

ON THE ORIGIN OF THE PHOTOCONVERTIBLE CHLOROPHYLL-PROTEIN COMPLEX $C_p668 \rightarrow C_p743$ IN *CHENOPODIUM* AND *AMARANTHUS* SPECIES*

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SUMMARY

The photoconvertible chlorophyll-protein complex $C_p668 \rightarrow C_p743$ could be observed in situ after a freeze-thaw treatment in *Chenopodium* and *Amaranthus*. The concentration of the complex was highest in sections of the upper part of the stem. Capacity for photoconversion developed gradually after thawing. With *Chenopodium* the yield of $C_p668 \rightarrow C_p743$ depended to a large extent on growing conditions of the plant, with *Amaranthus* much less variability was found. In preparations from *Amaranthus* efficient energy transfer between pigments attached to a common protein carrier could be demonstrated. The initial quantum efficiency of $C_p668 \rightarrow C_p743$ photoconversion was similar to that of protochlorophyll-chlorophyll photoconversion. A possible relation between various photoprocesses in the measured plants is discussed.

1. INTRODUCTION

A chlorophyll-protein complex isolated from leaves of *Chenopodium album* has the interesting property to convert chlorophyll *a* upon illumination into a pigment with absorption maximum at 743 nm (YAKUSHIJI et al. 1963, TERPSTRA 1966). The converted form is also fluorescent, its fluorescence maximum is located at 747 nm (TERPSTRA & GOEDHEER 1966). A similar chlorophyll-protein complex could be isolated from various other plants related to the *Chenopodium* family (TAKAMIYA 1973).

The illuminated pigment-protein complex shows new absorption bands at 566, 390 and 362 nm. The absorption spectrum therefore is markedly different from that of the non-fluorescing chlorophyll aggregate with also an absorption maximum around 740 nm (DIJKMANS 1973).

Photoconversion occurred only under oxidising conditions (YAKUSHIJI et al. 1963). It was prevented by removal of oxygen from the solution. In anaerobic solution metastable pigment states could be observed upon illumination (TAKAMIYA et al. 1968 HAGAR & FRENCH 1973).

Photoconversion was not prevented by heating the preparation several minutes to 100°C (OKU et al. 1972).

* Dedicated to Professor Dr. J. B. Thomas, upon his retirement from the chair of Biophysics.

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As was indicated by a change in the tryptophane fluorescence properties (HAGAR & BRIGGS 1974) the spectral changes are correlated with changes in the protein part of the complex. It was also found that high pH and S-S reagents inhibited photoconversion (OKU & TOMITA 1975, OKU et al. 1974). Inverse transformation of C_p743 into C_p668 could be brought about by illumination at high pH in the presence of sodium hydrosulfite or ascorbate (YAKUSHIJI et al. 1963). The C_p668, thus produced, however, was no longer transformable into C_p743 at low pH.

Recombination experiments showed that phototransformation was possible if chlorophyll *a* or pheophytin *a* was added to the protein carrier, but only if it chlorophyll *a* or pheophytin *a* was added to the protein carrier, but only if it was obtained from a non-illuminated suspension. When chlorophyll *b* was added, or when the suspension was illuminated before extraction, no transformation was observed (MURATA et al 1968).

The spectral changes, characteristic for the illuminated chlorophyll-protein complex, were not observed *in vivo*. Hence the active complex may be an artifact, formed after cell disintegration. In the living cell the complex may either be present in the chloroplast or elsewhere in the cell under conditions which make transformation impossible, or the complex may not yet be formed. In the latter case a protein present somewhere in the cell could come into contact with the chlorophyll carrying chloroplasts after cell denaturation and combine with the most easily extractable chlorophyll fraction to constitute the C_p668→C_p743 complex.

The presence of chlorophyll *a* on the complex in this case is more or less fortuitous and the protein changes might be activated by different pigment molecules. A possible relation of the complex with other photoactive pigment-protein complexes, such as the protochlorophyll holochrome or phytochrome, could exist.

We therefore investigated the *in situ* occurrence of C_p668→C_p743 and some properties of it in solution to obtain information about the origin of this pigment-protein complex.

