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PHOTO-BLEACHING OF CAROTENOIDS RELATED TO THE ELECTRON TRANSPORT IN CHLOROPLASTS

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SUMMARY

Aerobic photo-bleaching of carotenoids in isolated spinach chloroplasts was investigated by measuring the absorption spectrum of chloroplasts and by analysing carotenoid components by means of thin-layer chromatography on microcrystalline cellulose. Carotenoids were irreversibly bleached when chloroplasts were illuminated by red light in the presence of ferricyanide or an inhibitor of the Hill reaction such as azide, hydroxylamine and carbonylcyanide *m*-chlorophenylhydrazone, while carotenoids in the chloroplasts suspended in a phosphate buffer in the absence of these agents underwent no bleaching on illumination. The photo-bleaching of carotenoids in the presence of these agents was suppressed by another group of inhibitors of the Hill reaction such as *o*-phenanthroline, substituted phenylureas and symmetrical triazines, or by reducing agents such as ascorbate and ascorbate *plus* *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Chromatographic analysis of carotenoids extracted from the chloroplasts illuminated in the presence of carbonylcyanide *m*-chlorophenylhydrazone or ferricyanide showed that three major carotenoid components, carotenes, lutein and violaxanthin, were bleached to similar and considerable extents. Neoxanthin was much more resistant to the action of light. The photo-bleaching of lutein and violaxanthin proceeded more rapidly in air than in a nitrogen atmosphere, but the bleaching of carotenes proceeded at an equal rate under these conditions. These results are discussed in relation to the photochemical electron transport in chloroplasts.

INTRODUCTION

DOROUGH AND CALVIN[1] first pointed out the possibility that carotenoids might be involved in the oxygen evolution in photosynthesis. Attempts have since been made to demonstrate the participation of carotenoids in the photosynthetic oxygen metabolism. One of the results obtained along this line of investigation may be

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulphate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichlorophenolindophenol.

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mutual conversion of xanthophylls in photosynthetic organisms[2–4]. YAMAMOTO, NAKAYAMA AND CHICHESTER[3] showed that violaxanthin is reversibly converted by light into antheraxanthin and zeaxanthin in green leaves, and KRINSKY[4] found a similar conversion of antheraxanthin into zeaxanthin in Euglena. Backward conversion, epoxidation of antheraxanthin and zeaxanthin, proceeded in the presence of molecular oxygen. Furthermore, isotope enrichment of epoxy xanthophylls in the presence of $H_2{}^{18}O$ (ref. 5) or $^{18}O_2$ (ref. 6) was observed in the light in green plants. On the other hand, GRIFFITHS *et al.*[7], from the experiments with mutants of photosynthetic bacteria, proposed that carotenoids function as protectors against lethal photo-oxidations sensitized by chlorophylls in the presence of molecular oxygen.

It has been known for some time that light causes bleaching of photosynthetic pigments in plant cells[8–10] and in isolated chloroplasts[11,12]. According to FRENCH *et al.*[8] and BROWN[9], the three forms of chlorophyll *a* in algal cells having their red absorption maxima at 673, 683, and 694 mμ, respectively, are bleached to different extents under similar illuminating conditions, and the form with a maximum at 694 mμ is most sensitive to illumination. SAUER AND CALVIN[12] demonstrated the photo-bleaching of carotenoids and chlorophylls in spinach chloroplast fragments. Little is known, however, about the mechanism of photo-bleaching of carotenoids.

In the present study, the photo-bleaching of carotenoids in isolated spinach chloroplasts was investigated under well-controlled conditions in order to see the relationship, if it exists, between the bleaching and the photochemical electron transport in chloroplasts. The light-induced changes of carotenoids were observed both by absorption spectrophotometry of chloroplasts and by thin-layer chromatography of carotenoid components. The thin-layer chromatography provided the data on each carotenoid component, while the spectrophotometry gave the changes in the total carotenoid content more precisely.

EXPERIMENTAL

Preparation of chloroplasts

Chloroplasts were squeezed out of spinach (*Spinacia oleracea*) leaves through cotton cloth into 0.04 M phosphate buffer (pH 7.2). The green juice was centrifuged in a refrigerated centrifuge at 100 × *g* for 2 min, the sediment was discarded, and the supernatant was recentrifuged at 1000 × *g* for 15 min to obtain whole chloroplasts. The chloroplasts were suspended in the same phosphate buffer and were stored in the dark at 0° until used.

Measurement of photo-bleaching by difference spectrophotometry

The changes in the total carotenoid content induced by light were followed by difference spectrophotometry between two chloroplast suspensions in 1.0-cm cells, one illuminated in the sample compartment, and the other in the dark in the reference compartment, of a Shimadzu multipurpose recording spectrophotometer model MPS-50 of the two-detector type. The suspensions for the spectrophotometry contained chloroplasts equivalent to 18 μg chlorophylls per ml, and illumination was made at 20 ± 2° in air by the red light above 680 mμ (170 lux) from a projector lamp through a fan-cooled heat-absorbing filter and a Toshiba-V-R68 filter at right angles to the measuring beam. The sample (or the reference) was placed close to the

large photo-cathode of one of the photomultipliers of the end-on type, so that all the diffuse transmitted light could be captured. The spectrum thus obtained is the difference spectrum in terms of semi-integral attenuance[13] and indicates the correct absorption difference between the two translucent suspensions.

