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# ROLE OF β-CAROTENE IN THE REACTION CENTRES OF PHOTOSYSTEMS I AND II OF SPINACH CHLOROPLASTS PREPARED IN NON-POLAR SOLVENTS

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## Summary

Spinach chloroplasts have been prepared nonaqueously using non-polar solvents (*n*-hexane, $CCl_4$, *n*-heptane) and the β-carotene content extracted in a controlled manner. This procedure is reproducible and does not result in large structural or spectral changes of the chloroplasts. The organisation of the chlorophyll-proteins is unaltered, as fragmentation with digitonin results in the appearance of the same fractions as found previously for aqueously-prepared chloroplasts, including the pink zone containing cytochromes *f* and $b_6$ in the ratio 1 : 2. The chloroplasts possess both Photosystem I activity (*P*-700 photobleaching, and $NADP^+$ photoreduction) and Photosystem II activity (parabenzoquinone reduction with $Mn^{2+}$ as electron donor, and chlorophyll fluorescence induction). Use of moderate intensity red illumination has allowed a study of the role of β-carotene in photochemistry separate from its roles in energy transfer and photoprotection.

Removal of the fraction of β-carotene closely associated with the Photosystem I reaction centre caused the rate of $NADP^+$ photoreduction to fall to a low, but significantly non-zero level. Thus, in the complete absence of β-carotene, photochemistry can still be observed, however the specific association of β-carotene with the reaction centre is required for maximal rates. We propose that β-carotene bound at the reaction centre decreases the rate of transfer of excitation energy away from the reaction centre, and increases the rate of photochemistry. It is possible that this occurs via formation of an exciplex between ground state β-carotene and chlorophyll in the first excited state.

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Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DCPIP, dichlorophenolindophenol; Chl, chlorophyll.

## Introduction

β,β-Carotene (β-carotene) and lutein are the major carotenoids in the thylakoid membrane of higher plant chloroplasts [1]. Whereas lutein appears to be primarily associated with the light-harvesting pigment complex, it is found that purified Photosystem I preparations have a higher β-carotene content compared to unfractionated chloroplasts [2]. More recently it has been possible to isolate purified Photosystem II particles free from light-harvesting chlorophyll *a/b* protein, and to show that these particles are also enriched in β-carotene relative to lutein (for a recent review see ref. 3). Thus β-carotene appears to be associated with the antenna pigments of the photosystems, whilst the xanthophylls (lutein, violaxanthin and neoxanthin) are located primarily in the light-harvesting accessory pigment system. This suggests that β-carotene could play a role in photochemistry, as initially proposed by Lynch and French [4], in addition to its function in energy transfer [5]. It has also been suggested that β-carotene is involved in photoprotection: both in the deactivation of chlorophyll triplets (the valve reaction of Witt, ref. 6) and the deactivation of singlet oxygen [7].

It appears that, although most of the β-carotene is present in the antenna pigments, a small proportion is associated closely with the reaction centres. Indeed about 1 mol β-carotene per Photosystem I reaction centre is found in Triton X-100-prepared Photosystem I fractions [8,9]; and in reaction centre preparations from photosynthetic bacteria, one carotenoid molecule per reaction centre is also seen [10].

In Photosystem II, the C-550 change first discovered by Knaff and Arnon [11] has been shown to be closely associated with the reaction centre and furthermore to require the presence of β-carotene [12]. However, Cox and Bendall [12] and Knaff et al. [13] have demonstrated that C-550 is not directly involved in electron transport, but acts rather as an indicator of the redox state of the primary acceptor of Photosystem II. In contrast, recent fluorescence lifetime studies on mixtures of chlorophyll *a* and β-carotene in organic solvents appear to suggest the possibility that the primary charge separation at the reaction centre could be obligatorily mediated by a β-carotene molecule [14].

To date little is known about the way in which β-carotene is bound at the reaction centre. It is likely that it is associated with protein, and indeed a minor specific β-carotene-protein conjugate has been isolated [15], which might represent reaction centre β-carotene [16]. Junge and coworkers have provided evidence from circular dichroism studies on chloroplast fragments that the large β-carotene pool associated with the Photosystem I antenna pigments is highly orientated [17], which suggests a high degree of organisation, indicative of specific binding to protein.

In this report we have investigated the requirement for β-carotene in spinach chloroplast photoreactions by preparing chloroplasts depleted in β-carotene, using the 'non-aqueous' chloroplast preparation technique [18]. We believe that this method largely overcomes the technical problems inherent in the solvent extraction of aqueously-prepared chloroplasts.

## Materials and Methods

Spinach (*Spinacea oleracea*) was grown in a greenhouse at 24°C under a 16-h light/8-h dark regime and used after 3—4 weeks. Before harvesting, the starch content of the chloroplasts was reduced by placing the plants in the dark for 20 h. The leaf material was stripped from the stems and large veins, placed in a mortar, and liquid nitrogen added. After lyophilisation, the dry leaves were powdered using a pestle and the last residues of water removed by storing the leaf powder in vacuo over $P_2O_5$ at −20°C for several days.

Chloroplasts were isolated from this powder by homogenisation at a low temperature in a *n*-hexane/$CCl_4$ mixture followed by density gradient centrifugation. Great care was taken to prevent atmospheric moisture from condensing on the dried leaf powder or on the dry isolated chloroplasts. To achieve this, all manipulations with dry material were carried out inside a deep-freeze cabinet at constant temperature and low humidity.

