Biochimica et Biophysica Acta, 396 (1975) 310–319

BBA 46955

LIGHT-INDUCED TURNOVER OF CHLOROPLAST CYTOCHROME *b*-559 IN THE PRESENCE OF *N*-METHYLPHENAZONIUM METHOSULPHATE

P. HORTON and W. A. CRAMER*

Department of Biological Sciences, Purdue University, West Lafayette, Ind. 47907 (U.S.A.)

(Received January 7th, 1975)

SUMMARY

In the presence of 0.1–5 μM *N*-methylphenazonium methosulphate approx. 50–70 % oxidation of cytochrome *b*-559 can be induced by far-red light. The oxidation is best observed with long wavelength far-red light (732 nm) of moderate intensities (approx. 10^4 ergs/cm^2 per s) and is reversed by subsequent illumination with red light. Concentrations of *N*-methylphenazonium methosulphate above 5 μM are inhibitory probably due to cyclic electron flow. The far-red oxidation is inhibited by low concentrations of the plastoquinone antagonist 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, while 3-(3,4-dichlorophenyl)-1,1-dimethylurea inhibits red light reduction and increases the amplitude of far-red oxidation. The effect of *N*-methylphenazonium methosulphate is mimicked by *N*-methyl-phenazonium ethosulphate, but not by pyocyanine or diaminodurene. Low concentrations (2–3 μM) of *N*-methylphenazonium methosulphate also stimulate a 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone-inhibitable red light reduction of cytochrome *f*.

INTRODUCTION

The role of chloroplast cytochrome *b*-559 in photosynthetic electron transport remains unclear mainly due to the inability to observe its turnover under physiological conditions. The assignment of this cytochrome as an electron carrier between the two photosystems was first implied by the observation of its oxidation by far-red light and its reduction by red light in fragments of the green alga *Chlamydomonas reinhardi* [1]. However, its location close to Photosystem II [2–4] with an approximate midpoint potential of +350 mV [5–8] argue against such a proposal. On the basis of this value for the midpoint potential and the Photosystem II-mediated photooxidation at 77 °K

PMS, *N*-methylphenazonium methosulphate; PES, *N*-methylphenazonium ethosulphate; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; FCCP, 4-trifluoromethoxyphenyl-hydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TMPD, *N,N,N',N'*-tetramethyl-phenylaminediamine.

* On leave of absence 1974–75 at Laboratory for Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Amsterdam-C., The Netherlands.

[4, 6, 8, 9] and in Tris-washed chloroplasts [4], it was suggested that it functions as an electron carrier around Photosystem II. At 77 °K and in Tris-washed chloroplasts the normal steps in water oxidation are blocked. Reversible far-red light-induced oxidation and red light reduction of cytochrome *b*-559 in higher plant chloroplasts in the presence of FCCP indicates that Photosystem I mediates the oxidative pathway and that the cytochrome functions in the main chain under these conditions [10–12]. The pathway of oxidation through Photosystem I must by-pass the plastoquinone pool if the midpoint potential of the cytochrome *b*-559 remains near 350 mV in the presence of actinic light and FCCP. However, under these conditions, the far-red oxidation is inhibited by low concentrations of the plastoquinone analogue DBMIB [12], which inhibits electron transport at the level of plastoquinone. This suggests the possibility that cytochrome *b*-559 undergoes a significant change in redox potential in the light which allows it to function near the redox level of plastoquinone. The mechanism of action of FCCP in these experiments is not understood, but does not relate to proton conduction [13]. The effects of FCCP on cytochrome *b*-559 are complicated by the fact that under somewhat different conditions, where higher FCCP concentrations, longer incubation times, or lower actinic light intensities are used, the photooxidation of cytochrome *b*-559 is preferentially mediated by Photosystem II [14, 15].