2. MATERIALS AND METHODS

Chenopodium album was harvested on the campus of the University and stored at -20°C or used immediately. *Amaranthus caudatus* was grown in the laboratory at room temperature in white fluorescent light (about 100 Wm⁻²). The seeds were kindly provided by dr. Spruit in Wageningen.

Absorption spectra were determined with a Shimadzu MPS 50 recording spectrophotometer, which enabled the use of strongly scattering preparations (leaves or stem sections).

Fluorescence emission spectra and fluorescence excitation (action) spectra were determined with a laboratory built apparatus. For emission spectra the excitation occurred with a slide projector. Light of the desired wavelength was

isolated with interference filters ($\lambda_{1/2} \cong 8$ nm). A copper sulfate solution was used to remove far red radiation. For action spectra a 1800 W Xenon arc was focused on the entrance slit of the monochromator. Trace amounts of $C_p668 \rightarrow C_p743$, which could not be detected in the absorption spectra, could be observed clearly in fluorescence action spectra when these were measured with a 749 nm interference filter transmitting fluorescence emitted by C_p743 .

Fluorescence action spectra of C_p668 were determined with a 677 nm interference filter.

Solutions containing C_p668 were prepared by macerating leaves or parts of the stems in a Sorval omnimixer or in a mortar at 4°C in weak green light. Leaves or stem sections were suspended in 0.1 M phosphate buffer pH = 7.3. Macerating was followed by 10 minutes centrifugation at 5000 r.p.m. to remove intact chloroplasts and large fractions, and 40 minutes at 50,000 r.p.m. to remove small chloroplast fractions. The supernatant was filtered and concentrated with an Amicon PM-30 filter.

RESULTS

The phototransformation $668 \rightarrow 743$ could not be observed in healthy young leaves or stem sections of the *Chenopodium* or *Amaranthus* species. After plant sections were frozen to -20°C and subsequently thawed, however, an increase around 740 nm could be detected. The value of this absorption increase, considered relative to the absorption values in the main red chlorophyll maximum, varied in *Chenopodium* plants picked on a same location markedly with age, growing season of the plant and location in the plant. The highest relative absorption increase values were observed in stem sections immediately below the flower buds. In the lower and middle part of the stem the increase around 740 nm is usually less than 10% of that in the upper part. The absorption spectrum measured before and after illumination with red light of the upper part of the stem of a *Chenopodium album* plant is given in *fig. 1*. Illumination occurred 30 minutes after thawing. The fluorescence emission spectra, resulting from excitation with light of 437 nm (absorption in the Soret band of chlorophyll *a*) or 565 nm (absorption in the maximum of the yellow band of C_p743) are given in *fig. 2*.

It is essential that the frozen samples are thawed in order to observe formation of C_p743 upon illumination. In frozen samples no C_p743 is formed, while only a small fraction is formed immediately after thawing. The amount of transformable chlorophyll increases during storage at room temperature. The far red part of the absorption spectra, made of a sample which was illuminated at various times after thawing, is given in *fig. 3*. Each illumination was sufficient to transform all C_p668 present into C_p743 . When a second section of the same stem is kept in the dark at room temperature for two hours and subsequently refrozen, immediate formation of C_p743 occurs after illumination. No additional C_p743 is formed when this sample is stored in the dark.

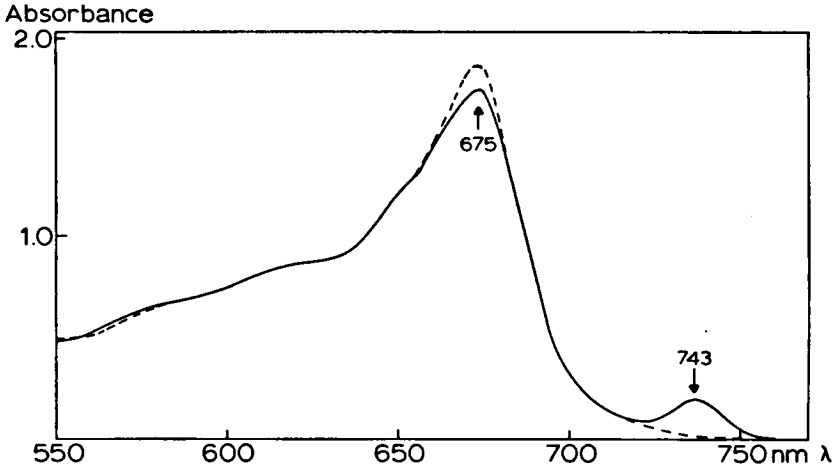


Fig. 1. Absorption spectrum of an approx. 0.5 mm thick stem section of *Chenopodium album* before (----) and after (—) a 30 sec. illumination with yellow light (OG_2 filter) from a 150 W projector. The stem had been stored overnight at $-2^{\circ}C$ and thawed in the dark. Spectra were taken 15–20 minutes after thawing.

