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THE EFFECTS ON CYTOCHROME *b*-559$_{HP}$ AND P546 OF TREATMENTS THAT INHIBIT OXYGEN EVOLUTION BY CHLOROPLASTS

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SUMMARY

We have studied the effects on the oxidation–reduction potential of cytochrome *b*-559$_{HP}$ of a variety of treatments known to inhibit oxygen evolution by isolated chloroplasts. NH_2OH and 0.8 M Tris buffer had no effect on the cytochrome. Treatment of a chloroplast suspension with heptane, Triton X-100, $NaClO_4$, KI, 2,4,6-triiodophenol or trypsin all caused the conversion of the cytochrome to a low-potential form not reducible by hydroquinone. However, there was no consistent relation between the effect on the cytochrome and the effects on photochemical activities.

Three main conclusions were drawn from the results:

(i) Cytochrome *b*-559$_{HP}$ cannot be directly involved in the process of water oxidation.

(ii) Cytochrome *b*-559$_{HP}$ cannot be directly involved in the oxidation of diphenylcarbazide through Photosystem II.

(iii) The conformation of the cytochrome, and hence its redox potential, depends in part on hydrophobic lipid protein interactions which would be disturbed by several of the treatments mentioned above and also by some others known to effect the potential of the cytochrome.

The photoreduction of the component P546 (C550) at 77 °K was shown to be relatively resistant to treatment of chloroplasts with perchlorate, trypsin or triiodophenol. The results supported the view that P546 is either a primary electron acceptor of Photosystem II or else provides an indication of the redox state of the primary acceptor.

INTRODUCTION

Among the cytochrome components of the chloroplasts of higher plants are two which have α-bands at 559 nm[1]. They can be clearly distinguished by their redox potentials. The high-potential component, cytochrome *b*-559$_{HP}$ ($E_0' = +0.37$ V at pH 7.0)[2] is the only cytochrome that appears to be closely associated with Photo-

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine *N*-2-ethanesulphonate; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris-(hydroxymethyl)methylglycine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

system II. This cytochrome can be oxidised by Photosystem II at the temperature of liquid nitrogen[3-7] and it follows Photosystem II when the chloroplasts are fractionated with digitonin by the procedure of Boardman and Anderson[8]. When etiolated barley leaves are exposed to light, cytochrome b-559$_{HP}$ appears in parallel with the capacity to photooxidize water, in contrast to the other cytochromes and plastocyanin which are already fully formed in the etiolated leaf[9]. Bendall and Sofrová[3] suggested that cytochrome b-559$_{HP}$ is directly involved in oxygen evolution and showed how its redox potential might be compatible with such a role.

A remarkable change in the redox potential of cytochrome b-559$_{HP}$ can be induced by a variety of treatments such as gentle heating, exposure to acetone or detergents[1,10] or to carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) in the light[11], or extraction of freeze–dried chloroplasts with heptane[12]. These treatments cause the potential to fall by about 300 mV.

A change of potential may be taken as an indication of a conformational change of the protein, but this is not so extensive as to represent denaturation in the ordinary sense, as the cytochrome does not combine with CO after exposure to these conditions. The changes induced by heptane extraction have been shown to be reversible[12].

The capacity of chloroplasts to oxidize water is also sensitive to the treatments mentioned above and this suggested a similarity, superficially at least, between the chemistry of cytochrome b-559$_{HP}$ and of Photosystem II as a whole. We have therefore made a comparison between the effects of various treatments on the activity of Photosystem II and on the potential of cytochrome b-559$_{HP}$. We hoped that the results would give information not only on the possible role of the cytochrome in oxygen evolution but also on the general chemistry of the cytochrome and of Photosystem II.

Wada and Arnon[10] reported a correlation between the high-potential form of cytochrome b-559 and Photosystem II activity. Since our experiments were completed Cramer and Böhme[13] have published observations from which they conclude that cytochrome b-559$_{HP}$ cannot be involved in water oxidation. Although, as shown below, there is some discrepancy between the two sets of results, our findings confirm the conclusion of Cramer and Böhme.