Chromatography of carotenoids in illuminated chloroplasts

A chloroplast suspension (2 ml, chloroplasts equivalent to 264 μg chlorophylls) in a glass vessel was illuminated for 10 min at 20 $\pm$ 2° by the red light above 600 mμ (600 lux) from a white fluorescent lamp through a red cellophane filter. The illumination was made in air unless otherwise noted. To the illuminated suspension were added 10 ml of a methanol–acetone mixture (1:1, v/v) in order to extract the pigments. The extract was saponified by the addition of 0.5 ml of 60% aq. KOH. After the saponification of chlorophylls, carotenoids were extracted from the mixture with diethyl ether and washed with water, and the solvent was evaporated at room temperature under reduced pressure. The carotenoid sample thus obtained was dissolved in 1 ml of acetone, and a portion (0.15 ml) was applied to a thin layer of microcrystalline cellulose (Avicel SF). Four major components, carotenes, lutein, violaxanthin and neoxanthin, and two minor xanthophylls, X-443 and X-439 (the number denotes the maximal wavelength in mμ)[14], were clearly separated by the ascending one-dimensional chromatography, using light petroleum (b.p. 30–50°) containing 10% acetone as the developer. Carotenes were eluted from the chromatogram with 3 ml of light petroleum, and xanthophylls with 3 ml of ethanol. The absorbance values of the solutions at their main absorption maxima, which ranged from 0.06 to 0.17, were determined precisely on an expanded scale of the spectrophotometer. The molar extinction coefficients of 1.35, 1.45, 1.46 and $1.38 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ (refs. 15 and 16) were used to estimate the molar contents of carotenes, lutein, violaxanthin and neoxanthin, respectively.

Measurements of evolution and uptake of oxygen

Oxygen evolved by or incorporated in chloroplasts on illumination was monitored with a Clark-type oxygen electrode. A chloroplast suspension (chloroplasts equivalent to 77 μg chlorophylls per ml) was introduced into a semi-closed vessel made of lucite, and was illuminated by the red light above 680 mμ (170 lux) in the same manner as for the difference spectrophotometry. During illumination the temperature of the system was kept at 20° with running water.

RESULTS

Photo-bleaching of carotenoids as analysed by difference spectrophotometry

Carotenoids in the chloroplasts freshly prepared and suspended in 0.04 M phosphate without added agents did not change on illumination by red light. Addition of an agent such as hydroxylamine, CCCP, azide or ferricyanide induced the change in carotenoids on illumination. In the experiment, an aliquot of a chloroplast suspension with one of these agents was divided into two parts. One was illuminated for a certain period, 1.0–15 min, and the other was kept in the dark for the same period, and the spectral difference between these samples was measured immediately in the dark. Fig. 1 shows the light-*minus*-dark difference spectra thus obtained in the presence of

1 mM hydroxylamine hydrochloride (Curves A, B and C) or 10 μM CCCP (Curve D). In the presence of hydroxylamine, the absorbance of the chloroplast suspension decreased upon illumination in the wavelength range above 390 mμ. Difference minima were located in the spectrum at 425, 457, 490, 630 and 680 mμ for the chloroplasts illuminated for 15 min in the presence of hydroxylamine (Curve C in the figure). The exact positions of the minima were dependent on the period of illumination. After the first 1 min of illumination, the main negative peak was at 500 mμ (Curve A) but shifted gradually with time toward a shorter wavelength of 490 mμ (Curves B and C). The spectral change on illumination obtained in the presence of sodium azide was similar in wavelength dependency to that obtained with hydroxylamine, but was smaller than that obtained with hydroxylamine at the same concentration. The illumination for 15 min in a more concentrated (5 mM) azide solution gave a difference spectrum similar to Curve C in Fig. 1 obtained with 1 mM hydroxylamine after the same period of illumination. The difference minima found for the chloroplasts illuminated for 10 min in the presence of 10 μM CCCP were at 400, 457, 490, 595, 630 and 680 mμ (Curve D). The difference spectrum obtained in the presence of 1 mM potassium ferricyanide after 10 min of illumination showed a negative peak at 490 mμ. The spectrum was, however, greatly different from that obtained in the presence of CCCP or hydroxylamine. The former showed a negative peak at 420 mμ due to photoreduction of ferricyanide (the Hill reaction) in addition to the change in carotenoids found at 490 mμ. The absorbance drop at 420 mμ was 2.6 times greater than the drop at 490 mμ when compared after 10 min of illumination.