The C_p743 pigment form could be less easily extracted with acetone from the stem section than the bulk of chlorophyll. The ratio: absorption C_p743 / total chlorophyll absorption, increased from 0.1 to 0.4 during a six hours extraction. Prolonged extraction, however, results in a decrease of both chlorophyll and C_p743 in the stem section. In the acetone extract no far red absorption band could be detected, even if C_p743 was an appreciable fraction of total chlorophyll. This suggests that the pigment molecules themselves are not changed. With methyl-ethyl ketone used as extracting agent, however, C_p743 disappeared first during extraction.

With frozen and thawed leaves of *Chenopodium album* only a slight absorption increase around 740 nm is found after illumination.

Also with laboratory grown *Chenopodium* plants the occurrence of the

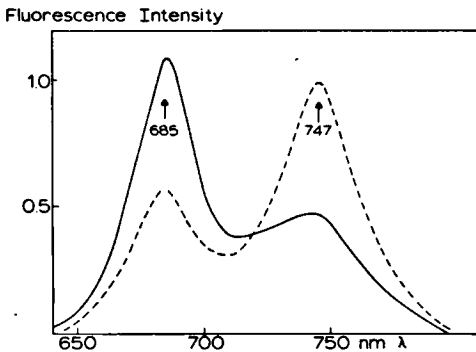


Fig. 2. Fluorescence emission spectrum of a thin (~ 0.2 mm) section of the upper part of the stem of *Chenopodium* after a freeze-thaw treatment.

---- spectrum determined with exciting light of 565 nm
 — spectrum determined with exciting light of 437 nm

Absorbance

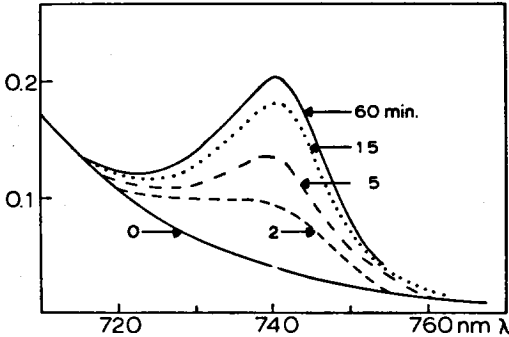


Fig. 3. Far red part of the absorption spectrum of a freeze-thawed *Chenopodium* stem section, illuminated after 2, 5, 15 and 60 minutes storage in the dark. Illumination was sufficient to transform all C_p668 present at the moment of irradiation. The 0 minutes curve was illuminated when the section was still frozen.

$C_p668 \rightarrow C_p743$ complex could be demonstrated. Germination of seeds with a high content of the chlorophyll-protein complex did not occur in the dark. Efficient (> 95%) germination of these seeds was obtained in 8 hours illumination days (100 W m^{-2} from fluorescent tubes) combined with a temperature shock of 10°C . No germination occurred in the dark control.

Storage of the extracted (non-purified) complex in the dark at 4°C during a week resulted in a considerable decline of capacity to form C_p743 , although the absorption decrease at 688 nm was little affected. Heating freshly prepared preparations or stem sections containing the complex, for one minute to 90°C , resulted in a considerable loss of phototransformation capacity.

Intact leaves of *Chenopodium album*, stored at -40°C for 12 years, contained about as much of the $C_p668 \rightarrow C_p743$ complex as at the time of storage.