Previous work has shown a close connection, both chemically and functionally[5,12,14,15], between cytochrome b-559$_{HP}$ and the component referred to as C550 by Knaff and Arnon[16] or P546 by Bendall and Sofrová[3]. We have therefore studied the effects of several treatments on the photooxidation of cytochrome b-559$_{HP}$ and photoreduction of P546 that occur in chloroplasts at the temperature of liquid nitrogen.

METHODS

Chloroplast preparations

Chloroplasts were prepared essentially according to the method of Cockburn *et al.*[17] using peas (*Pisum sativum* var. Laxton's Superb) grown in the laboratory for 3–4 weeks in moist vermiculite or market spinach (*Spinacea oleracea*). 25–50 g of leaves were ground in a "Polytron" (Kinematica GmbH, Luzern) with 200 ml of a medium containing 0.33 M mannitol, 5 mM $MgCl_2$ and 10 mM sodium pyrophosphate adjusted to pH 6.5 with HCl at 0 °C. The chloroplasts were resuspended in

a medium containing 0.33 M mannitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM *N*-2-hydroxyethylpiperazine *N*-2-ethanesulphonate (HEPES) or *N*-tris(hydroxymethyl)methylglycine (Tricine) adjusted to pH 7.6.

Occasionally experiments were repeated with chloroplasts which had been stored frozen according to the method of Wasserman and Fleischer[18]. With respect to electron transport and cytochromes they seemed identical to freshly prepared chloroplasts.

Cytochrome assays

Cytochromes were assayed according to the methods of Bendall *et al.*[1] in a medium containing 0.33 M mannitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM potassium phosphate buffer, pH 6.5. Difference spectra were recorded with a Johnson Foundation split-beam spectrophotometer. Denaturation of cytochromes was checked by determination of the effect of CO on the spectrum in the presence of dithionite.

Photoinduced absorbance changes at 77 °K

These were followed at the temperature of liquid nitrogen according to the method fo Bendall and Sofrová[3]. If necessary, mannitol to a final concentration of 0.33 M was added to the medium before it was frozen.

Photochemical activities

Measurements of oxygen evolution with ferricyanide as electron acceptor were made with an oxygen electrode (Rank Bros., Bottisham, Cambs.). Chloroplasts were suspended in the Tricine or HEPES medium at pH 7.6 to give 30 μg chlorophyll/ml. The actinic beam from a slide projector was passed through a red filter (Schott, RG 610) to give an intensity of approximately 90 mW/cm^2. An uncoupled rate was found by addition of 3.3 mM NH_4Cl. When measurements were made in weak light Balzer Neutral Density Filters were placed between the light source and the chloroplast suspension.

The reduction of ferricyanide was measured spectrophotometrically by the method of Krogmann and Jagendorf[19]. Chloroplasts were suspended in the medium used for the measurement of oxygen evolution.

Photoreduction of 2,6-dichlorophenolindophenol was measured spectrophotometrically at 600 nm on a Unicam SP500 Spectrophotometer. The chloroplasts were usually suspended in 50 mM potassium phosphate buffer, pH 7.0, to a final concentration of 20 μg chlorophyll/ml. 1,5-Diphenylcarbazide, which was used as an alternative donor to Photosystem II[20], was present at a final concentration of 0.75 mM. The rate of reduction of DCIP in the presence of diphenylcarbazide was corrected by subtraction of the rate insensitive to 1.0 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Photochemical activities were measured at 20–22 °C.

Other methods

Chlorophyll was assayed by the method of Arnon[21]. All reagents used were AR grade.

RESULTS

A variety of inhibitory treatments were tested for their effects on the potential of cytochrome b-559$_{HP}$ and on reactions associated with Photosystem II.

Hydroxylamine[22]

In order to study the effects of hydroxylamine, pea chloroplasts, suspended in the medium used for the cytochrome assays (pH 6.5), were incubated in the dark at approx. 21 °C with 2 mM NH_2OH for 30 min. The evolution of oxygen, measured in the presence of ferricyanide as electron acceptor, was completely inhibited by this treatment. In contrast, 2 mM NH_2OH had no effect on cytochrome b-559$_{HP}$. We have been unable to observe the conversion of the cytochrome to a low potential form as claimed by Cramer and Böhme[13].