The absorbance drop caused by red light at 457 and that at 490 mμ are interpreted from the shapes of the curves in Fig. 1 as being due to the bleaching of carotenoids, whereas the changes at 595, 630 and 680 mμ are due to the bleaching mostly of chlorophyll *a*. The extent of photo-bleaching of carotenoids was greater than that of chlorophyll *a* as estimated from the absorbance changes at 490 and 680 mμ, respectively. The light-induced absorbance change at 490 mμ was 9.3 % of the absorbance (= 0.74) at 490 mμ of the suspension kept in the dark, while the change at 680 mμ was 3.3 % of the absorbance (= 0.80) at 680 mμ in the dark (Curve D in Fig. 1). The maximum at 650 mμ close to the zero line found on Curve D in Fig. 1 indicates that chlorophyll *b* is highly resistant to the action of light. The maximum found around 470 mμ on the same spectrum supports this interpretation. The negative band around 400 mμ obtained on illumination in the presence of CCCP (Curve D)

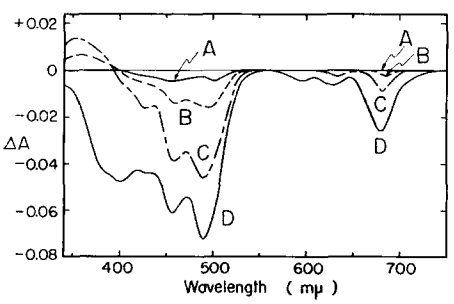
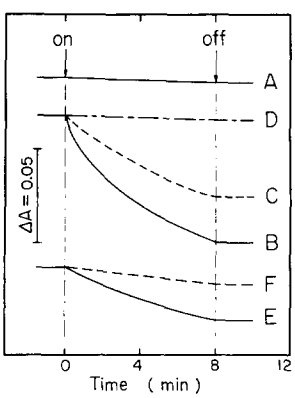


Fig. 1. The light-*minus*-dark difference spectra of chloroplasts in the presence of 1 mM hydroxylamine (Curves A, B and C) or 10 μM CCCP (Curve D). These difference spectra were observed in the dark after 1 min (Curve A), 5 min (B), 15 min (C) or 10 min (D) of illumination by red light.

is mainly due to the photo-bleaching of CCCP; the difference spectrum between the two supernatant solutions obtained by centrifugation of the suspension incubated in the light and that in the dark showed a single negative peak at 390 mμ which agrees with the band position of CCCP in a neutral phosphate buffer.

The time profile of the light-induced absorbance change was observed at 490 mμ during illumination, and the result is shown in Fig. 2. The absorbance decreased rapidly upon illumination without appreciable time lag (Curve B obtained with 10 μM CCCP and Curve E with 1 mM ferricyanide). Curve A in Fig. 2 indicates that fresh chloroplasts suspended in 0.04 M phosphate buffer without added agents underwent only a slight decrease in absorbance at 490 mμ. The rate of the light-induced absorbance change in the presence of CCCP, which was estimated from the absorbance drop at 490 mμ during the first 3 min of illumination, was enhanced by increasing the concentration of CCCP. As shown by Curve A in Fig. 3, the rate started to increase at 1 μM of CCCP and reached a constant level above 10 μM. These spectral changes observed on illumination with various added agents were not reversed in the succeeding dark period.

Azide[17], hydroxylamine[17] and CCCP (ref. 18) are known to inhibit the Hill reaction. Fig. 3 shows the relationship between the inhibitory action of CCCP on the oxygen evolution (Curve B) and its bleaching action on carotenoids estimated from the absorbance change at 490 mμ (Curve A). The oxygen evolution was observed in the presence of 3 mM *p*-benzoquinone at varied concentrations of CCCP, and the absorbance change was observed with CCCP without quinone. The Hill reaction activity observed for the chloroplasts without CCCP was 21 μmoles of O_2 evolved per mg of chlorophylls per h. As seen from the figure, there exists a striking correlation



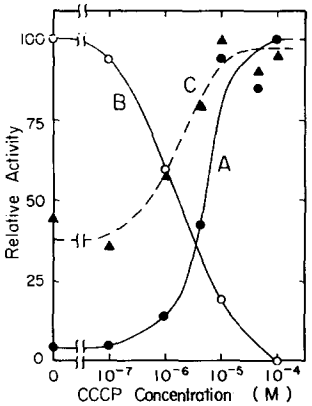


Fig. 2. The time profiles of the light-induced absorbance changes at 490 mμ as observed for the chloroplasts suspended in 0.04 M phosphate without any addition (Curve A), and for the chloroplasts mixed with 10 μM CCCP (Curve B), 10 μM CCCP + 1 mM ascorbate (C), 10 μM CCCP + 1 mM ascorbate + 20 μM PMS (D), 1 mM ferricyanide (E) or 1 mM ferricyanide + 10 μM DCMU (F). Illumination by red light as indicated by arrows.