Other conditions for observing light-induced turnover of cytochrome *b*-559 are required before considering further the possibility of light-induced formation of a low potential component. In this communication we report that cytochrome *b*-559 can be observed to be oxidised by far-red light and reduced by red light in the presence of low concentrations of *N*-methylphenazonium methosulphate and *N*-methylphenazonium ethosulphate.

MATERIALS AND METHODS

Spinach chloroplasts were prepared as previously described [16]. Incubations were carried at out 22–23 °C in a reaction medium containing 25 mM Tricine–NaOH, pH 7.8, 5 mM K_2HPO_4, 5 mM NaCl, 2 mM $MgCl_2$ and 0.1 mM methyl viologen. Cytochrome oxidation and reduction were measured by dual wavelength spectrophotometry with a reference wavelength at 570 nm [17]. Room temperature absorption spectra were recorded continuously using one monochromator of the dual wavelength instrument and an on-line computer to correct for the system response as previously described [17]. Steps of 1/4 nm were made from 542 to 574 nm with a scanning time of 64 s. The band width was 1.4 or 3 nm. Difference spectra were computed from sums of several identical experiments. The monochromators were calibrated with the 546.1 nm Hg line from a Pen-ray quartz lamp (Ultra-violet products). Light intensities were adjusted using Balzers neutral density filters and were measured with a YSI-Kettering Radiometer. PMS was purchased from Calbiochem and PES from Sigma. DBMIB and diaminodurene were kind gifts from Professor A. Trebst.

RESULTS

Fig. 1A shows the absorbance changes at 560 nm recorded before and after the addition of 1 μM PMS. The presence of PMS enabled a large absorbance decrease to

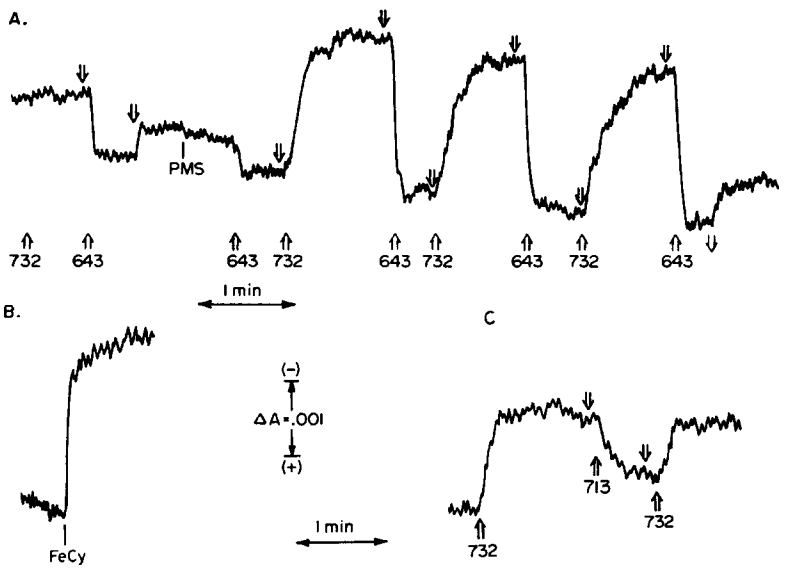

Fig. 1. Light-induced absorbance changes at 560 nm. (A) Before and after addition of 1 μM PMS, and (C) using different wavelengths of far-red light after addition of 1 μM PMS. (B) Oxidation by 0.5 mM ferricyanide. Reference, 570 nm. Chlorophyll concentration, 80 μg/ml. Light intensities, $6.3 \cdot 10^4$ ergs/cm^2 per s. Upward arrows indicate actinic light on; downward arrows, off.

be observed upon illumination with 732 nm light. Subsequent exposure to red light caused an increase in absorbance, part of which was reversed in darkness. Fig. 1B shows the total amplitude of oxidative change upon adding 0.5 mM ferricyanide and indicates that the far-red light-induced absorbance decrease in the presence of PMS represents approx. 70 % of the total change. This proportion is somewhat variable and depends on the wavelength and intensity used for the far-red illumination. Fig. 1C shows that the shorter wavelength of far-red illumination (713 nm) used in earlier experiments (e.g. ref. 11) gives smaller changes than obtained with 732 nm light. Fig. 2 shows the amplitude of the 560-nm change as a function of incident far-red light intensity. A maximum level is reached at about 10^4 ergs/cm^2 per s and at higher intensities the amplitude decreases. With low intensities of 732 nm light an oxidative change of small amplitude can often be observed without PMS (see Fig. 5A), whose origin is difficult to establish from difference spectra.