Tris buffer[23,24]

Washing pea chloroplasts with Tris buffers containing either 0.8 M Tris and 0.46 M HCl, or 0.8 M Tris, 0.75 M HCl and 30 mM ferricyanide, caused more than 80 % inhibition of oxygen evolution. We could not observe any effect on cytochrome b-559$_{HP}$ under these conditions. However the recent paper of Erixon *et al.*[25] suggests that washing with Tris buffer may cause a moderate change in potential of the cytochrome which would not be detectable under our conditions.

Triton X-100[1,20,26]

The effects of various concentrations of the non-ionic detergent Triton X-100 on pea chloroplasts are shown in Fig. 1. As found by Vernon and Shaw[26], the photoreduction of DCIP was inactivated by lower concentrations of the detergent when water was the electron donor than when diphenylcarbazide was the donor. Inhibition of the reaction with diphenylcarbazide did develop, however, at high concentrations of the detergent. Cytochrome b-559$_{HP}$ was found to be more resistant than was photoreduction of DCIP by diphenylcarbazide.

It is noteworthy that the change in potential of the cytochrome was correlated roughly with the clearing of the chloroplast suspension, and thus presumably with a general breakdown of membranes.

Heptane

Laber and Black[27] showed that treatment of an aqueous chloroplast suspension with heptane caused uncoupling. Under the more rigorous conditions used here successive exposures of chloroplast suspensions to heptane caused a decline in both the rate of oxygen evolution with ferricyanide as acceptor and in the amount of cytochrome b-559$_{HP}$. However the decline of oxygen evolution was more rapid (Fig. 2).

It is uncertain whether these effects are due to extraction of essential components or to the addition of heptane to the chloroplast membranes.

Chaotropic agents

Solutions of certain salts (chaotropic agents) weaken hydrophobic interactions because of their greater disordering effect on the structure of water[28]. Lozier *et al*[29] have reported the inhibition of electron transport in chloroplasts after incubation