Fig. 3. The light-induced absorbance decrease at 490 mμ (Curve A), the oxygen evolution in the presence of 3 mM *p*-benzoquinone (B) and the light-induced oxygen uptake (C) as observed as a function of CCCP concentration. The initial rates of these processes were estimated from the change of absorbance (Curve A) or oxygen tension (Curves B and C) obtained during the first 3 min of red light illumination, and are expressed in relative units.

between these actions of CCCP. The concentration of CCCP for 50 % inhibition of the Hill reaction and that for 50 % activation of the absorbance change were of the same order, 1.8 and 5.3 μM, respectively. According to DE KIEWIET, HALL AND JENNER[18], the CCCP concentration for 50 % inhibition of the oxygen evolution was 4.6 μM in the presence of ferricyanide and was 6.4 μM in the presence of NADP. The same authors also showed that CCCP can act as an uncoupler of electron flow from photophosphorylation. The uncoupling action of CCCP may not be responsible for the bleaching, since another well-known uncoupler, 4 mM ammonium chloride, did not cause any absorbance change at 490 mμ on illumination.

A remarkable fact found in the present study is that the photo-bleaching of carotenoids in the presence of these inhibitors of the Hill reaction is suppressed by another group of inhibitors of the Hill reaction. The light-induced absorbance change at 490 mμ was strongly inhibited by substituted phenylureas such as DCMU and CMU and by symmetrical triazines such as propazine (2-chloro-4,6-bis-isopropylamino-*s*-triazine), simazine (2-chloro-4,6-bis-ethylamino-*s*-triazine) and prometryn (2-methylthio-4,6-bis-isopropylamino-*s*-triazine). The effects of the latter type of inhibitor on the absorbance change in the presence of CCCP are summarized in Table I. DCMU (Curve A in Fig. 4) was more effective than CMU (Table I), their concentrations for 50 % inhibition being 1.0 and 10 μM, respectively. Among the three triazine derivatives examined, prometryn was most effective, the inhibition obtained with 20 μM being as much as 86 % (Table I). These inhibitory effects on the absorbance change are closely related to their effects on the Hill reaction. DCMU is known to exhibit a stronger inhibitory action on the Hill reaction than CMU (refs. 19 and 20). In an experiment in the present study with *p*-benzoquinone as the oxidant, prometryn more effectively inhibited the Hill reaction than did simazine or propazine. *o*-Phenanthroline suppressed the absorbance change, but the effect of this weak inhibitor of the Hill reaction was lower than those of phenylurea or triazine derivatives (Table I). The rate of the photo-bleaching of carotenoids decreased as the Hill reaction activity decreased with increasing concentration of DCMU (Curves A and B in Fig. 4). The absorbance change observed at 490 mμ in the presence of 1 mM ferricyanide was also inhibited by DCMU or prometryn, the degree of inhibition being 80 % with 10 μM DCMU (Curve F in Fig. 2) and 70 % with 20 μM prometryn. The absorbance change in the presence of 1 mM hydroxylamine or 5 mM azide was inhibited almost completely by 10 μM

TABLE I

EFFECTS OF PHENYLUREAS, TRIAZINES AND *o*-PHENANTHROLINE ON THE RATE OF THE PHOTO-BLEACHING OF CAROTENOIDS IN THE PRESENCE OF 10 μM CCCP

The rate was estimated from the absorbance drop at 490 mμ during the first 3 min of illumination with red light.

Inhibitor	*Inhibition* (%)
None	0
10 μM DCMU	89
100 μM CMU	79
20 μM prometryn	86
20 μM propazine	68
20 μM simazine	68
100 μM *o*-phenanthroline	57

DCMU. DCMU (10 μM), CMU (100 μM) and prometryn (20 μM), when added alone in the absence of CCCP or other inducers of photo-bleaching, exhibited no effect in the light on the absorbance reading at 490 mμ.

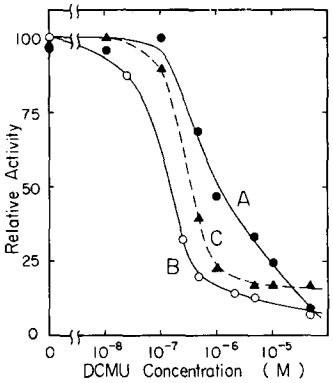


Fig. 4. The light-induced absorbance decrease at 490 mμ in the presence of 10 μM CCCP (Curve A), the oxygen evolution in the presence of 3 mM *p*-benzoquinone (B) and the light-induced oxygen uptake in the presence of 10 μM CCCP (C) as observed as a function of DCMU concentration. Others are the same as for Fig. 3.