The spectra for the absorbance changes observed during a PMS experiment are shown in Fig. 3 and clearly show that cytochrome *b*-559 oxidation is predominantly responsible for the PMS-stimulated far-red change. Before PMS addition the spectrum indicates largely cytochrome *f* oxidation (Fig. 3B), but with PMS the maximum is shifted to 559 nm (Fig. 3C), the shift being due to the oxidation of a component with a maximum at 560 nm (Fig. 3D). No change in the amount of cytochrome oxidation occurs upon adding PMS without illumination (Fig. 3A). In this experiment low intensity light was used so that some cytochrome *b*-559 oxidation seems to have occurred in the absence of PMS so as to slightly red-shift the maximum away from the cytochrome *f* maximum (Fig. 3B). The optimum concentration for the PMS-induced increase in cytochrome *b*-559 photooxidation is 0.5–5 μM as shown in

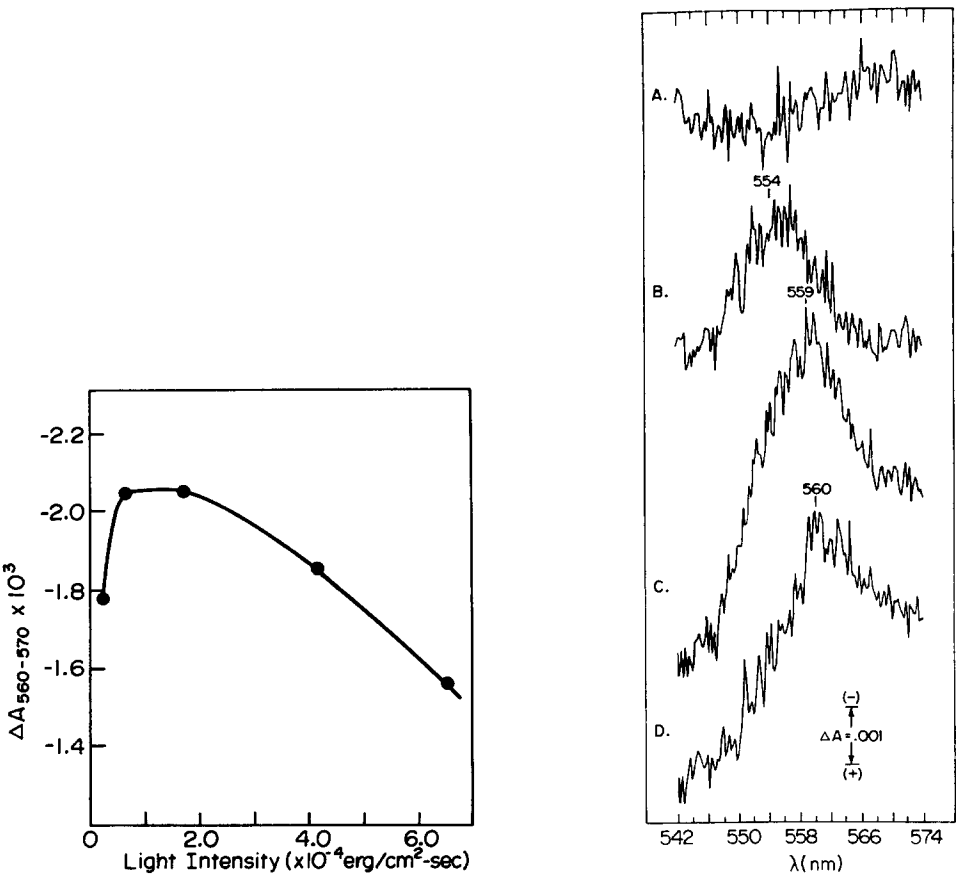

Fig. 2. Absorbance changes at 560 nm induced by different intensities of 732 nm light in the presence of 1 μM PMS. Reference, 570 nm. Chlorophyll, 85 μg/ml. Light intensities were adjusted using neutral density filters.