Lyophilised leaf powder (10 g) was homogenised for four times 1 min in 200 ml *n*-hexane/$CCl_4$ (50 : 50, v/v) using a Sorvall Omnimixer fitted with Teflon end bearings and a Teflon sealing ring. Immersion of the stainless-steel homogenisation vessel in a salt/ice mixture kept the temperature below −10°C. The homogenate was layered over an equal volume of *n*-hexane/$CCl_4$ (25 : 75, v/v) and centrifuged in a swing-out rotor at 2000 × g for 10 min. After the yellow upper zone was pipetted off, the chloroplasts at the interface could be decanted, together with the lower zone, away from the green precipitate into an equal volume of *n*-hexane. The chloroplasts were centrifuged at 2000 × g for 10 min, and then treated further with *n*-hexane/$CCl_4$ mixtures below 0°C in order to extract the larger part of the β-carotene. These chloroplasts, retaining about 10% of the original β-carotene content will be referred to as 'low-temperature extracted chloroplasts'. To remove β-carotene completely, the chloroplasts were further extracted with *n*-heptane for 4 h at 24°C [12]; these chloroplasts contained no detectable β-carotene (<1 mol β-carotene/1000 mol chlorophyll) and will be referred to as 'room-temperature extracted chloroplasts'. These is no detectable loss of chlorophyll from the chloroplasts during the preparation. Finally, the chloroplasts were resuspended in a minimal volume of *n*-hexane, placed in a desiccator over $P_2O_5$ and the solvent was removed under vacuum. The dry chloroplasts could be stored in the dark at −20°C in vacuo for several days without significant change in their properties. In a typical preparation 100 g fresh leaves would give 10 g dried leaves, which on homogenisation would yield 600 mg chloroplasts equivalent to approx. 30 mg chlorophyll.

Extracted chloroplasts could be resuspended in buffer and fractionated by treatment with digitonin followed by sucrose density gradient centrifugation as described previously [19]. The chloroplasts were incubated with 1.3% digitonin (w/v) at a digitonin/chlorophyll ratio of 10 : 1 (w/w) in 50 mM Tris-HCl (pH 7.8) buffer, containing 2 mM sodium EDTA and 5 mM $MgCl_2$.

The pigment composition of chloroplasts and digitonin-derived fractions was analysed by thin-layer chromatography on silica gel plates [20], and estimation of the separated chlorophylls and carotenoids was carried out as described previously [2].

The activity associated with Photosystem I in the chloroplasts and in $F_I$, the digitonin-derived Photosystem I fraction, was measured by the photoreduction of $NADP^+$ using ascorbate and 2,6-dichlorophenolindophenol (DCPIP) as electron donor couple. Plastocyanin, ferredoxin and ferredoxin : $NADP^+$ reductase were prepared by the method of Borchert and Wessels [21]. NADPH was estimated either enzymatically with oxidised glutathione and glutathione reductase [22], or spectrophotometrically by the increase in absorbance at 340 nm in a Cary 14 recording spectrophotometer adapted for side illumination with red light [23].

Photosystem II activity in the extracted chloroplasts was measured on the addition of 2,5-cyclohexadiene-1,4-dione (parabenzoquinone) as Hill oxidant ($E_m$ at pH 6.5 = 325 mV) and $Mn^{2+}$ as electron donor. The reaction mixture was contained in a Thunberg cuvette, which was carefully evacuated and gassed with nitrogen several times in dim green light in order to remove all traces of oxygen. The absorbance increase at 290 nm was observed in the Cary 14 spectrophotometer. Using freshly sublimed parabenzoquinone, and sodium acetate buffer, pH 6.5, both parabenzoquinone and 1,4-benzenediol (hydroquinone) were stable in the presence of $Mn^{2+}$.

Absorption spectra were measured on a Cary 14 or Aminco-Chance dual beam spectrophotometer. *P*-700 and cytochromes were estimated spectrophotometrically on the Aminco-Chance dual beam spectrophotometer using a molar absorbance at the absorption maximum of 64 000 $cm^{-1}$ for *P*-700 [24], and the molar absorbances for cytochromes *f* and $b_6$ given by Heber et al. [25]. The apparatus used to study the chlorophyll fluorescence induction of dark-adapted chloroplasts has been described previously [26]. Chlorophyll concentrations were measured using the method of Bruinsma [27].

## Results

*The characterisation of extracted chloroplasts*

Fig. 1 shows the structure of the thylakoid membranes of extracted chloroplasts, fixed in 50% acetone and stained with $OsO_4$ according to the procedure of Stocking et al. [28]. It is seen that distinct membranes can still be observed in an electron-dense stroma, although the stacked membranes in the grana regions are less well resolved.

Fig. 2A gives the absorption spectrum of extracted chloroplasts, resuspended in 50 mM Tris-HCl pH 7.8 containing 2 mM sodium EDTA and 5 mM $MgCl_2$. Apart from the shift of the maximum of the chlorophyll *a* long-wavelength absorption envelope by approx. 2 nm to shorter wavelengths, no changes in chlorophyll absorption are apparent after extraction of β-carotene.

Extracted chloroplasts show a shift of the chlorophyll maximum in aqueous media from 677 to 681 nm when a diffusing plate is not placed between the sample and the photodetector. This could arise from wavelength-selective scattering [29] due to a larger difference between the refractive indices of the chloroplasts and the suspending medium for lipid-depleted compared to untreated chloroplasts.

When the dry extracted chloroplasts are resuspended at 0°C in a *n*-hexane/$CCl_4$ mixture (with a density close to that of the chloroplasts in order to mini-

Fig. 1. An electron micrograph, of spinach chloroplasts prepared in *n*-hexane/$CCl_4$, after fixation and staining with $OsO_4$; magnification X32 500.

mise settling), the long-wavelength chlorophyll *a* absorption envelope is different from that in aqueous media. The absorption maximum is shifted to 666 nm and furthermore a fine structure can be observed (Fig. 2B). It should be stressed that the chlorophyll is not solubilised under these conditions even though the wavelength of the absorption maximum is comparable to that reported for chlorophyll *a* in non-polar solvents [30]. The fine structure of the absorption band is lost and the absorption maximum shifted slightly to 665 nm on solubilisation of the chlorophyll on standing the suspension in *n*-hexane/$CCl_4$ at room temperature for several hours. As extracted chloroplasts suspended in aqueous media do not show these inflections, the resolution of