Further evidence supporting the correlation between the photo-bleaching of carotenoids and the Hill reaction activity was obtained from the experiments with aged chloroplasts. In contrast to fresh materials, chloroplasts that had been aged for 24 h at 0° in the dark in 0.04 M phosphate buffer underwent a spectral change on illumination without added agents, and the change was similar in wavelength dependency to that observed for fresh chloroplasts treated with hydroxylamine or CCCP. However, the rate of change at 490 mμ observed for aged chloroplasts without added agents was low[18], at most 30 % of the rate observed in the presence of 10 μM CCCP. The light-induced absorbance change at 490 mμ observed for aged chloroplasts was inhibited by DCMU. Interestingly, the change was inhibited as much as 50 % by the addition of 2 mM NaCl; NaCl is known to restore the decreased Hill activity of aged or washed chloroplasts[21]. Fresh chloroplasts that had been treated for 3 min in the dark with a detergent such as sodium dodecylsulphate (1 mM) or Triton X-100 (0.1 %), or fresh chloroplasts heated at 50° for 10 min, underwent the photo-bleaching of carotenoids in the presence of 10 μM CCCP or 1 mM ferricyanide, but the rate was lower (10–40 %) than the control rate observed for untreated chloroplasts in the presence of CCCP or ferricyanide.

The light-induced decrease of absorbance at 490 mμ was suppressed by various redox agents. The effects of 1 mM ascorbate + 20 μM PMS, 1 mM ascorbate + 20 μM TMPD or 1 mM cysteine were the highest of those of various agents listed in Table II and examined in the presence of 10 μM CCCP. Cysteine is known to reverse the inhibitory effect of CCCP on photophosphorylation[18], and other agents in Table II activate the photochemical electron transport in chloroplasts. Ascorbate itself showed a weaker inhibitory effect than ascorbate + a cofactor (Table II and Curves C and D in Fig. 2). The light-induced absorbance change in the presence of hydroxylamine or azide was inhibited by more than 90 % by the addition of 1 mM ascorbate + 20 μM TMPD; and *p*-benzoquinone, which is known to serve as a Hill oxidant,

inhibited the absorbance change in the presence of CCCP (Table II). The redox agents in Table II seem to act as reducing agents rather than as oxidizing agents, because the change was inhibited by ascorbate or by trimethyl-*p*-benzohydroquinone, and because benzohydroquinone exhibited a stronger inhibitory effect than benzoquinone.

TABLE II

EFFECTS OF REDOX AGENTS ON THE RATE OF THE PHOTO-BLEACHING OF CAROTENOIDS IN THE PRESENCE OF 10 μM CCCP

The rate was estimated from the absorbance drop at 490 mμ during the first 3 min of illumination with red light.

Redox agent	*Inhibition* (%)
None	0
1 mM ascorbate	55
1 mM ascorbate + 20 μM PMS	95
1 mM ascorbate + 20 μM TMPD	95
1 mM ascorbate + 20 μM DCIP	87
1 mM trimethyl-*p*-benzohydroquinone	90
1 mM *p*-benzohydroquinone	60
1 mM *p*-benzoquinone	30
1 mM cysteine	97

Photo-bleaching of carotenoids as assayed by chromatography

The spectroscopic study of illuminated chloroplasts showed that carotenoids were bleached in the light in the presence of hydroxylamine, CCCP, azide or ferricyanide, and that the bleaching was suppressed by redox agents and some inhibitors of photosynthetic oxygen evolution such as DCMU and prometryn. The study was extended by chromatographic analysis of carotenoids in the chloroplasts illuminated in the presence of CCCP or ferricyanide. The results on aerobic photo-bleaching of carotenoids are summarized in Tables III and IV. In these tables, the light-induced changes of carotenoid contents are expressed as moles of carotenoids changed per 100 moles of chlorophylls. The chlorophyll content used as the reference was determined for the chloroplasts incubated in the dark for a given period without CCCP or ferricyanide, although the incubation under such conditions did not appreciably alter the chlorophyll content. Chloroplasts that had been stored in the dark in 0.04 M phosphate, contained 6.9 moles carotenes, 7.8 moles lutein, 4.8 moles violaxanthin and 2.8 moles neoxanthin per 100 moles of chlorophylls (the first column of Table III), which agreed with previously reported data[22]. Totally 8.1 and 6.1 moles of these carotenoids were bleached during 10 min of illumination in the presence of 15 mM ferricyanide and 20 μM CCCP, respectively, whereas these were almost unaltered without ferricyanide or CCCP (Table III). An interesting feature of the photo-bleaching is that neoxanthin was highly resistant to the action of light. The content of neoxanthin was almost unaffected by 10 min illumination in the presence of 20 μM CCCP (at most 10 % of the changes of other carotenoids) while the contents of carotenes, lutein and violaxanthin decreased by a similar extent of 2 moles (Table III). The decrease of neoxanthin in the presence of 5 mM ferricyanide after 10 min illumination was similarly small (the first column of Table Vb). The two minor xanthophylls designated previously[14] as X-443 and X-439 underwent no change on illumination in the presence of

20 μM CCCP or 15 mM ferricyanide. Further study was therefore directed to the four major carotenoids.

The two types of inhibition of the photo-bleaching by redox agents and by inhibitors of oxygen evolution were confirmed by chromatographic analysis. The photo-bleaching of each carotenoid component in the presence of 20 μM CCCP was inhibited by 20 μM PMS, and the bleaching in the presence of 15 mM ferricyanide or 20 μM CCCP was inhibited by 10 μM DCMU as seen from comparison of the results in Tables III and IV.