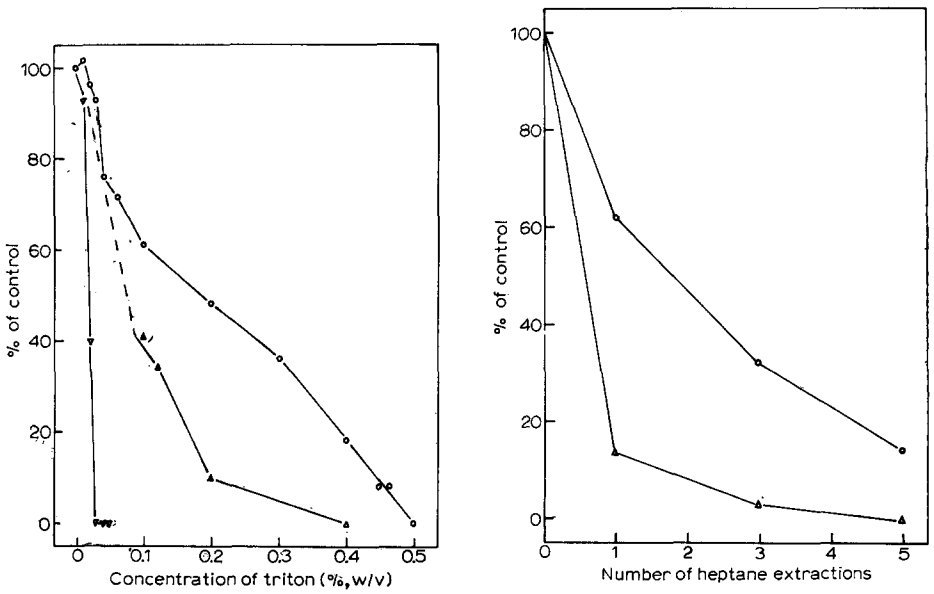

Fig. 1. Effect of incubation with different concentrations of Triton X-100 on the potential of cytochrome *b*-559$_{HP}$ and photochemical activities in spinach chloroplasts. Chloroplasts (200 μg chlorophyll/ml) were incubated in the dark for 2–3 h at 0 °C with various concentrations of Triton X-100 in a medium containing 0.5 M sucrose and 50 mM Tricine, pH 7.5. ○—○, cytochrome *b*-559$_{HP}$; ▽—▽, rate of photoreduction of DCIP (initial concn 40 μM) in the absence of added electron donor; △—△, rate of photoreduction of DCIP in the presence of 0.75 mM diphenylcarbazide.

Fig. 2. Effect of repeated extractions of a chloroplast suspension with heptane on the potential of cytochrome *b*-559$_{HP}$ and oxygen evolution in pea chloroplasts. Chloroplasts (170 μg chlorophyll/ml) were suspended in a medium containing 20 mM Tricine buffer and 10 mM NaCl at pH 7.9 and shaken with 4 vol. of heptane for 2 min. The layers were separated by brief centrifugation. ○—○, cytochrome *b*-559$_{HP}$; △—△, rate of oxygen evolution with ferricyanide (1.33 mM) as acceptor in the presence of 3.3 mM NH_4Cl.

with chaotropic agents at 0°C for 20 min. Oxygen evolution was completely inhibited by 400 mM perchlorate. The photooxidation of water was reported to be more sensitive than the photooxidation of a mixture of ascorbate and hydroquinone which can donate electrons more directly to Photosystem II.

Under the conditions used here, which involved incubation with perchlorate at 30 °C, lower concentrations were needed for the inhibition of electron transport. To avoid confusion due to light-induced oxygen uptake at the higher concentrations of perchlorate the reduction of both DCIP and ferricyanide were followed spectrophotometrically. Fig. 3 confirms that DCMU- sensitive photooxidation of diphenylcarbazide is more resistant than oxidation of water when DCIP is used as electron acceptor.

Incubation of chloroplasts with 250 mM perchlorate caused the conversion of cytochrome *b*-559$_{HP}$ to a low-potential form without a significant effect on any of the other cytochromes (Table I). With increasing concentrations of perchlorate cytochrome *b*-559$_{HP}$ and photochemical activities involving water as donor declined in parallel. It should be noted that after treatment with 150 mM perchlorate over 50 % of the activity with diphenylcarbazide remained but only about 5 % of the high-

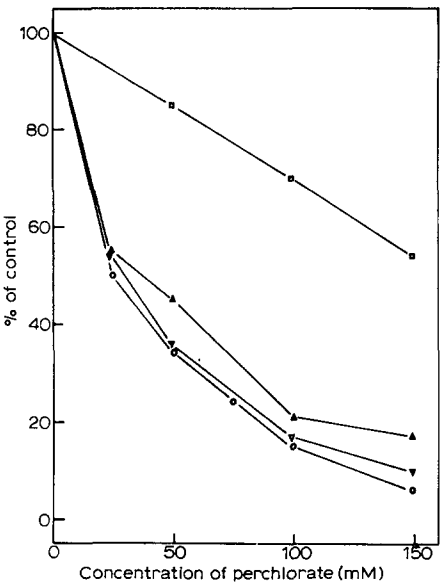

Fig. 3. Effect of incubation with different concentrations of $NaClO_4$ on the potential of cytochrome *b*-559HP and photochemical activities in pea chloroplasts. Chloroplasts (170 μg chlorophyll/ml) suspended in 10 mM potassium phosphate buffer, pH 7.0, containing 10 mM NaCl and 1 mM $MgCl_2$, were incubated with various concentrations of chaotropic agents at 30 °C in the dark for 10 min. ○—○, cytochrome *b*-559HP; △—△, rate of photoreduction of DCIP (initial concn 40 μM) in the absence of added electron donor; ▽—▽, rate of oxygen evolution with 1.33 mM ferricyanide as acceptor in the presence of 3.3 mM NH_4Cl; □—□, rate of photoreduction of DCIP in the presence of 0.75 mM diphenylcarbazide.