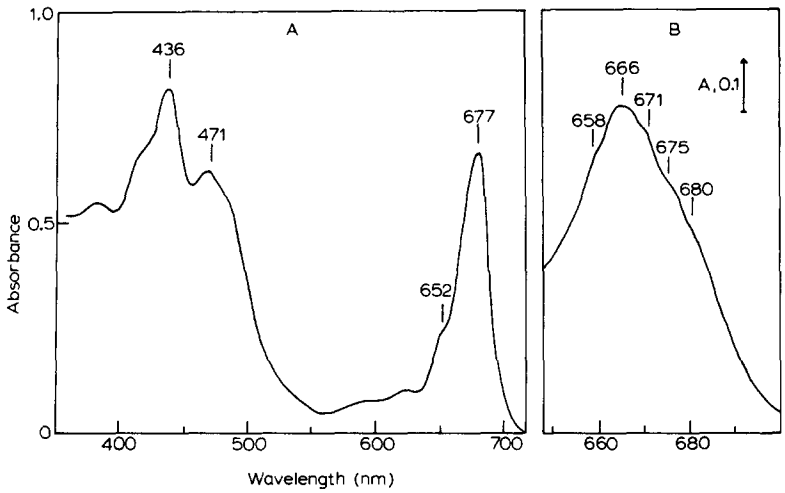


**Fig. 2. Absorption spectra of extracted chloroplasts. (A) The complete visible spectrum after resuspension in buffer. (B) The long-wavelength chlorophyll *a* maximum after resuspension in *n*-hexane/$CCl_4$ at 0°C. The spectra were obtained on a Cary 14 spectrophotometer with a diffusing plate to prevent wavelength-selective scattering artefacts.**

the envelope fine structure can probably be attributed to the diffusion of a solvent of low dielectric constant into the thylakoid membrane. It is possible that under these conditions the different spectral forms of chlorophyll *a* can be resolved directly, without the use of first derivative spectra or cryogenic temperatures. The locations of the broad absorption maximum (666 nm) and shoulders (658, 671, 675 and 680 nm; see Fig. 2B) are similar to the forms derived by Elgersma and Voorn [31] by deconvolution of room temperature absorption spectra of spinach chloroplasts (661, 668, 676, 682, 689 and 699 nm). It is apparent that the presence of non-polar solvent can alter the relative proportions of these spectral forms.

*Digitonin fractionation of extracted chloroplasts*

In order to compare chlorophyll organisation in extracted chloroplasts with that in untreated chloroplasts, the chloroplasts were fractionated with digitonin as described in Materials and Methods. As shown in Fig. 3A, the pattern of bands on the sucrose density gradient is apparently similar to that for unextracted chloroplasts [3], suggesting that the characteristic chlorophyll-containing complexes are still present. The upper band of solubilised pigment lacks any yellow carotenoid zone and does not contain a higher proportion of chlorophyll than is found for unextracted chloroplasts. The light-harvesting $F_{III}$ complex is yellow-green and easily identified from its pronounced chlorophyll *b* content (Fig. 3). The pink zone contains cytochromes *f* and $b_6$ in the 1 : 2 ratio normally found for the cytochrome-containing particle prepared with digitonin (Fig. 4). There is no indication of a large change in the redox potential of these cytochromes from chloroplasts extracted with non-polar solvents. Compared to fractions from unextracted chloroplasts the two lower chlorophyll-containing bands appear to have reversed their positions, although definite identification has not yet been possible. It was found to be more diffi-

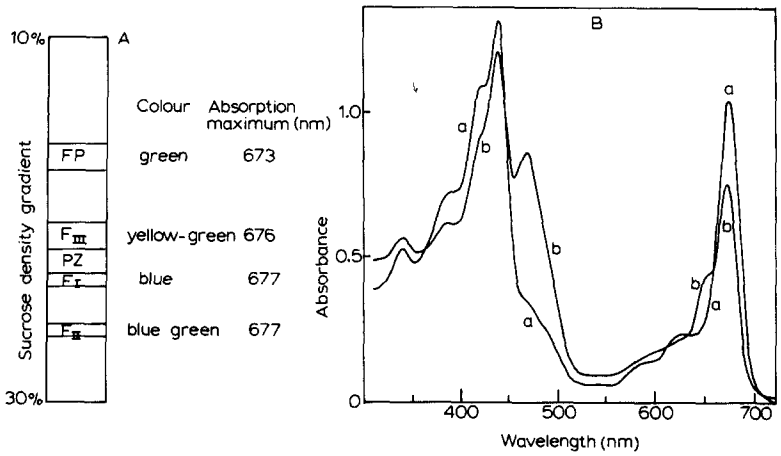


**Fig. 3. Digitonin-derived fractions from extracted chloroplasts. (A) The pattern of coloured bands on a 10—30% (w/w) sucrose density gradient. (B) The absorption spectrum of the fractions (a) $F_I$ (Photosystem I) and (b) $F_{III}$ (light-harvesting). FP, free pigment; PZ, pink zone containing cytochromes *f* and $b_6$.**

cult to obtain the band denoted $F_{II}$ free of chlorophyll *b*, as might be expected for a Photosystem II fraction; and also $F_{II}$ was a minor component compared with $F_I$. The $F_I$ contains 1 mol *P*-700/95 mol chlorophyll, with a ratio of chlorophyll $a/b > 10$; its absorption maximum is at 677 nm compared to 679 nm for $F_I$ from unextracted chloroplasts. This small spectral shift could be caused by the removal of β-carotene, but in general the chlorophyll organisation is disturbed to a remarkably small extent in chloroplasts prepared in non-polar solvents.

*Photosystem I activity*

In agreement with previous reports (e.g., ref. 32) the complete extraction of β-carotene does not prevent the reversible photobleaching of *P*-700 in chloroplasts (Fig. 5). *P*-700 represents either the reaction centre chlorophyll, or an electrochromic indicator closely associated with the reaction centre [33], and its photobleaching therefore depends upon an active Photosystem I. It is clear that β-carotene cannot be required absolutely for the primary charge separation as suggested by Beddard and coworkers [14].

Using the artificial donor couple, ascorbate/DCPIP, the Photosystem I-dependent reduction of $NADP^+$ can be studied in extracted chloroplasts and $F_I$ (Fig. 6). In low-temperature extracted chloroplasts, which we believe contain only the reaction centre β-carotene, the rate of $NADP^+$ photoreduction in red light is still high (Table I). Addition of ferredoxin is found to be essential, and plastocyanin addition stimulates the rate compared to that with DCPIP alone; however, the addition of ferredoxin : $NADP^+$ reductase is not necessary.