Fig. 3. Difference spectra for the absorbance changes in the presence of 1 μM PMS recorded at room temperature using single beam scanning. (A) Dark (+PMS) minus dark control. (B) Far-red minus dark. (C) Far-red minus dark in the presence of PMS. (D) C minus B. Far-red 732 nm, $1.3 \cdot 10^4$ ergs/cm^2 per s. Chlorophyll, 100 μg/ml. Spectra are the sums of six identical experiments.

Fig. 4. At concentrations above 5 μM, inhibition of the PMS effect on cytochrome *b*-559 oxidation occurs, so that at 20 μM the effect is only marginal. It should be noted that at higher PMS concentrations (5–20 μM) most of the stimulation of cytochrome *b*-559 oxidation occurs after a lag of 1–2 min in far-red light, and it is these final values that are shown in Fig. 4. The smaller amplitude of cytochrome *b*-559 oxidation occurring in the presence of higher concentrations of PMS (Fig. 4) is thought to be a consequence of reduction of the pool of acceptor for cytochrome *b*-559 by cyclic electron flow occurring at higher PMS concentrations. DBMIB at low concentrations inhibits Photosystem I-mediated photooxidation of cytochrome *b*-559 in a manner partly reversible by subsequent addition of benzoquinone (Fig. 5A). The effect of DBMIB is in contrast to the stimulation of oxidation and inhibition of red-light

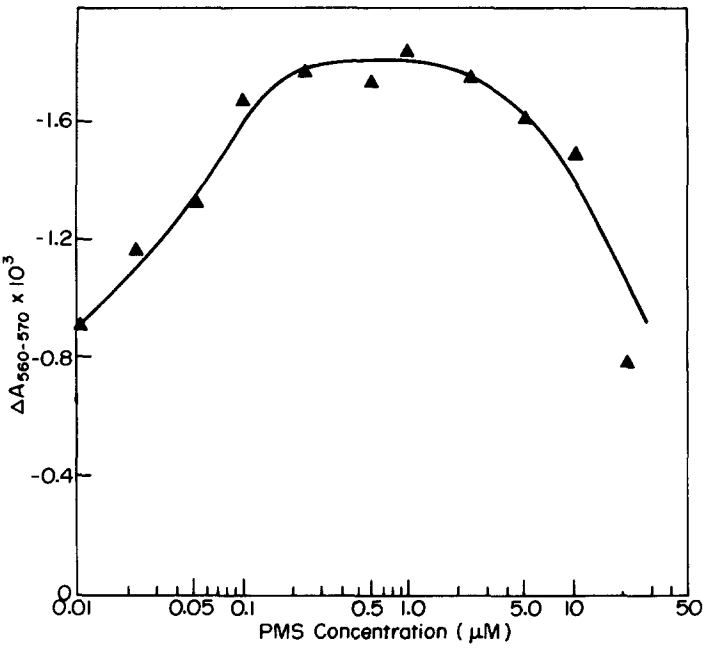

Fig. 4. Amplitude of cytochrome *b*-559 oxidation (560–570 nm) induced by far-red light after addition of different concentrations of PMS. Chlorophyll, 80 μg/ml. Light intensity, $1.3 \cdot 10^4$ ergs/cm^2 per s.

reduction of cytochrome *b*-559 observed in the presence of 5 μM DCMU (Figs 5B and C).