Mature plants of *Amaranthus caudatus* were found to contain an appreciable amount of $C_p668 \rightarrow C_p743$. Also in this species the occurrence of the complex was confined to the upper part of the stem. In contrast to *Chenopodium*, little transformation was observed in situ after a freeze-thaw treatment in stem sections, but a slight transformation capacity could be detected after 1 minute heating to 90°C . Macerating stem sections and subsequent centrifugations showed that the supernatant contained, per unit of stem weight, more $C_p668 \rightarrow C_p743$ than *Chenopodium* samples. The presence of the complex in *Amaranthus* seems largely independent of age and condition of the plant. However, in very young plants (14–30 days) the complex was present in the leaves, and not in the stems. In the 30 days old plants it could be detected in the leaves in situ by a freeze-thaw treatment. In the 14 days old plants it could be clearly seen in the fluorescence action spectra of extracts prepared from leaves, while the presence of a large band ascribed to an anthocyanin pigment prevents detection in the absorption spectra. Although anthocyanin was found to be also weakly fluorescent, its emission spectrum differed appreciably from that of C_p743 .

No indication about the occurrence of the complex, however, could be obtained if three days old *Amaranthus* seedlings were used for extraction. This holds also with seedlings germinated in the dark. Germination could be

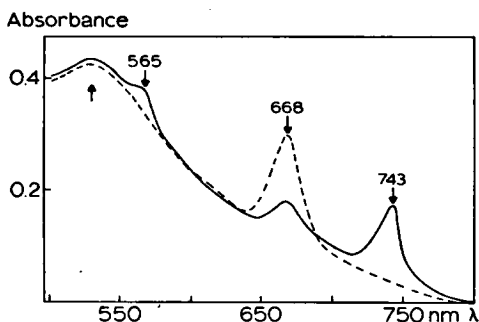


Fig. 4. Absorption spectrum of the supernatant containing $C_p668 \rightarrow C_p743$ from leaves of young (20 days) *Amaranthus* plants, before (----) and after (—) illumination. About 75% of C_p668 is transformed upon illumination. The band at 530 nm is due to anthocyanin.

prevented by irradiation of the seeds with far red light ($\lambda > 710$ nm).

Photoconversion in *Amaranthus* extracts affected a higher percentage of chlorophyll on the protein carrier than with extracts of *Chenopodium*. As shown in fig. 4, about 70% of the 668 nm band disappears upon illumination, and the increase at 743 nm was about 1.15 that of the decrease at 668 nm. In our best *Chenopodium* preparations we found only a decrease of 50%, and an increase at 743 nm that about equalled the decrease at 668 nm.

An appreciable energy transfer from chlorophyll *a* and chlorophyll *b* to C_p743 was found in extracts of *Amaranthus*. In view of the very dilute suspensions used for the determination of fluorescence action spectra, this energy transfer can occur only when the pigments are located on a common protein carrier. In fig. 5 the fluorescence action spectra of C_p743 (measured with a 749 interference filter for exciting wavelengths shorter than 680 nm and with a 820 nm cut-off filter for the far red band) are given before and after illumination. The bands assumed to belong to C_p743 , C_p688 and chlorophyll *b* are indicated by arrows. Also the absorption difference spectrum, measured between a non-diluted illuminated and unilluminated sample, is given in the figure. The fluorescence action spectrum of C_p743 shows that light absorbed by both chlorophyll *a* and chlorophyll *b* is partly effective in producing C_p743 fluorescence. With 565 nm light, C_p743 fluorescence is predominant. This is also shown in fig. 6, where the fluorescence emission spectra resulting from excitation in the Soret band of chlorophyll *a* (437 nm) and from excitation in the yellow band of C_p743 (565 nm) are given.

The rate of formation of C_p743 is highest at the onset of illumination. In fig. 7 fluorescence values, taken from fluorescence action spectra measured at various stages of formation are given. After a short period of illumination with weak red (671 nm) light an appreciable increase in 749 nm (C_p743) emission excited at 565 nm occurs. At the same time a decrease in 677 nm (C_p668) emission, excited at 437 nm is found. Comparison of this initial rate of transformation $C_p668 \rightarrow C_p743$ with that of protochlorophyll-chlorophyll in bean leaf extracts (excited at 645 nm) showed that the initial quantum efficiency for both processes was of the same order of magnitude.