Measurements of $NADP^+$ photoreduction by $F_I$ isolated from room-temperature extracted chloroplasts show that even in the complete absence of β-carotene a significant rate can still be observed: 12 μmol/mg chl per h (Table I). The $NADP^+$ photoreduction by $F_I$ requires the addition of all three enzymes, and its total dependence on plastocyanin distinguishes this photo-

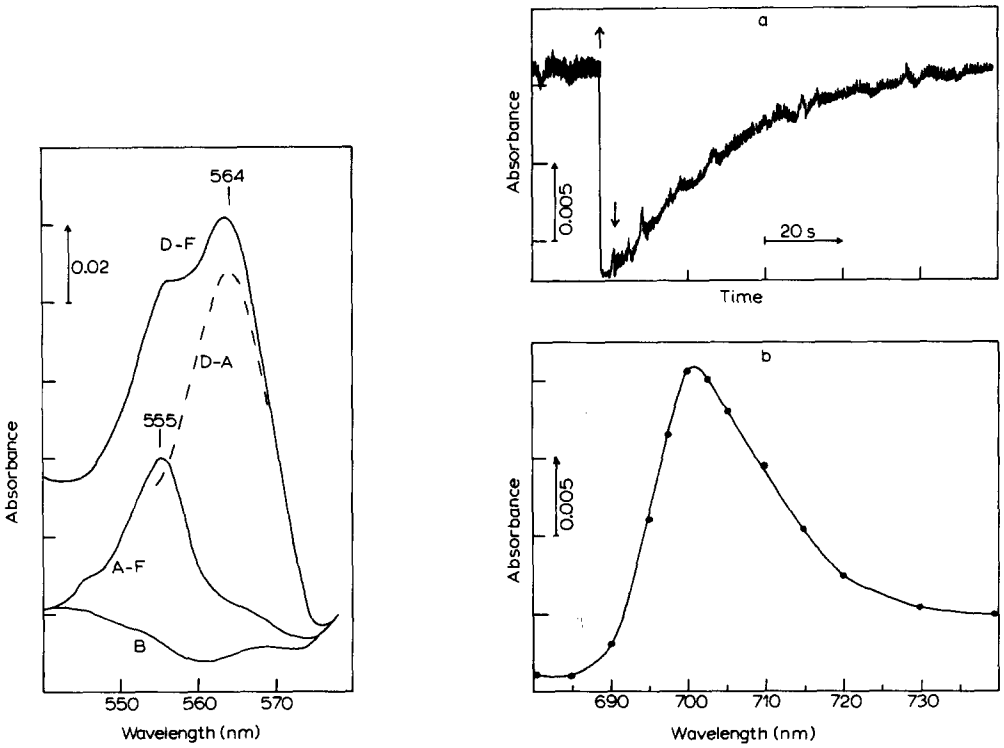


**Fig. 4. Cytochromes of the pink zone from the sucrose density gradient. The absorption spectra were measured on an Aminco-Chance dual beam spectrophotometer in the split beam mode. Additions were: F, ferricyanide; A, ascorbate; and D, dithionite. The dashed line (D-A, cytochrome $b_6$) represents the calculated difference spectrum between the two experimental curves: A-F (cytochrome *f*), and D-F. B is the baseline.**

**Fig. 5. The light-induced *P*-700 absorption change in room-temperature extracted chloroplasts. The extent of the slow absorption increase after the side-illumination was turned off (for example $A_{705}-A_{740}$, (a)), is plotted as a function of monitoring wavelength ($A_\lambda-A_{740}$, (b)); the reference wavelength was 740 nm. The spectra were obtained on an Aminco-Chance dual beam spectrophotometer at a chlorophyll concentration of 90 μg/ml; pathlength 10 mm. The green side-illumination had an intensity of 80 W/m$^2$. Upward pointing arrow light on, downward pointing arrow light off.**

activity from the NADP$^+$ photoreduction carried out by detergent-solubilised chlorophyll [23].

In Photosystem I, it appears that β-carotene is not essential for either *P*-700 photobleaching or for NADP$^+$ photoreduction, but the removal of the smaller, more tightly bound fraction of β-carotene (which we believe to be the reaction centre β-carotene) does greatly reduce the NADP$^+$ photoreduction rate.

*Photosystem II activity*

Non-aqueously prepared chloroplasts cannot oxidise water, however artificial electron donors and acceptors can be used to demonstrate the activity of Photosystem II. Fig. 7 illustrates the absorbance increase which can be observed at 290 nm on illumination of low-temperature extracted chloroplasts in the presence of parabenzoquinone. The change is completely light-dependent, and requires the presence of both $MnCl_2$ and parabenzoquinone.

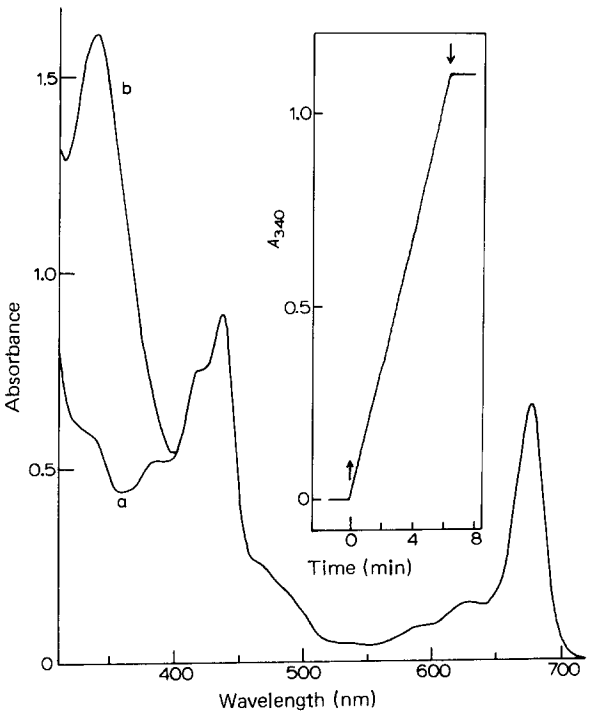


Fig. 6. $NADP^+$ photoreduction by $F_I$ from low-temperature extracted chloroplasts. Curves a and b are spectra of the reaction mixture before and after 6 min red light illumination, respectively. The inset shows the increase in $A_{340}$ due to the formation of NADPH (upward arrow light on; downward arrow light off). The composition of the reaction mixture is the same as in Table I. The spectra were taken on a Cary 14 spectrophotometer.