TABLE III

CONTENTS OF CAROTENOIDS IN THE DARK AND THEIR PHOTO-BLEACHING IN THE PRESENCE OF FERRICYANIDE OR CCCP AS ASSAYED BY CHROMATOGRAPHY

The contents in the dark and their changes (light-*minus*-dark) on 10 min illumination by red light are expressed in moles of carotenoids per 100 moles of chlorophylls in the chloroplasts kept in the dark without added agents.

Carotenoids	*Content in darkness*	*Light-induced change*		
		None	*15 mM* $Fe(CN)_6^{3-}$	*20 μM CCCP*
Carotenes	6.9	−0.1	−2.2	−2.0
Lutein	7.8	+0.1	−2.8	−1.8
Violaxanthin	4.8	0	−2.1	−2.1
Neoxanthin	2.8	0	−1.0	−0.2
Total	22.3	0	−8.1	−6.1

TABLE IV

PHOTO-BLEACHING OF CAROTENOIDS IN THE PRESENCE OF 20 μM CCCP + 20 μM PMS, 20 μM CCCP + 10 μM DCMU OR 15 mM FERRICYANIDE + 10 μM DCMU AS ASSAYED BY CHROMATOGRAPHY

Changes of carotenoid contents (light-*minus*-dark) on 10 min illumination by red light are expressed in moles of carotenoids per 100 moles of chlorophylls in the chloroplasts kept in the dark without added agents.

Carotenoids	*Light-induced change*		
	CCCP + PMS	*CCCP + DCMU*	$Fe(CN)_6^{3-}$ *+ DCMU*
Carotenes	−0.5	−0.4	0
Lutein	−0.4	−0.2	−0.3
Violaxanthin	+0.2	−0.2	0
Neoxanthin	+0.3	+0.2	+0.1
Total	−0.4	−0.6	−0.2

The carotenoid content of the chloroplasts incubated for 10 min in the dark in the presence of 20 μM CCCP or 5 mM ferricyanide was compared with that of the chloroplasts incubated for the same period in the dark without the agents. The result showed that CCCP in the dark did not affect the contents of the four carotenoids. By contrast, ferricyanide in the dark lowered the content of lutein, although the contents of the other carotenoids were unaltered. The rate of this dark-bleaching of lutein was approximately one half of that produced by illumination. The dark-

TABLE V

PHOTO-BLEACHING OF CAROTENOIDS IN AIR AS COMPARED WITH THAT IN A NITROGEN ATMOSPHERE

Chloroplasts were illuminated by red light for 10 min in air and under nitrogen flow in the presence of CCCP or ferricyanide. Differences in carotenoid contents (air-*minus*-nitrogen) are expressed in moles of carotenoids per 100 moles of chlorophylls in the chloroplasts kept in the dark without added agents.

Carotenoids	*Air*	*Nitrogen*	*Difference*
(a) 20 μM CCCP			
Carotenes	−2.0	−2.0	0
Lutein	−1.8	−0.3	−1.5
Violaxanthin	−2.1	−0.1	−2.0
Neoxanthin	−0.2	−0.2	0
(b) 5 mM ferricyanide			
Carotenes	−1.3	−1.3	0
Lutein	−1.1	−0.1	−1.0
Violaxanthin	−1.2	−0.2	−1.0
Neoxanthin	−0.1	−0.1	0

bleaching was suppressed by the addition of ascorbic acid in excess of the ferricyanide. This indicates that the dark-bleaching of lutein is due to oxidation by ferricyanide. The experiment on the light-induced change of carotenoids in the presence of ferricyanide was therefore performed with caution, the bleaching being terminated by the addition of two equivalents of ascorbate immediately after illumination. The dark-bleaching of lutein by 5 mM ferricyanide was not inhibited at all by 10 μM DCMU, whereas the photo-bleaching was inhibited by this reagent.

An experiment was conducted to see whether or not the photo-bleaching of carotenoids is due to oxidation by molecular oxygen. Chloroplasts were illuminated in air and under nitrogen flow in the presence of CCCP or ferricyanide, and the carotenoid contents of these samples were compared. Table V shows that lutein and violaxanthin were bleached more rapidly in air than in a nitrogen atmosphere either with CCCP or with ferricyanide. By contrast, the photo-bleaching of carotenes proceeded with an equal rate under these conditions. When estimated after 10 min of red light illumination, the difference in the total carotenoid content expressed in air-*minus*-nitrogen was 3.5 moles in the presence of 20 μM CCCP, and 2.0 moles in the presence of 5 mM ferricyanide. If the photo-bleaching of lutein or violaxanthin were due to the photo-oxidation caused by molecular oxygen, oxygen uptake would be observed during the bleaching. This was demonstrated in an experiment in the presence of CCCP (Curve C in Fig. 3). The rate of oxygen uptake in the light was enhanced by increasing the CCCP concentration, and reached a maximal value of 5.7 μmoles O_2 per mg of chlorophylls per h at 10 μM of CCCP. Furthermore, the oxygen uptake increased in parallel with the carotenoid bleaching determined by spectrophotometry (Curve A in Fig. 3). The oxygen uptake in the presence of CCCP was inhibited by DCMU as expected (Curve C in Fig. 4). These results indicate that the photo-bleaching of lutein and violaxanthin is coupled to a photo-oxidation reaction by molecular oxygen.