If no energy transfer from C_p668 to C_p743 occurred, the fluorescence emission at 749 nm, excited in the C_p668 bands, would decrease similarly to fluorescence

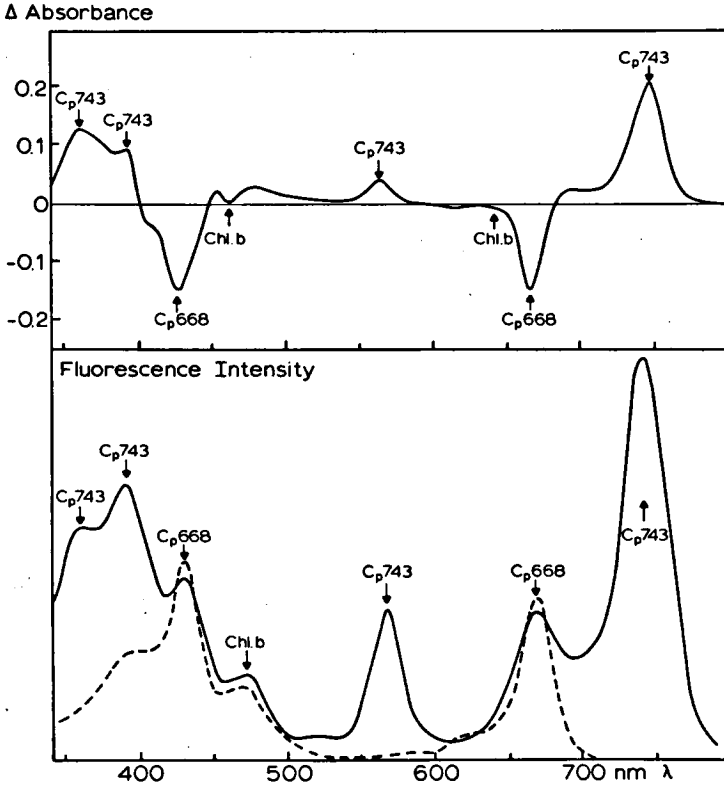


Fig. 5. Fluorescence excitation spectrum of C_p743 before (---) and after (—) 30 sec. illumination of a preparation obtained from *Amaranthus* stems. In the upper part the absorption difference spectrum between an illuminated and non-illuminated sample is given. The band at 465 nm in the absorption difference spectrum ascribed to chlorophyll *b* is absent when much lower intensities are used for phototransformation.

emitted at 677 nm. As shown in *fig. 7* however, no decrease, but an increase is measured, which indicates that an efficient energy transfer from C_p668 as well as from chlorophyll *b* to C_p743 occurs once this pigment is formed. Due to this energy loss the transformation efficiency $C_p668 \rightarrow C_p743$ may drop considerably during illumination.

4. DISCUSSION

The relatively large absorption changes around 740 nm, occurring upon illumination of freeze-thawed stem sections of *Chenopodium*, show that an appreciable fraction of total chlorophyll (estimated up to 10%) may be present in the $C_p668 \rightarrow C_p743$ form. The occurrence of a marked fluorescence band at 747 nm, when excitation occurs at 565 nm, suggests that the photoconvertible

Fluorescence Intensity

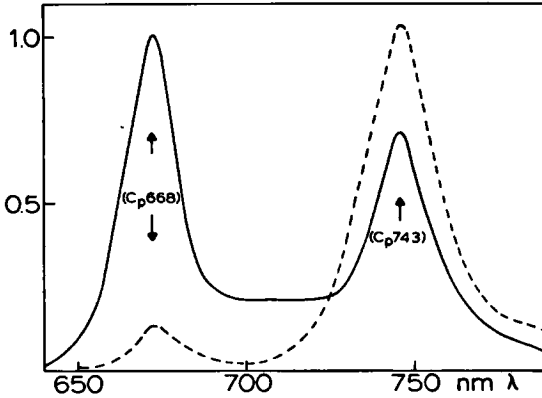


Fig. 6. Fluorescence emission spectrum of an illuminated $C_p668 \rightarrow C_p743$ preparation of *Amaranthus*. The spectra are excited with light of 437 nm (—) and light of 565 nm (----).