TABLE I

$NADP^+$ PHOTOREDUCTION BY EXTRACTED SPINACH CHLOROPLASTS AND THE DIGITONIN-DERIVED PHOTOSYSTEM I COMPLEX ($F_I$)

The sample volume of 3 ml contained: 4 μmol $NADP^+$, 10 μmol ascorbate, 0.1 μmol DCPIP, 6 μmol $NH_4Cl$, 0.2 mg ferredoxin, 0.2 unit ferredoxin : $NADP^+$ reductase, 0.1 mg plastocyanin, 100 μmol Tris · HCl buffer, pH 7.3, and chloroplasts or $F_I$ equivalent to 30 μg chlorophyll. The red side-illumination had an intensity of 55 $W/m^2$, which gave a rate for $F_I$ in the complete system which was 85% of the maximal rate at saturating light intensity. Temperature: 21°C. The samples were stirred continuously. Pcy, plastocyanin; Fd, ferredoxin; and Rd, ferredoxin: $NADP^+$ reductase.

| | Additions | | | $NADP^+$ photoreduction (μmol/mg chl per h) |
|---|---|---|---|---|
| | Pcy | Fd | Rd | |
| Chloroplast * | + | + | + | 18 |
| | — | + | + | 6 |
| | + | — | + | 0 |
| | + | + | — | 17 |
| $F_I$ ** | + | + | + | 165 |
| | — | + | + | 0 |
| | + | — | + | 6 |
| | + | + | — | 10 |
| $F_I$ *** | + | + | + | 12 |

* Low-temperature extracted chloroplasts.
** Prepared from low-temperature extracted chloroplasts.
*** Prepared from room-temperature extracted chloroplasts.

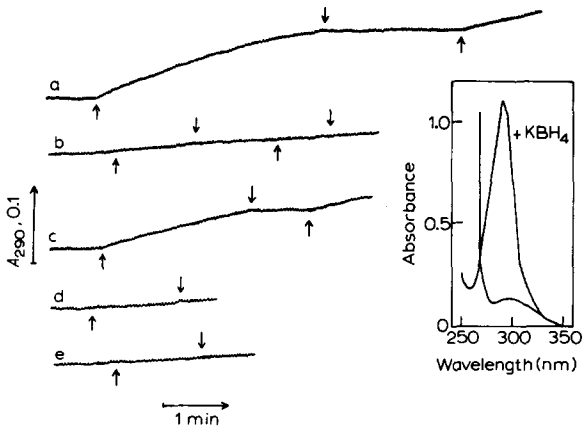


Fig. 7. Parabenzoquinone reduction by Photosystem II of extracted chloroplasts with $Mn^{2+}$ as electron donor. The sample (3 ml) in a Thunberg cuvette (pathlength 10 mm) contained unless otherwise stated 0.1 M sodium acetate buffer, pH 6.5, 1 mM parabenzoquinone, 3.3 mM $MnCl_2$ and low-temperature extracted chloroplasts equivalent to 50 $\mu$g chlorophyll. All samples were anaerobic with nitrogen as gas phase, and were stirred continuously. Temperature: 21°C. The absorbance changes were measured on a Cary 14 spectrophotometer, with a Schott UG 11 filter to protect the photomultiplier. The intensity of the side illumination (100 W/$m^2$) was saturating. The curves shown are for (a) the complete reaction mixture; (b) addition of 13 $\mu$M DCMU; (c) addition of 0.16% ethanol (v/v), equivalent to that added in (b), ethanol control; (d) $MnCl_2$ omitted; and (e) parabenzoquinone omitted. Upward pointing arrows indicate light on and downward pointing arrows light off. The inset shows the change in the absorption spectrum of 0.5 mM parabenzoquinone on reduction with solid potassium borohydride; the molar absorbance increase at 290 nm for parabenzoquinone on reduction to hydroquinone is 2250 $cm^{-1}$.

This absorbance change appears to represent the photoreduction of parabenzoquinone by the chloroplasts. Addition of 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea (DCMU) inhibited the reaction, showing its dependence on Photosystem II. Control experiments demonstrated the stability of parabenzoquinone and its reduction product, hydroquinone, anaerobically in the presence of $MnCl_2$, both in the dark and on illumination, when sodium acetate buffer is used.

A steady decrease in the rate of change of $A_{290}$ occurs during several minutes illumination, which under these anaerobic conditions is probably not due to photoinactivation, but rather to the build-up of hydroquinone. The hydroquinone can then compete effectively with $Mn^{2+}$ as donor to Photosystem II [34], giving no net parabenzoquinone reduction. This is seen in Table II, where

TABLE II

EFFECT OF HYDROQUINONE ON PARABENZOQUINONE PHOTOREDUCTION BY LOW-TEMPERATURE EXTRACTED CHLOROPLASTS

Parabenzoquinone reduction was monitored by the increase in absorption at 290 nm on illumination. The photoreduction rates shown are initial rates. Other conditions as for Fig. 7.

| Hydroquinone added (mM) | Parabenzoquinone photoreduction ($\mu$electronequiv./mg chl per h) |
|---|---|
| None | 98 |
| 0.05 | 42 |
| 0.25 | 26 |

addition of hydroquinone at the start of the experiment causes an immediate decrease in the rate of absorbance change observed.

Electron flow rates through Photosystem II of approx. 100 μelectronequiv./mg chl per h are found for low-temperature extracted chloroplasts using this electron donor/acceptor system (Table II). It was found difficult to obtain reproducible rates of $MnCl_2$-supported parabenzoquinone reduction by unextracted control chloroplasts (isolated from the lyophilised leaf powder after rehydration). However, electron flow rates similar to those of low-temperature extracted chloroplasts have been reported for $MnCl_2$-supported photoreductions in aqueously-prepared chloroplasts, in which oxygen evolution has been inhibited. For example, Izawa found a rate of 100—150 μelectronequiv./mg chl per h for DCPIP reduction in EDTA-washed heat-inactivated chloroplasts at pH 6.5 [35]. It therefore appears that low-temperature extraction has a minimal effect on Photosystem II activity.

The results with room-temperature extracted chloroplasts are more variable, and rates between 0—30 μelectronequiv./mg chl per h are seen. Difficulty in consistently demonstrating a small residual rate of parabenzoquinone reduction in the complete absence of β-carotene could arise from concomitant extraction of the specialised plastoquinone molecule R, assuming that parabenzoquinone reduction is mediated by this species (Knaff et al. [13] reported that extraction with *n*-hexane at 24°C for 4 h removed about 85% of the plastoquinone, leaving only approx. 1 mol plastoquinone per Photosystem II reaction centre, the primary acceptor Q).