Table I shows the capability of other compounds to replace PMS ($E_{m7} = +0.08$ V) [18] in the stimulation of cytochrome *b*-559 oxidation. *N*-Methylphenazonium ethosulphate (PES, $E_{m7} = +0.055$ V) [18] was equally as active as PMS. Menadione ($E_{m7} = -0.010$ V) [18] at high concentrations and TMPD ($E_{m7} \approx +0.24$ V; Cramer, W. A., unpublished titration) at low concentrations could partially replace PMS, but diaminodurene and pyocyanine caused enhancement of the 560-nm change only in very low light intensity. The rates of change were also much slower than with PMS or PES.

If PMS can increase the rate of electron transfer between Photosystem I and Photosystem II, this effect might be manifested in an increased level of cytochrome *f* reduction. Addition of 2 μM PMS in the presence of red actinic light does cause an increase in reduction of cytochrome *f* (Fig. 6). The difference spectrum for the spectral change caused by PMS addition in red light has a maximum at 554 nm (Fig. 7). The spectrum decreases fairly sharply towards longer wavelength, presumably as a result of increased oxidation of cytochrome *b*-559 and/or cytochrome b_6 when PMS is added in red light. The interpretation of these cytochrome *f* experiments is complicated by PMS-induced cyclic electron flow which also causes reduction of cytochrome *f* [19]. However, it may be possible with DBMIB to separate the two effects of PMS on cytochrome *f* reduction. DBMIB inhibits reduction of cytochrome *f* by non-cyclic electron flow, but does not inhibit PMS-induced cyclic electron flow, at least with

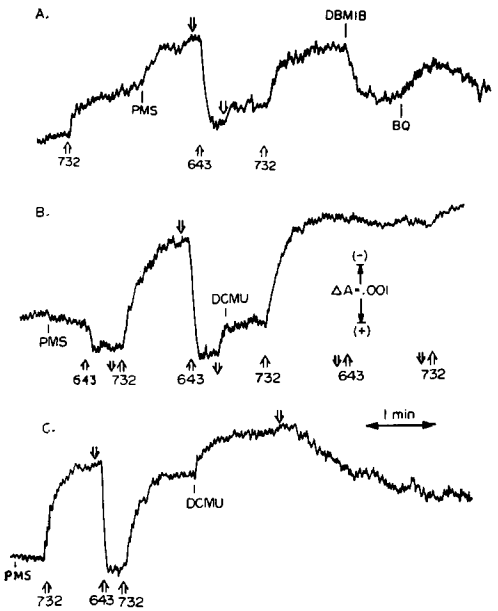

Fig. 5. Effect of addition of 2 μM DBMIB (A) and 5 μM DCMU (B and C) on the redox level of cytochrome *b*-559 in far-red light. PMS, 0.5 μM. Benzoquinone, 20 μM. Chlorophyll, 75 μg/ml. Light intensity, $1.3 \cdot 10^4$ (732 nm) and $1.6 \cdot 10^4$ (643 nm) ergs/cm^2 per s. Absorbance changes at 560 nm with 570 nm reference. Upward arrows indicate actinic light on; downward arrows, off.

TABLE I

THE AMPLITUDE OF FAR-RED OXIDATION OF CYTOCHROME *b*-559 IN THE PRESENCE OF DIFFERENT LIPOPHILIC REDOX COFACTORS

Chloroplasts (80 μg chlorophyll/ml) were illuminated with 732 nm light at $1.6 \cdot 10^4$ (I) and $2.4 \cdot 10^3$ (II) ergs/cm^2 per s in the presence of 1 or 10 μM oxidised electron carriers. No difference was found between reduced diaminodurene and TMPD and their ferricyanide-oxidised forms. Cytochrome *b*-559 oxidation was measured at 560 nm with a 570 nm reference and is not corrected for cytochrome *f* or b_6 changes. Cofactors listed in order of effect in stimulating far-red oxidation of cytochrome *b*-559.