TABLE I

EFFECT OF VARIOUS TREATMENTS ON PEA CHLOROPLAST CYTOCHROMES

The conditions of treatment with triiodophenol, $NaClO_4$ and trypsin were as given in the legends to Figs 4, 3 and 5, respectively.

Conditions	*Amount of cytochrome (natoms haem/mg chlorophyll)*			
	f	*b*-559HP	*b*-559LP	*b*-563
Control	1.0	2.2	0.9	2.1
500 μM triiodophenol	0.9	0	2.4	2.2
250 mM $NaClO_4$	1.0	0	2.7	2.0
After trypsin digestion	1.2	0	2.2	1.8

potential form of the cytochrome. The effect of iodide on the cytochrome was similar to that of perchlorate. It therefore seems probable that perchlorate was acting as a chaotropic agent rather than as a specific inhibitor.

Triiodophenol

The inhibitory effect of 2,4,6-triiodophenol on oxygen evolution has been discovered by Desmet[30]. He suggested that triiodophenol inhibits on the high-poten-

tial side of Photosystem II on the basis of its effects on the variable fluorescence. Its effect on cytochrome *b*-559$_{HP}$ was to convert it to a low-potential form but it had no significant effect on the other cytochromes (Table I).

Inhibition by triiodophenol depended on the concentration of chloroplasts as well as on the concentration of inhibitor and the time of incubation (Table II). The effects of 5 min of preincubation with various concentrations of triiodophenol under identical conditions are shown in Fig. 4.

TABLE II

EFFECT OF CHLOROPLAST CONCENTRATION ON INHIBITION OF DCIP PHOTOREDUCTION BY 2,4,6-TRIIODOPHENOL

Chloroplasts were suspended in the HEPES medium at pH 7.6 at a concentration equivalent to 170 μg chlorophyll/ml and preincubated in the dark for 5 min at room temperature with different concentrations of triiodophenol. Before assay of DCIP photoreduction with 50 μM DCIP in the presence of 3.3 mM NH_4Cl the chloroplasts were diluted to 17 μg chlorophyll/ml in the HEPES medium.

Concentrations during preincubation		*Rate of DCIP reduction (% of untreated chloroplasts)*
Chloroplasts (μg chlorophyll/ml)	*Triiodophenol (μM)*	
17	200	0
17	500	0
170	200	66
170	500	33

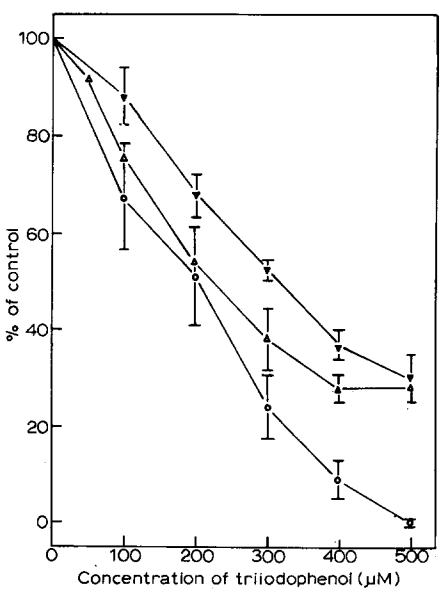

Fig. 4. Effect of incubation with 2,4,6-triiodophenol on the potential of cytochrome *b*-559$_{HP}$ and photochemical activities in pea chloroplasts. Chloroplasts suspended in the HEPES medium at pH 7.6 at a concentration of 170 μg chlorophyll/ml were preincubated for 5 min in the dark with different concentrations of triiodophenol. ○—○, cytochrome *b*-559$_{HP}$; ▼—▼, rate of photoreduction of DCIP (initial concn 40 μM) in the absence of added electron donor; △—△, rate of oxygen evolution with 1.33 mM ferricyanide as acceptor. Both photochemical activities were measured at pH 7.6 in the HEPES medium in the presence of 3.3 mM NH_4Cl.