The yield of the fraction $F_{II}$ from the sucrose gradient (Fig. 3) was too small for a study of its photochemical activity to be possible, although a DCMU-sensitive parabenzoquinone photoreduction supported by $MnCl_2$ is detectable in the unfractionated digitonin extract of low-temperature extracted chloroplasts.

Our conclusion therefore is that extraction of the larger part of the β-carotene does not affect Photosystem II activity. Room-temperature extraction of the smaller, more tightly bound fraction of β-carotene diminishes the activity to a low non-zero level, although some preparations give zero activity probably as the result of extraction of R.

*Chlorophyll fluorescence*

In order to demonstrate more conclusively Photosystem II activity in chloroplasts from which the β-carotene has been completely extracted we have monitored chlorophyll fluorescence. As shown above (Fig. 3), there is no evidence for any disruption of the chlorophyll organisation in extracted chloroplasts, and the steady state fluorescence yield is comparable to that of unextracted chloroplasts (Fig. 8).

The increase in the probability for transfer of the excitation energy out of the reaction centre to the antenna chlorophylls (see ref. 36), when the open reaction centres become closed on illumination, would be expected to be smaller for dark-adapted extracted chloroplasts compared to unextracted chloroplasts if the removal of reaction centre β-carotene caused the efficiency of photochemical trapping to become less than 100%. This would give rise to a decrease in the amount of variable fluorescence, and an increased $F_0$ level.

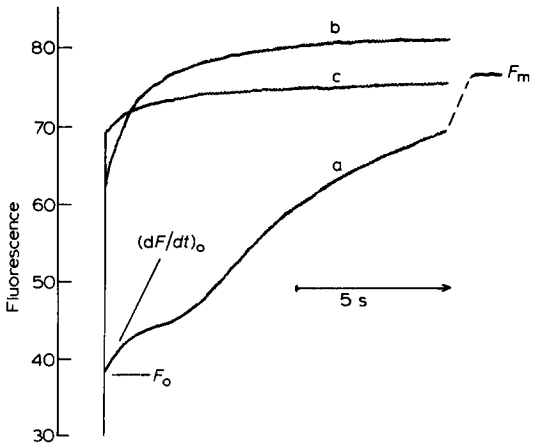


Fig. 8. Chlorophyll fluorescence induction in dark-adapted chloroplasts. The samples, in a 10 X 10 mm cuvette, were illuminated with 530 nm light of low intensity (1 W/m$^2$) and the increase in fluorescence yield was monitored at 90°. For curve a unextracted chloroplasts were suspended in 50 mM potassium phosphate buffer pH 7.0 containing 10 mM NaCl; for curve b low-temperature extracted chloroplasts were resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM NaCl, and 60 mM $NH_2OH$ as electron donor; and for curve c room-temperature extracted chloroplasts were resuspended in 50 mM sodium acetate buffer, pH 6.5, and 1.0 mM $MnCl_2$ added. The concentration of the samples was 2.7 μg chlorophyll/ml. The amount of variable fluorescence ($F_v$) is given by $F_m - F_0$. $(dF/dt)_0$ is the initial rate of increase of the fluorescence yield.

A smaller variable fluorescence ($F_v$) and larger $F_0$ value is indeed observed (Fig. 8): $F_v$ is 10% of $F_0$ for room-temperature extracted chloroplasts (curve c), compared to 100% of $F_0$ for unextracted chloroplasts (curve a) under the same conditions. The presence of some variable fluorescence indicates that the rate of photochemistry does not fall to zero on complete removal of β-carotene. In low-temperature extracted chloroplasts (curve b), $F_v$ appears to be about 30% of $F_0$, although it may be underestimated by the time resolution of the detecting system. In contrast to unextracted chloroplasts [37], addition of DCMU does not change either the value of the initial slope $(dF/dt)_0$ or the amount of $F_v$, owing to the absence of the bulk plastoquinone pool. The effectiveness of $MnCl_2$ as electron donor is seen in Table III by the increase in both $F_v$ and

TABLE III

CHARACTERISTICS OF THE CHLOROPHYLL FLUORESCENCE YIELD INDUCTION CURVE FOR DARK-ADAPTED EXTRACTED CHLOROPLASTS

Room-temperature extracted chloroplasts were resuspended in 50 mM sodium acetate buffer, pH 6.5. Fluorescence is expressed in arbitrary units, and the maximum level, seen with dithionite added, was 93 units. $F_v$ is the amount of variable fluorescence, and $(dF/dt)_0$ is the initial rate of increase of the fluorescence yield.

| Addition | $F_v$ | $(dF/dt)_0$ |
|---|---|---|
| None | 6 | 3.2 |
| 0.1 mM $MnCl_2$ | 6 | 4.3 |
| 1.0 mM $MnCl_2$ | 11 | 8.3 |
| Dithionite | 0 | 0.0 |

$(dF/dt)_0$ on its addition. When dithionite is present no induction is observed and the fluorescence rises directly to the maximum level.

## Discussion

Although chlorophyll *a* is the most important pigment of photosynthetic systems, some accessory pigments also play vital roles. Of the carotenoids, β-carotene appears to be the most essential in higher plant chloroplasts, as it can transfer excitation energy to chlorophyll in the antenna pigment complexes [5], and is also closely associated with the photochemical reaction centres of Photosystem I [9] and II [12].

A useful experimental technique in the study of the role of β-carotene has been to observe the effect of its removal by non-polar solvents, although complications can arise from simultaneous extraction of quinones (e.g., ref. 13). It is possible that readdition of purified β-carotene to chloroplasts extracted in the way described in this report would be more specific than that normally observed in extracted aqueously-prepared chloroplasts, where considerably more β-carotene must often be added back than was originally present, in order to obtain maximum restoration [12,16]. Our conclusion (see below), that only the loss of reaction centre β-carotene affects photochemical activity, would be supported if it could be shown that full restoration of activity to room-temperature extracted chloroplasts occurred on the readdition of only 1—2 mol β-carotene per reaction centre.