Reagent	E_{m7} (mV)	Concentration (μM)	$\Delta A_{560-570} \times 10^3$	
			I	II
None	—	—	0.5	1.1
PMS	+ 80	1	2.1	1.5
		10	0.3	0
PES	+ 55	1	2.0	1.0
		10	0.5	0
TMPD	≈ +240	1	1.5	1.3
		10	0.3	0.2
Menadione	− 10	1	0.5	0.9
		10	1.3	1.8
Pyocyanine	− 38	1	0.8	1.4
		10	0.6	1.6
Diaminodurene	≈ +240	1	0.5	1.4
		10	0	0

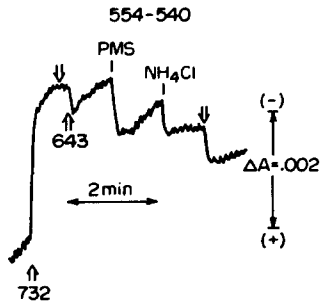

Fig. 6. Absorbance increases at 554 nm caused by PMS (5 μM) and NH_4Cl (3 mM) in red light. Chlorophyll, 85 μg/ml. Light intensities, $1.3 \cdot 10^4$ ergs/cm^2 per s. Reference, 540 nm. Upward arrows indicate actinic light on; downward arrows, off.

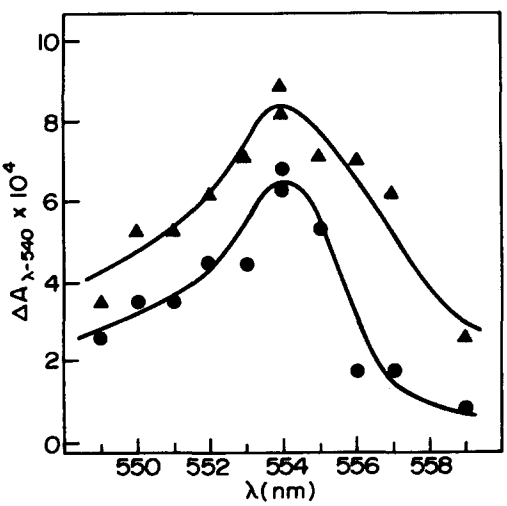

Fig. 7. Point-by-point difference spectrum for the PMS- (●) and NH_4Cl- (▲) stimulated absorbance increase. Conditions as in Fig. 8 except that PMS concentration was 1 μM and chlorophyll, 80 μg/ml.

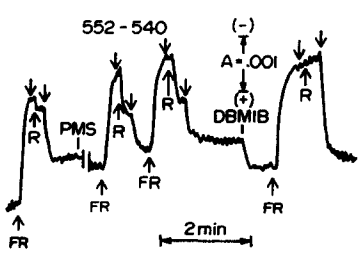

Fig. 8. Inhibition of red light reduction of cytochrome *f* (552–540 nm) by DBMIB (1 μM). PMS concentration, 1 μM. Far-red (FR) and red (R) light intensities at 732 and 643 nm, $1.3 \cdot 10^4$ ergs/cm^2 per s. Chlorophyll, 70 μg/ml. Upwards arrows indicate light on; downward arrows, off.