The uncoupled rate of electron transport from water was progressively inhibited by concentrations of triiodophenol in the range 100–400 μM. Activity with ferricyanide as acceptor appeared to be more sensitive than with DCIP as acceptor. However the most notable result was that cytochrome b-559$_{HP}$ was significantly more sensitive than activity; in particular no cytochrome b-559$_{HP}$ could be detected after treatment with 500 μM triiodophenol although about 30% of the normal uncoupled rate of electron transport remained. It is therefore very unlikely that cytochrome b-559$_{HP}$ is directly involved in oxygen evolution. Neither the modification of cytochrome b-559$_{HP}$ nor the inhibition of electron transport by triiodophenol could be reversed by washing.

Trypsin

Mantai[31] has shown that incubation of chloroplasts with trypsin inhibits the reactions of Photosystem II. The first effect is an uncoupling of electron transport from phosphorylation followed by an inhibition of electron flow. Selman and Bannister[32] reported that the photooxidation of water was more rapidly inhibited than the photooxidation of diphenylcarbazide.

Trypsin digestion was found to lower the potential of cytochrome b-559$_{HP}$ without a significant effect on any of the other cytochromes (Table I). This change was found to occur more rapidly than inhibition of oxygen evolution. This result again suggested that cytochrome b-559$_{HP}$ could not be directly involved in oxygen evolution.

The assumption that a component of the electron transport chain cannot be more sensitive to inhibitory treatments than electron flow is only valid if the chains are separate and independent. There is some evidence that this is not the case at high light intensities at which the activity of the photocentres is not limiting[33]. Under these conditions the rate-limiting step appears to be at the exit of a carrier pool into which several electron transport chains feed. The observations of Stiehl and Witt[34] suggest that this carrier is plastoquinone.

In weak light, however, the limiting factor is the number of active photocentres. The flash yield experiments of Joliot *et al.*[35] and Kok *et al.*[36] show that there can be no pool of carriers and no cooperation of chains on the high-potential side of Photosystem II. Hence no component involved in water oxidation should be more sensitive to an inhibitory treatment than electron flow in weak light.

Light intensity was found to have little effect on the general shape of the curve relating the rate of oxygen evolution to the time of exposure to trypsin. Cytochrome b-559$_{HP}$ disappeared several times more rapidly than activity and about 30% of the activity remained at a time when no cytochrome was detectable. Fig. 5 shows the results obtained with an intensity of 4.3 mW/cm^2; the curve was similar with 0.7 mW/cm^2, at which the control rate was less than 10% of that observed with 90 mW/cm^2.

Trypsin has been shown to induce an ATPase activity in the coupling factor CF_1[37] and uncoupling is probably a consequence of this effect. However the high potential character of cytochrome b-559$_{HP}$ does not depend on the presence of CF_1 because its removal by washing with EDTA[38] had no detectable effect on the potential of the cytochrome.