The extraction of chloroplasts prepared in aqueous media by non-polar solvents is not an easy technique, especially if sugars are present as osmoticum. On the other hand, the non-aqueous procedure for chloroplast isolation described in this study is a relatively simple method for the reproducible extraction of β-carotene. We have shown that chloroplasts prepared in this way retain their chlorophyll organisation; both from the absorption spectrum (Fig. 2) and the digitonin-derived chlorophyll fractions (Fig. 3).

Chloroplasts prepared in non-polar solvents possess both Photosystem I activity (ascorbate/DCPIP → $NADP^+$; Fig. 6 and Table I) and Photosystem II activity ($Mn^{2+}$ → parabenzoquinone, inhibited by DCMU; Fig. 7 and Table II). With the moderate intensity red light used in these experiments the functions of β-carotene in energy transfer and in photoprotection need not be considered; the effect of removal of β-carotene on the above activities then reflects the role of β-carotene in the photochemistry of the two photosystems.

We suggest that partial extraction with *n*-hexane/$CCl_4$ at low temperature only removes the bulk β-carotene of the antenna pigment systems; and the rate of $NADP^+$ photoreduction by $F_I$, for example, is comparable to that seen in $F_I$ from unextracted chloroplasts (Table I). On complete extraction (*n*-heptane at 24°C for 4 h) the β-carotene associated with the reaction centre is also lost and this results in a large decrease in the $NADP^+$ photoreduction rate. However, it is important to note that in completely extracted chloroplasts the activity does not fall to zero. This is supported by the photobleaching of *P*-700, a primary photochemical reaction of Photosystem I, in chloroplasts which completely lack β-carotene (Fig. 5). However, we cannot rule out the possibility that together with the extraction of reaction centre β-carotene a component of the

electron transport sequence, such as a quinone, is also lost. Thornber and coworkers have observed a quinone (not a plastoquinone) in Photosystem I fractions [9], although there is no evidence at present for its involvement in electron transport. It is possible that, as in Photosystem II, effects of extraction of β-carotene and quinone are seen together. However, our main conclusion is not affected: that the complete removal of the β-carotene specifically associated with the reaction centre does not prevent photochemistry.

A survey of literature data on Photosystem I gives support to the above conclusions. *Anabaena variabilis* grown in the presence of diphenylamine has an altered carotenoid composition with a much reduced β-carotene content [38]. The H*P*-700 fraction (a Photosystem I fraction highly enriched in *P*-700) from these cells, prepared without the use of solvent extraction, showed a slight absorption shoulder at 495 nm indicating a trace of carotenoid, probably associated with the reaction centre, and gave high rates of $NADP^+$ reduction [39]. On the other hand, the *Scenedesmus* mutant 6E, which yields a H*P*-700 fraction completely free of carotenoid absorption, showed a $NADP^+$ photoreduction rate too low to be measured, although the activity of Photosystem I could be demonstrated by *P*-700 photobleaching [39]. H*P*-700 fractions from solvent-extracted systems, which completely lack β-carotene, also show low rates of $NADP^+$ photoreduction: from *Anabaena*, 20 μmol/mg chl per h [39]; and from spinach chloroplast, 8 μmol/mg chl per h [40]. These rates are comparable to that reported above for chloroplast $F_I$ in the complete absence of β-carotene (Table I).

Fig. 8 and Table III show that photoactivity in the absence of β-carotene is also seen in Photosystem II: chlorophyll fluorescence induction, indicative of active reaction centres, is observed in room-temperature extracted chloroplasts. Knaff et al. [13] also demonstrated Photosystem II photochemical activity (the photo-oxidation of *P*-680, detected by electron paramagnetic resonance) in the absence of β-carotene. Cox and Bendall [12] found that β-carotene was not absolutely required for activity, but that it could stimulate the rather low rates of $O_2$ evolution, when added back, together with plastoquinone, to extracted chloroplasts. It also stimulated the photo-oxidation of cytochrome *b*-559, (H*P*), although this may have been a non-specific effect of non-polar lipid. They concluded that the stimulatory effect of β-carotene was possibly due to a more efficient use of excitation energy at the reaction centre. Okayama and Butler [16] had also observed that β-carotene could stimulate the rate of ferricyanide photoreduction restored by the readdition of plastoquinone. Sadewasser and Dilley have recently reported that β-carotene alone can significantly restore methyl viologen and 2,3-dimethyl-5,6-methylene dioxy-*p*-benzoquinone reduction by hexane-extracted chloroplasts with $I^-$ as artificial electron donor to Photosystem II [41].

It therefore appears true of both Photosystems I and II that although β-carotene is not absolutely essential for photochemical activity, it is required in order to obtain maximal rates.

The conclusion of Beddard and coworkers [14] that β-carotene is involved directly in the primary charge separation at the reaction centre must be considered very unlikely in view of the evidence that photochemistry is possible, albeit at a reduced rate, in the complete absence of β-carotene. However, their

observation that a chlorophyll molecule in the excited singlet state ($S_1$) could form a non-emissive exciplex [42] with a β-carotene molecule in the ground state ($S_0$) in vitro suggests the possibility that in photosynthetic reaction centres a long-lived exciplex could be formed between carotenoid and chlorophyll:

$$\beta\text{-carotene } (S_0) + \text{Chl } (S_1) \rightleftharpoons (\beta\text{-carotene}^+ \cdot \text{Chl}^-)$$

In a rigid system such as the photoreaction centre, an exciplex can be defined as a stoichiometric complex stabilised by charge transfer in the excited electronic state, but dissociative in the ground electronic state [43]. The chlorophyll involved could be either the reaction centre special pair, or another chlorophyll molecule closely associated with it. The occurrence of 1 carotenoid molecule attached closely to each reaction centre [9,10] certainly provides circumstantial evidence for this hypothesis.

As mentioned above in our description of the chlorophyll fluorescence data of Fig. 8, the removal of β-carotene from the reaction centre appears to cause the trapping at an open reaction centre to become less than 100% efficient. The concomitant increase in energy transfer away from the reaction centre is reflected in a higher $F_0$ chlorophyll fluorescence yield. It could be argued that the β-carotene extraction procedure produces a disorganisation of Photosystem II, and that the decrease in photochemistry is not necessarily a consequence of a specific function for β-carotene in photochemistry. However, we suggest that the presence of 1 mol β-carotene at the Photosystem II reaction centre acts to reduce the rate of transfer of excitation energy away from the reaction centre into the antenna chlorophylls, possibly via formation of an exciplex. It would appear reasonable to assume similar models for Photosystem I and possibly also for photosynthetic bacterial reaction centres.