Trials were made to detect the photo-bleached product of carotenoids by means of thin-layer chromatography. Chloroplasts were illuminated in air by red light for

10 min in the presence of 20 μM CCCP or 5 mM ferricyanide, and carotenoids were extracted with diethyl ether from the saponified material. The chromatogram when inspected under ultraviolet irradiation at 365 mμ revealed a substance(s) showing yellow-green fluorescence near the origin. The absorption spectrum of this substance in ethanol is shown in Fig. 5, which indicates a typical three-banded spectrum of carotenoid. The three bands were located at much shorter wavelengths, 375, 397 and 421 mμ. The molar content of this carotenoid-like substance in the chloroplasts illuminated for 10 min in the presence of 20 μM CCCP was estimated to be 35 % of the molar content of neoxanthin in the dark, the molar extinction coefficient of this substance being assumed equal to that of neoxanthin. The content of this substance in the chloroplasts stored in complete darkness was low, being only 9 % of the neoxanthin content. This unidentified carotenoid, which is designated as xanthophyll-397 or simply as X-397, is inferred from its chromatographic behaviour to be most polar carotenoid in chloroplasts, containing more oxygen atoms than neoxanthin. Light petroleum containing acetone more than 20 % was required to move X-397 from the origin whereas the petroleum with 10 % acetone was sufficient to move neoxanthin. Addition of oxygen atoms to coloured carotenoids to produce X-397 may result in cleavage of their long conjugated double-bond system, thereby causing the great blue shift of the spectrum.

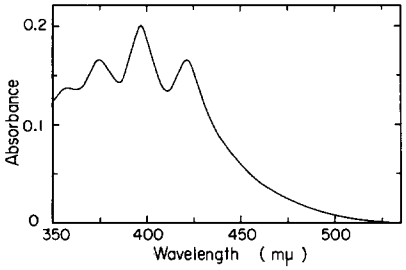


Fig. 5. The absorption spectrum of a photo-bleached product (X-397 in ethanol) extracted from the chloroplasts illuminated by red light for 10 min in the presence of 20 μM CCCP.

DISCUSSION

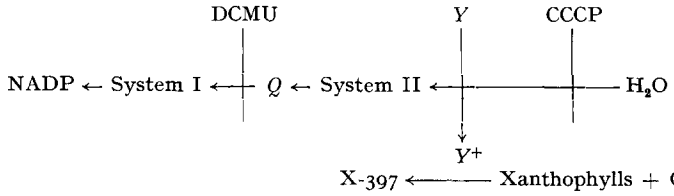
In the present study carotenoids in chloroplasts were bleached when illuminated by red light in the presence of azide, hydroxylamine, CCCP or ferricyanide. The fact that the photo-bleaching of carotenoids was suppressed by substituted phenylureas, symmetrical triazines and *o*-phenanthroline indicates its relevance to the Hill reaction. It must be stressed that the substances which induced the photo-bleaching of carotenoids are also the inhibitors of the Hill reaction, and that the rate of the bleaching increased in parallel with the lowering of the oxygen evolution caused by inhibitors or by ageing. Ferricyanide induced the photo-bleaching, despite the fact that it is not an inhibitor but is one of the most effective Hill oxidants. The action of ferricyanide on carotenoids may, therefore, be different in mechanism from that of azide, hydroxylamine or CCCP. This seems to be reflected in the observation that ferricyanide bleaches lutein in the dark whereas CCCP does not. Two specific forms of chlorophyll *a* in chloroplasts showing the red bands at 683 (ref. 23) and 700 mμ (ref. 24), respectively, are known to be bleached in the dark in the presence of ferricyanide. Ferricyanide

seems, therefore, to interact directly both with carotenoids and with chlorophyll *a*. The carotenoid bleaching is not necessarily effected by the Hill reaction, since an alternative Hill oxidant, *p*-benzoquinone (10 mM), was found in the present experiment not to cause photo-bleaching of carotenoids.