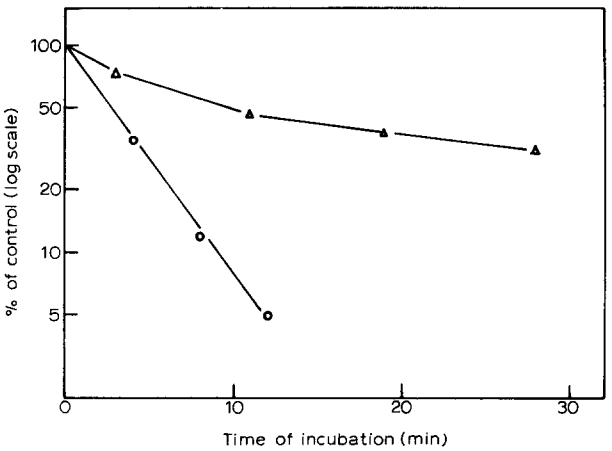

Fig. 5. Effects of incubation with trypsin on cytochrome b-559$_{HP}$ and the rate of oxygen evolution in weak light. Chloroplasts at a concentration of 340 μg chlorophyll/ml in a medium containing 20 mM Tricine buffer at pH 7.9 and 10 mM NaCl were incubated at room temperature with trypsin (20 μg/ml). At various times samples were withdrawn and diluted with equal volumes of a medium containing 0.66 M mannitol, 4 mM EDTA, 2 mM $MgCl_2$, 2 mM $MnCl_2$ and 100 mM potassium phosphate buffer, pH 6.5, for measurement of cytochrome b-559$_{HP}$ (○—○). Other samples were withdrawn and the rate of oxygen evolution in weak light (4.3 mW/cm^2) was measured with ferricyanide as acceptor in the presence of 3.3 mM NH_4Cl (△---△).

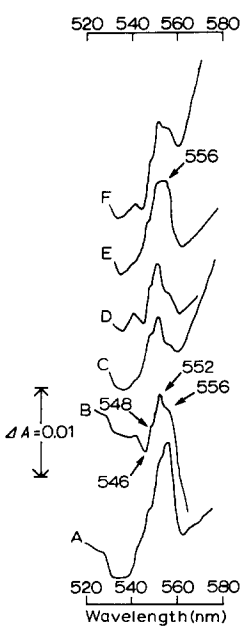

Fig. 6. The effect of trypsin on the light-induced absorbance changes of pea chloroplasts at 77 °K. A suspension of chloroplasts in 20 mM Tricine, 10 mM NaCl, pH 7.9 (400 μg chlorophyll/ml) was treated with trypsin (40 μg/ml). At intervals samples were withdrawn and diluted with equal volumes of a medium containing 0.66 M mannitol, 4 mM EDTA, 2 mM $MgCl_2$, 2 mM $MnCl_2$ and 100 mM potassium phosphate buffer, pH 6.5. The suspension was divided into two halves, to the negative sample was added 2.5 mM $K_3Fe(CN)_6$ and to the positive sample was added either 2.5 mM hydroquinone (A and C) or 5 mM ascorbate (E). The cuvette was frozen in darkness. A, the difference spectrum (reduced *minus* oxidized) of untreated chloroplasts; B, the spectrum of the same suspension after actinic illumination of the positive sample (100 μW/cm^2 at 580 nm for 2 min); C, the difference spectrum of chloroplasts after treatment with trypsin for 40 min; D, the same after actinic illumination of the positive sample; E, the difference spectrum after 60 min exposure to trypsin; F, the same after actinic illumination of the positive sample.

Light-induced absorbance changes at 77 °K

Several treatments which modify cytochrome *b*-559HP were found to have little effect on P546 as judged by its photoreducibility at low temperature. Treatment of chloroplasts with 150 mM perchlorate modified nearly all the cytochrome *b*-559HP. Under these conditions 60 % of the control activity of Photosystem II can be obtained with diphenylcarbazide as electron donor. The amount of P546 that could be photoreduced was not significantly less than this. Fig. 6 shows that P546 was not affected by trypsin treatment even when all the cytochrome *b*-559HP had been modified. Triiodophenol was similar to trypsin in this respect.

Alteration of the redox potential of cytochrome *b*-559HP by treatment of chloroplasts with trypsin was found not to prevent its photooxidation at the temperature of liquid nitrogen. Fig. 6 shows that the modified component could be reduced by ascorbate, although not by hydroquinone, and could then be photooxidized with concomitant reduction of P546.

DISCUSSION

The nature of the chemical components involved in the oxidation of water is one of the major unsolved problems of photosynthesis. There is strong evidence that cytochrome *b*-559HP is intimately connected with Photosystem II, on which the oxidation of water depends. In a previous paper Bendall and Sofrová[3] suggested that cytochrome *b*-559HP could be identical with the carrier S that plays the key part in the linear four-step mechanism postulated by Kok *et al.*[36] for oxygen evolution. However the results reported in this paper make it very unlikely that this cytochrome could be directly involved in the water oxidation process. Significant amounts of oxygen evolution (30 % of the rate in untreated chloroplasts) can still be observed when no cytochrome *b*-559HP is detectable. The minimum amount of cytochrome *b*-559HP that can be detected in the presence of cytochrome *f* is perhaps 5 % of that in untreated chloroplasts. The discrepancies between the amounts of cytochrome and of photochemical activities after treatment with either trypsin or 2,4,6-triiodophenol therefore effectively exclude the participation of cytochrome *b*-559HP in oxygen evolution.