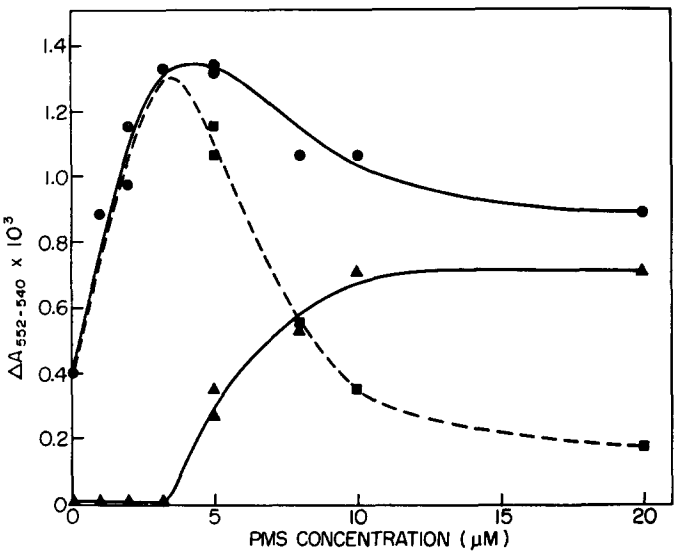

Fig. 9. Amplitude of cytochrome *f* reduction (552–540 nm) in red light caused by addition of different PMS concentrations in the absence (●) and presence (▲) of DBMIB (2 μM). Amplitude of DBMIB-sensitive change is represented as —■—. Light intensity as in Fig. 6. Chlorophyll, 80 μg/ml.

higher PMS concentrations [19, 20]. Fig. 8 shows that DBMIB (1 μM) totally inhibits cytochrome *f* reduction by red light in the presence of PMS (1 μM). The amplitude of the increase in cytochrome *f* reduction caused by addition of PMS in red light is shown as a function of PMS concentration in the absence (solid circles) and presence (solid triangles) of DBMIB in Fig. 9. The reduction of cytochrome *f* by PMS is totally sensitive to DBMIB up to PMS concentrations of approx. 3 μM. At higher PMS concentrations (5–20 μM) PMS stimulates a DBMIB-insensitive cytochrome *f* reduction implying that cyclic electron flow is involved in this reduction. The amplitude of the DBMIB-sensitive cytochrome *f* reduction is optimal at low (≈ 2–3 μM) PMS concentrations, but has a somewhat different concentration dependence than does cytochrome *b*-559 photooxidation (Fig. 4). A "cross-over" effect involving a PMS short-circuit of non-cyclic electron flow is consistent with the 30 % stimulation of state 4 electron transport to ferricyanide observed by others [21]. We have also observed a stimulation of state 2 oxygen evolution in the presence of PMS and high red actinic light intensities (≈ $8 \cdot 10^5$ ergs/cm^2 per s), although a problem with the above interpretation is that this effect has not been reproducible in our hands.

DISCUSSION

The antagonistic effects of far-red and red light on the oxidation state of cytochrome *b*-559 observed in the presence of low concentrations of PMS are comparable to those reported for components of the main electron transport chain, cytochrome *f* and plastoquinone. This would imply that cytochrome *b*-559 can also function in the main electron transport chain under such conditions, although there is a problem with other properties of the cytochrome (low temperature photooxida-

tion by Photosystem II [4, 6, 9] and very positive E_{m7} [5–8] being not easily compatible with such a function). This same incompatibility has been previously noted for the oxidation of cytochrome *b*-559 by Photosystem I in the presence of FCCP [12, 13]. The oxidation pathway to Photosystem I must either by-pass plastoquinone and this intermediate potential region of the main chain, or the effective cytochrome *b*-559 potential in the presence of actinic light must shift to a more negative value. A midpoint potential of the functioning cytochrome of approx. +100 mV, a potential at which it could equilibrate with plastoquinone, is in the range of most determinations of *b*-cytochrome potentials [22]. A midpoint of approx. +350 mV makes cytochrome *b*-559 the most atypical *b*-cytochrome in the literature in terms of this parameter.