Fluorescence Intensity

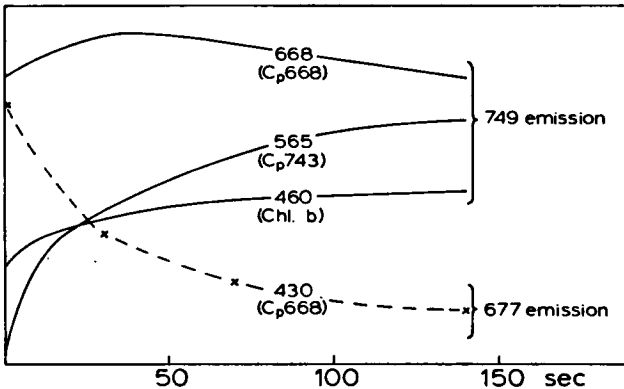


Fig. 7. Fluorescence intensity at 749 nm and 688 nm as a function of time of illumination with weak red (671 nm) light used for phototransformation. The curves are given for various bands in the fluorescence excitation spectrum. In the 749 nm emission the bands of C_p743 increase, while the bands of C_p668 and chlorophyll *b* are little affected.

chlorophyll-protein complex isolated after macerating and high speed centrifugation, and the form giving the 743 nm absorption band in situ, are identical. As the complex is formed only after the frozen sample is thawed, it is most likely produced by structural changes in the cell.

The gradual appearance of the capacity to form the far red band after thawing may suggest that its occurrence is coupled to a diffusion limited process. This seems to exclude the possibility that complex as such exists somewhere in the cell outside the chloroplast and cannot be transformed due to highly anaerobic or alkaline conditions. As the main fluorescence band of C_p668 is located at 673 nm, and the main band of chlorophyll in the plant is located at 685 nm, an asymmetry in the fluorescence band in those frozen stem sections,

which contain much C_p668 , should be expected when the complex is present but somehow not transformable. Such an asymmetry was not found. The complex then could originate either in the chloroplast and diffuse out when the chloroplast envelope is ruptured, or it could be formed after denaturation by some special cell protein which combines with those chloroplast pigments which are most easily released from their original pigment carriers. Fluorescence action spectra suggest that chlorophyll *a* (bands at 668 and 430 nm) and chlorophyll *b* (shoulder at 650 and band at 465 nm) are present on a common protein carrier in a ratio similar to that given by YAKUSHIJI et al. (1963). This ratio is comparable to that in photosystem I particles, which are assumed to occur in grana as well as in stroma lamellae (PARK & SANE 1971). As system I in grana is supposed to be located at the outside of the membranes (BRIANTAIS 1969), both stroma and grana lamellae appear to be candidates for production of the complex. The predominant existence of the complex in a small fraction of the stem in mature plants immediately below the flower buds might be correlated with the chloroplast structure as well as with the presence of a required protein in the cells.

As pigment extraction from illuminated stem sections and C_p668 preparations shows that the extracted pigments do not differ quantitatively from those of non-illuminated samples, the change in absorption and fluorescence properties of the complex should be brought about only by changes in the protein configuration. The experiments of MURATA et al. (1968) showed that phototransformation was possible only with chlorophyll *a* and pheophytin *a*, but not with chlorophyll *b*. Absorption, fluorescence and fluorescence polarisation spectra of the C_p743 form suggest (GOEDHEER 1966) that the spectral properties of the pigment-protein complex are changed upon illumination from a dihydroporphin type (chlorophyll *a*) into a tetrahydroporphin type (resembling bacteriochlorophyll). Such a change could indicate that the double bond around the carbon atoms 3 and 4 (Fischer notation, cf. RABINOWITCH 1945; 3b and 4b in ARONOFF 1966) of chlorophyll *a* is affected by a change in the protein.

The carbonyl group in chlorophyll *b*, present on carbon atom 3, could prevent phototransformation.

Two other phototransformable pigment systems occur in the investigated species. Photoactivity on germination of *Chenopodium* species was confirmed (cf. CUMMING 1959, 1963; KARSEN 1967), and, though in opposite direction, in *Amaranthus caudatus* (cf. KENDRICK et al. 1969), suggesting phytochrome activity in these species. Also the occurrence of protochlorophyll in dark germinated *Amaranthus* could be demonstrated. The amount of protein present in the $C_p668 \rightarrow C_p743$ complex is, however, a large multiple of that expected to be present in either phytochrome or the protochlorophyll holochrome, though the basic quantum efficiency of the processes seems to be of the same order of magnitude.

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