 was at least 95 % reduced. Ascorbate reduced *minus* ferricyanide oxidized difference spectra closely resembled corresponding untreated *minus* ferricyanide oxidized difference spectra. This is in agreement with our previous observations with spinach chloroplasts[10]. Since cytochrome *b*-559 is already reduced in our isolated chloroplasts, we could not expect to observe a photoreduction of cytochrome *b*-559 in 640 nm actinic light as reported by BEN HAYYIM AND AVRON[5] for lettuce chloroplasts.

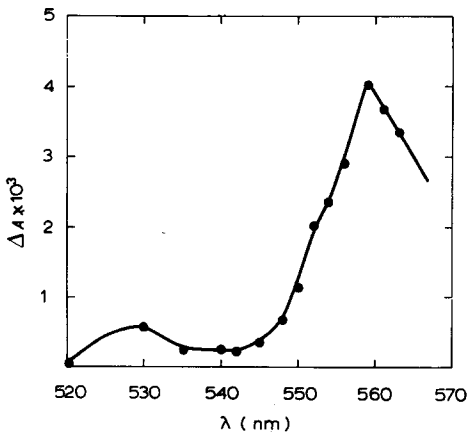


Fig. 7. Light *minus* dark difference spectrum of mutant pea chloroplasts, after ageing for 1 day at 0°. Reference wavelength, 570 nm. Actinic light of wavelength 650 nm and intensity $0.95 \cdot 10^4$ erg·cm^{-2}·sec^{-1}. Chlorophyll concentration, 50 μg/ml.

If cytochrome *b*-559 is a component of the electron transport chain between the light reactions, it should be at least partly photooxidized in untreated chloroplasts by far-red light (Photosystem I). It might be argued that the failure to observe photooxidation of cytochrome *b*-559 in far-red light is due to it being kept reduced by Photosystem II even in actinic light absorbed predominantly by Photosystem I. This seems unlikely in view of the antagonistic effect of red versus far-red light on the fluorescence yield of chloroplasts[11]. The level of fluorescence is thought to be determined by the redox state of a quencher (*E*) in close proximity to the reaction-centre chlorophyll of Photosystem II (ref. 11). *E* is more oxidized in far-red light than in red light, and therefore we would expect that all electron carriers between *E* and Photosystem I would be more oxidized in far-red light than in red light.

We suggest, therefore, that cytochrome *b*-559 is not a component of the electron transport chain between the light reactions. In view of its redox potential[12–15], it is highly unlikely that it is a component of the pathway between water and Photosystem II.

Previously[16], we suggested that cytochrome *b*-559 is on a sidepath from the oxidizing side of Photosystem II in order to explain the photooxidation of cytochrome *b*-559 at liquid nitrogen temperatures by Photosystem II (refs. 7, 16, 20). KNAFF AND ARNON[7] also observed a photooxidation of cytochrome *b*-559 by Photosystem II in chloroplasts at room temperature when electron flow from water was inhibited by treatment of the chloroplasts with Tris buffer[17]. Heat treatment is also considered to inhibit water oxidation which may explain the photooxidation of cytochrome *b*-559

in the heat-treated pea leaf. In other words, we are suggesting that cytochrome *b*-559 is able to donate an electron either to the excited reaction centre chlorophyll of Photosystem II or to the primary oxidant formed in Photosystem II, provided normal electron flow from water is largely inhibited (Fig. 8).

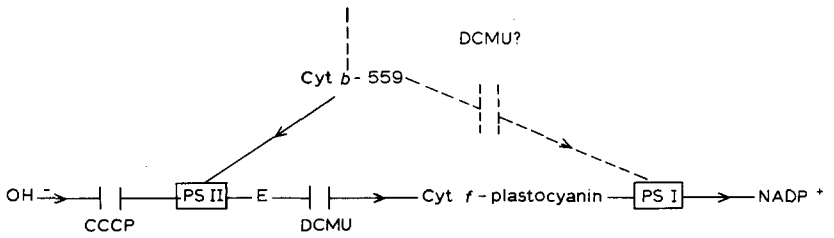


Fig. 8. Scheme for photosynthetic electron transport, showing possible location of cytochrome *b*-559. The pathway indicated by the broken line is included to explain the photooxidation of cytochrome *b*-559 by far-red light in the presence of CCCP. PS I and PS II, Photosystem I and II, respectively.