DBMIB inhibition of the far-red oxidation of cytochrome *b*-559 indicates that plastoquinone participates as an electron acceptor of cytochrome *b*-559 and that it is a relatively low potential form of the cytochrome which is observed to be oxidized in the presence of PMS. We note, however, that the mode of action of DBMIB is not completely understood. One anomalous property is its action as a very potent dark reductant of photooxidized cytochrome *f* [12]. If a low potential form of cytochrome *b*-559 does function in the light, then PMS could either facilitate the necessary potential shift, or could alternatively alter the relative rate constants for oxidation and reduction. The latter possibility is strongly suggested by the known ability of PMS to create artificial electron transport pathways in chloroplasts, to interact with cytochrome b_6 in chloroplasts [19], and to by-pass accessibility barriers for cytochrome *b* reduction by NADH and the antimycin block of cytochrome *b* oxidation in mitochondria [23–25]. The existence of such a bypass in chloroplasts is apparent in the effect of PMS on Photosystem II reduction of cytochrome *f* (Figs 6 and 7) However, the fact that PMS stimulates oxidation of cytochrome *b*-559 with relatively low intensities of far-red light, and that this effect saturated at moderate light intensities makes it difficult tc understand how such a bypass mechanism could so markedly alter the far-red light response of cytochrome *b*-559.

ACKNOWLEDGEMENTS

We would like to thank J. J. Donnell for her participation in some of these experiments, Dr R. A. Dilley for helpful discussions and Mona Imler for typing the manuscript. This research was supported by N.S.F. grant GB-34169X and N.I.H. Research Career Development Award I K04 29735.

REFERENCES

1 Levine, R. P. and Gorman, D. S. (1966) Plant Physiol. 41, 1293–1300
2 Boardman, N. K. and Anderson, J. M. (1967) Biochim. Biophys. Acta 143, 187–203
3 Ke, B., Vernon, L. P. and Chaney, T. H. (1972) Biochim. Biophys. Acta 254, 345–357
4 Knaff, D. B. and Arnon, D. I. (1969) Proc. Natl. Acad. Sci. U.S. 63, 956–962
5 Bendall, D. S. (1968) Biochem. J. 109, 46P
6 Erixon, K. and Butler, W. L. (1971) Photochem. Photobiol. 14, 427–434
7 Knaff, D. B. and Arnon, D. I. (1971) Biochim. Biophys. Acta 226, 400–408
8 Boardman, N. K., Anderson, J. M. and Hiller, R. G. (1971) Biochim. Biophys. Acta 234, 126 136

9 Vermeglio, A. and Mathis, P. (1973) Biochim. Biophys. Acta 314, 57–65
10 Cramer, W. A. and Butler, W. L. (1967) Biochim. Biophys. Acta 143, 332–339
11 Cramer, W. A., Fan, H. N. and Böhme, H. (1971) J. Bioenerg. 2, 289–303
12 Böhme, H. and Cramer, W. A. (1971) FEBS Lett. 15, 349–351
13 Horton, P., Böhme, H. and Cramer, W. A. (1974) in Proceedings of the Third International Congress on Photosynthesis, (Avron, M., ed.), in the press
14 Hiller, R. G., Anderson, J. M. and Boardman, N. K. (1971) Biochim. Biophys. Acta 245, 439–452
15 Ben-Hayyim, G. (1972) FEBS Lett. 28, 145–148
16 Cramer, W. A. and Böhme, H. (1972) Biochim. Biophys. Acta 256, 358–369
17 Horton, P. and Cramer, W. A. (1974) Biochim. Biophys. Acta 368, 348–360
18 Clark, W. M. (1960) in Oxidation-Reduction Potentials of Organic Systems, p. 375, Williams and Wilkins, Baltimore
19 Böhme, H. and Cramer, W. A. (1972) Biochim. Biophys. Acta 283, 302–315
20 Böhme, H., Reimer, S. and Trebst, A. (1971) Z. Naturforsch. 26b, 341–352
21 Reeves, S. G. and Hall, D. O. (1973) Biochim. Biophys. Acta 314, 66–78
22 Cramer, W. A. and Horton, P. (1975) in The Porphyrins (Dolphin, D., ed.), in the press, Academic Press, New York
23 Wikström, M. K. F. and Lambowitz, A. M. (1974) FEBS Lett. 40, 149–153
24 Lee, I. Y. and Slater, E. C. (1972) Biochim. Biophys. Acta 283, 223–233
25 Lambowitz, A. M., Bonner, W. D. and Wikström, M. K. F. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1183–1187