The role of reaction centre carotenoid in deactivating chlorophyll triplets, demonstrated clearly in photosynthetic bacteria by Cogdell and coworkers [44,45], could be subsidiary at least in higher plant chloroplasts, to its postulated role in bringing about 100% efficient trapping of excitation energy at the reaction centre.

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## References

1 Goodwin, T.W. (1976) in Chemistry and Biochemistry of Plant Pigments, 2nd ed., (Goodwin, T.W., ed.), pp. 225—261, Academic Press, London
2 Wessels, J.S.C. (1968) Biochim. Biophys. Acta 153, 497—500
3 Wessels, J.S.C. (1977) in Encyclopedia of Plant Physiology, New Series, Photosynthesis I (Trebst, A.

and Avron, M., eds.), Vol. 5, pp. 563—573, Springer Verlag, Heidelberg
4 Lynch, V.H. and French, C.S. (1957) Arch. Biochem. Biophys. 70, 382—391
5 Goedheer, J.C. (1969) Biochim. Biophys. Acta 172, 252—265
6 Witt, H.T. (1971) Quart. Rev. Biophys. 4, 365—477
7 Foote, C.S. and Denny, R.W. (1968) J. Am. Chem. Soc. 90, 6233—6235
8 Thornber, J.P. (1975) Annu. Rev. Plant Physiol. 26, 127—158
9 Thornber, J.P., Alberte, R.S., Hunter, F.A., Shiozawa, J.A. and Kan, K.-S. (1977) Brookhaven Symp. Biol. 28, 132—148
10 Boucher, F., van der Rest, M. and Gingras, G. (1977) Biochim. Biophys. Acta 461, 339—357
11 Knaff, D.B. and Arnon, D.I. (1969) Proc. Natl. Acad. Sci. U.S. 63, 963—969
12 Cox, R.P. and Bendall, D.S. (1974) Biochim. Biophys. Acta 347, 49—59
13 Knaff, D.B., Malkin, R., Myron, J.C. and Stoller, M. (1977) Biochim. Biophys. Acta 459, 402—411
14 Beddard, G.S., Davidson, R.S. and Trethewey, K.R. (1977) Nature 267, 373—374
15 Nishimura, M. and Takamatsu, K. (1957) Nature 180, 699—700
16 Okayama, S. and Butler, W.L. (1972) Plant Physiol. 49, 769—774
17 Junge, W., Schaffernicht, H. and Nelson, N. (1977) Biochim. Biophys. Acta 462, 73—85
18 Heber, U. and Tyszkiewicz, E. (1962) J. Exp. Bot. 13, 185—200
19 Wessels, J.S.C. and van Leeuwen, M.J.F. (1971) in Energy Transduction in Respiration and Photosynthesis (Quagliariello, E., Papa, S. and Rossi, C.S., eds.), pp. 537—550, Adriatica Editrice, Bari
20 Hager, A. and Bertenrath, T. (1962) Planta 58, 564—568
21 Borchert, M.T. and Wessels, J.S.C. (1970) Biochim. Biophys. Acta 197, 78—83
22 Wessels, J.S.C. (1959) Biochim. Biophys. Acta 35, 53—64
23 Massini, P. and Voorn, G. (1968) Biochim. Biophys. Acta 153, 589—601
24 Hiyama, T. and Ke, B. (1972) Biochim. Biophys. Acta 267, 160—171
25 Heber, U., Boardman, N.K. and Anderson, J.M. (1976) Biochim. Biophys. Acta 423, 275—292
26 Brandon, P.C. and Elgersma, O. (1973) Biochim. Biophys. Acta 292, 753—762
27 Bruinsma, J. (1961) Biochim. Biophys. Acta 52, 576—578
28 Stocking, C.R., Shumway, L.K., Weier, T.E. and Greenwood, D. (1968) J. Cell Biol. 36, 270—275
29 Latimer, P. (1959) Plant Physiol. 34, 193—199
30 Cotton, T.M., Trifunac, A.D., Ballschmiter, K. and Katz, J.J. (1974) Biochim. Biophys. Acta 368, 181—198
31 Elgersma, O. and Voorn, G. (1974) in Proceedings of the Third International Congress on Photosynthesis (Avron, M., ed.), pp. 1943—1949, Elsevier, Amsterdam
32 Vernon, L.P., Yamamoto, H.Y. and Ogawa, T. (1969) Proc. Natl. Acad. Sci. U.S. 63, 911—917
33 van Gorkom, H.J., Tamminga, J.J. and Haveman, J. (1974) Biochim. Biophys. Acta 347, 417—438
34 Ben-Hayyim, G. and Avron, M. (1970) Eur. J. Biochem. 15, 155—160
35 Izawa, S. (1970) Biochim. Biophys. Acta 197, 328—331
36 Butler, W.L. (1978) Ann. Rev. Plant Physiol. 29, 345—378
37 Searle, G.F.W. (1977) Z. Naturforsch. 32c, 968—972
38 Ogawa, T. and Vernon, L.P. (1970) Biochim. Biophys. Acta 197, 292—301
39 Ogawa, T., Vernon, L.P. and Yamamoto, H.Y. (1970) Biochim. Biophys. Acta 197, 302—307
40 Vernon, L.P., Shaw, E.R., Ogawa, T. and Raveed, D. (1971) Photochem. Photobiol. 14, 343—357
41 Sadewasser, D.A. and Dilley, R.A. (1978) Biochim. Biophys. Acta 501, 208—216
42 Förster, Th. (1975) in The Exciplex (Gordon, M. and Ware, W.R., eds.), pp. 1—21, Academic Press, New York
43 Birks, J.B. (1975) in The Exciplex (Gordon, M. and Ware, W.R., eds.), pp. 39—73, Academic Press, New York
44 Cogdell, R.J., Parson, W.W. and Kerr, M.A. (1976) Biochim. Biophys. Acta 430, 83—93
45 Cogdell, R.J., Monger, T.J. and Parson, W.W. (1975) Biochim. Biophys. Acta 408, 189—199