A salient feature of the present study is that phenylureas, triazines and *o*-phenanthroline suppress the photo-bleaching in the presence of azide, hydroxylamine or CCCP. This implies the existence of two types of inhibitor of the Hill reaction. There is, however, much evidence that all these inhibitors suppress the Hill reaction in a similar manner specifically nullifying the photochemical oxygen evolution in photo-system II (see a review by KOK AND CHENIAE[25] for the details of the two photo-systems). For instance, addition of 1.1 μM DCMU, 0.5 mM hydroxylamine or 8 mM sodium azide abolishes the photo-reduction of *f*-like cytochrome (photo-act II) in Porphyridium, but these agents do not inhibit the photo-oxidation of this cytochrome (photo-act I)[26]. Furthermore, the photo-reduction of NADP takes place in the presence of DCMU, hydroxylamine or CCCP, provided chloroplasts are supplied with ascorbate and DCIP (refs. 18, 27). On the other hand, phenylureas, triazines and *o*-phenanthroline are known to intensify the fluorescence emission from chloroplasts. According to DUYSENS[28], this intensification is due to accumulation of the reduced form of a primary oxidant, Q, for System II. It has been assumed, on the basis of these results, that inhibitors of the Hill reaction block the electron transport at a site between the two photo-systems; more exactly, between Q and System I. By contrast, JOLIOT[29] found that hydroxylamine, unlike CMU, does not increase the fluorescence intensity of Chlorella cells even when the oxygen evolution is completely inhibited by this agent, and that addition of CMU to the cells inhibited by hydroxylamine increases the fluorescence to the same level as obtainable with CMU alone. She concluded from these results and others that hydroxylamine interacts with a component designated as Y which functions between water and System II (see the scheme shown below). Probably, the inhibition of the Hill reaction by azide or CCCP is analogous in mechanism to that by hydroxylamine. This is supported by the result obtained by BANNISTER[30] that carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, an analogue of CCCP, does not raise but rather diminishes the fluorescence intensity of algal cells at a concentration (10 μM) which suffices for the complete inhibition of oxygen evolution.

In these circumstances we would propose that azide, hydroxylamine and CCCP inhibit the reduction of Y by water in System II, and that the carotenoid bleaching is a result of oxidation by the oxidized form of Y, namely Y^+, which accumulates in the light in the presence of these inhibitors. The oxidized form, Y^+, will be consumed by some reducing agent such as ascorbate and ascorbate + TMPD, thus explaining the observed inhibition of the bleaching by reducing agents. This interpretation is consistent with the fact that ascorbate *plus* phenylenediamine can supply electrons to the electron transport chain of chloroplasts at a site on the water side of System II (ref. 31). On the other hand, the amount of Y^+ formed by illumination is dependent on the amounts of the oxidants for System II which accept electrons from System II. Therefore, the amount of Y^+ will be decreased when the electron transport in chloroplasts is blocked between the two photosystems by DCMU. The photo-bleaching of carotenoids may thus be inhibited by DCMU or by analogous substances. Other effects of DCMU have not been clarified, and the electron transport driven by System I

is completely resistant to a high concentration (0.5 mM) of DCMU, CMU or atrazine (a symmetrical triazine)[32]. The carotenoid bleaching based on the above interpretation is illustrated below in relation to the photochemical electron transport in chloroplasts; xanthophylls are oxidized by Y^+ with aid of molecular oxygen to give X-397. Carotenes may also be oxidized by Y^+, but the oxidation proceeds without participation of molecular oxygen (see Table V).



Hydrogen peroxide, which is known to be formed by the Hill reaction in the presence of molecular oxygen (the Mehler reaction[33]), may not be the substance, Y^+, causing the photo-bleaching of carotenoids, because the bleaching was observable even when the Hill reaction was completely inhibited. If hydrogen peroxide be responsible for the photo-bleaching, the bleaching would be enhanced in the presence of potassium cyanide, a strong inhibitor of catalase. The bleaching did not occur at all in the light with 5 mM cyanide, as estimated from the absorbance change at 490 mμ. Furthermore, spectroscopic measurements showed that the rate of the photo-bleaching observed with 1 mM hydrogen peroxide alone was as low as 20% of the rate observed with 10 μM CCCP but without hydrogen peroxide.

Chloroplast-mediated photo-oxidations of 2,3-diketogulonate[34,35] and manganese[34,36] are known to be inhibited by DCMU. The DCMU concentration for 50% inhibition of the diketogulonate oxidation[34] was 0.7 μM, being in agreement with the concentration (1.0 μM) required for the inhibition of carotenoid bleaching. The photo-oxidations are, therefore, related to the photo-bleaching of carotenoids found in the present study.

Xanthophylls are known to undergo light-induced mutual conversion[2–4]. Such conversion was, however, not observed in the present study of isolated spinach chloroplasts illuminated in the presence of an inhibitor of the Hill reaction. The epoxidation of zeaxanthin and antheraxanthin to give violaxanthin[3] proceeded in red light[37] in the presence of molucular oxygen, so that it may be related to the photo-bleaching of carotenoids. Exhaustive oxidation of carotenoids may result in bleaching. The light-induced de-epoxidation of violaxanthin and antheraxanthin in algal cells was inhibited by DCMU and *o*-phenanthroline[38], while the de-epoxidation was similarly inhibited by CCCP (ref. 38) and hydroxylamine[38,39], another group of inhibitors classified in the present study. Apart from the detailed mechanisms involved, the photo-bleaching of carotenoids in chloroplasts may offer a clue to elucidation of the photochemical events in photosynthesis.

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