The results reported here also suggest that cytochrome *b*-559HP is not involved in the oxidation of diphenylcarbazide, since the DCMU-sensitive photoreduction of DCIP in the presence of diphenylcarbazide is more resistant to treatment with perchlorate than the cytochrome.

A wide variety of treatments—heating, treatment with detergents or organic solvents[1,10], or chaotropic agents as well as trypsin, triiodophenol and FCCP[11]—will cause the lowering of the potential of the cytochrome *b*-559HP. The majority of these treatments would be expected to derange the hydrophobic portions of the membrane. This suggests that the conformation of the cytochrome, which determines its redox potential, is dependent on hydrophobic lipid–protein interactions. There is evidence from experiments involving extraction of freeze-dried chloroplasts with heptane that the high-potential character of cytochrome *b*-559HP can be reversibly altered by removal and readdition of the components of a heptane extract[12]. However, the potential can also be affected by triiodophenol or trypsin which have no obvious effect on the bulk hydrophobic environment but may act in a more specific manner.

It seems unlikely that trypsin can penetrate the thylakoid membrane, and hence the sensitivity towards trypsin attack may be evidence that cytochrome *b*-559$_{HP}$ is located on the outer surface of the thylakoid membrane.

It cannot be said whether the modified high-potential component after these treatments is the same as the original low-potential component. Wada and Arnon[10] have suggested that there are three interconvertible forms of cytochrome *b*-559 (high, middle and low potentials) on the basis of their reducibility by hydroquinone, ascorbate and dithionite. The M form is that which is derived from the H form by the treatments discussed above and by ageing of the chloroplast preparation. However, the results of Fan and Cramer[39] suggest that the potentials of the modified high-potential component (altered by the anti-foaming agent used in their technique) and the original low-potential component are close together. It should be noted that reduction by ascorbate tends to be slow and the low-potential components are liable to a slow autoxidation; also, the ascorbate/dehydroascorbate system is not truly reversible at neutral pH. Under these circumstances there may be no true equilibrium established between a cytochrome and ascorbate. Thus one cannot safely assume, as do Wada and Arnon, that ascorbate reduction readily distinguishes two low-potential components (their M and L forms). The normal low-potential component can be slowly and incompletely reduced by ascorbate in untreated chloroplasts (more extensive reduction might have been predicted from its potential) and the rate and extent of this reduction might be increased by the procedures that modify the high-potential cytochrome. Nor can one assume, with Cramer and Böhme[13], that the two low-potential forms are identical. The question of whether two distinct protein molecules exist, rather than one molecule that can adopt different conformations depending on its environment, will be answered only when the cytochromes have been isolated and purified.

P546 has been identified with the quencher of chlorophyll fluorescence Q (presumed to be the primary acceptor of Photosystem II) on the basis of identical titration curves and similar effects of inhibitors[5]. The absorbtion change at 546 nm has been shown by Butler and Okayama[40] to be due to a band shift. It seems unlikely that this represents the chemical oxidation and reduction of a component, and P546 probably indicates the redox state of the primary acceptor without itself gaining or loosing electrons. P546 has been shown to be closely associated with cytochrome *b*-559$_{HP}$, but photoreduction of P546 is still possible at 77 °K if the cytochrome is chemically oxidized before freezing[3,16]. As shown here, photoreduction of P546 is still possible if cytochrome *b*-559$_{HP}$ is modified by trypsin, triiodophenol or perchlorate. This demonstrates that it is possible to affect the cytochrome chemically without affecting the P546. Even though it now appears that cytochrome *b*-559$_{HP}$ is not involved directly in the water oxidation process there is no evidence against the absorbance changes due to P546 being at least an indication of the oxidation and reduction of the true primary acceptor.

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