It has been reported that CCCP, as well as being an uncoupler of photophosphorylation inhibits electron flow from water to Photosystem II (ref. 18). We confirmed the inhibitory effect of CCCP on electron transport by measuring the effect of CCCP on the Hill reaction of spinach chloroplasts under the same illumination conditions as used for the light-induced absorbance measurements. At $1 \cdot 10^{-5}$ M CCCP, photoreduction of 2,3',6-trichlorophenolindophenol (TCIP) with 675 nm actinic light was inhibited by 70 %.

In the present studies, it appears that the oxidation of cytochrome *b*-559 in leaves or chloroplasts after treatment with CCCP is driven either by Photosystem I or by Photosystem I + Photosystem II. As discussed above, the photooxidation of cytochrome *b*-559 by Photosystem II may be due to the inhibitory effect of CCCP on electron flow between water and Photosystem II. To explain the photooxidation of cytochrome *b*-559 in far-red light, we have included a link between cytochrome *b*-559 and Photosystem I (Fig. 8). The photooxidation of cytochrome *b*-559 by Photosystem I in the presence of CCCP may be related to the uncoupling of photophosphorylation by CCCP.

Several workers[2,4,6] have reported a photooxidation of cytochrome *b*-559 in CCCP-treated chloroplasts, driven by Photosystem I. But in contrast to our observations with pea leaves and spinach chloroplasts, these workers[2,4,6] observed a slow photoreduction of cytochrome *b*-559 in Photosystem II light. Aged mutant pea chloroplasts, but not freshly prepared ones, did show a slow photoreduction of cytochrome *b*-559 in Photosystem II light, and the dark decay (oxidation) was stimulated by far-red light. Similar observations have been reported for chloroplast fragments from *C. reinhardi*[3]. In those cases where a photoreduction of cytochrome *b*-559 is observed it seems possible that there is a change in the nature of cytochrome *b*-559 such that it is now able to act as a non-specific electron carrier between the light reactions. It may be relevant that two distinct oxidation–reduction potentials have been reported for cytochrome *b*-559 (refs. 12–15).

KNAFF AND ARNON[19] have recently proposed a scheme of three light reactions

of photosynthesis. In their scheme, Photosystem II consists of two "short wavelength" light reactions (IIb and IIa) operating in series and joined by an electron transport chain which includes cytochrome *b*-559 and plastocyanin. In untreated chloroplasts, cytochrome *b*-559 is thought to be kept reduced by electron flow from water via light reaction IIb. Inhibition of electron flow from water by Tris treatment permits photooxidation of cytochrome *b*-559 by light reaction IIa. KNAFF AND ARNON[19] consider that Photosystem I is in parallel with Photosystems IIa and IIb, and its role is confined to cyclic electron flow and cyclic phosphorylation. Cytochrome *f* and cytochrome b_6 are components of cyclic electron transport, and according to KNAFF AND ARNON[19] are not involved in non-cyclic electron flow from water to $NADP^+$. The well-known antagonistic effects of far-red and red light on the oxidation and reduction of cytochrome *f*[1] do not support the scheme of KNAFF AND ARNON[19]. It is also difficult to explain by their scheme the photooxidation of cytochrome *b*-559 in far-red light in CCCP-treated leaves or chloroplasts.

The published effects of DCMU on the light-induced absorbance changes of cytochrome *b*-559 are contradictory. In contrast to our own observations with pea leaves and spinach chloroplasts and those of KNAFF AND ARNON[19] with spinach chloroplasts, other workers[3–5] have reported that DCMU causes photooxidation of cytochrome *b*-559 in red light. The observations of BEN HAYYIM AND AVRON[5], however, appear to indicate a dual effect of DCMU. In red light, DCMU caused a more oxidized level of cytochrome *b*-559, but the rate of oxidation was lowered by increasing the DCMU concentration.

The extents of the light-induced absorbance changes reported for cytochrome *b*-559 in isolated chloroplasts[3–7] are highly variable and usually account for 20–50 % of the total cytochrome *b*-559 as determined from oxidized *minus* reduced difference spectra. In our experiments with CCCP-treated leaves, 60–90 % of the cytochrome *b*-559 was photooxidized in red light, compared with about 50 % in CCCP-treated chloroplasts.

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