

## Photothermal spectra of thylakoids isolated from cucumber cotyledons at various stages of greening

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### Abstract

The character of interaction between carotenoids (Cars) and chlorophylls (Chls) in thylakoids isolated from cucumber cotyledons at three stages of greening (3, 6, and 24 h of irradiation with  $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was studied. The shapes of the steady state photoacoustic spectra were changed with the change in time of greening and with the frequency of radiation modulation. The shapes show that changes not only in the contents of various pigments but also in pigment interactions with surrounding occur and that processes of thermal deactivation characterised by different kinetics take place. Slow processes of thermal deactivation are in most cases due to deactivation of triplet states. Long living triplet states are very often engaged in photochemical reactions that can destroy the tissue. Analysis of the time-resolved photothermal spectra shows that at later stage of greening, the chlorophyll (Chl) molecules are better shielded against photo-destruction because Cars more efficiently quench their triplet states. The yield of formation of the pigment triplet states measured by the time resolved photothermal method, always at the same energy absorbed by pigment mixture, declined during sample greening. The decay time of the slow component of pigment thermal deactivation, due predominantly to deactivation of the triplet state of Chl, decreases with the increase of time of greening from 6.2  $\mu\text{s}$  for the 3-h sample to 1.5  $\mu\text{s}$  for the 24 h sample. The energy taken by Cars from Chls is dissipated into heat, therefore the steady state and quick thermal deactivation values increased during the greening process. The Cars/Chls ratio in the thylakoids decreased during greening approximately 2 fold. Hence at a later phase of greening the Cars can quench the triplet states of Chls more efficiently than at an earlier phase of greening.

*Additional key words:* *Cucumis sativus*; laser-induced optoacoustic spectra; photoacoustic steady state spectra.

### Introduction

Irradiation of dark grown angiosperm plants leads to a gradual development of photosynthetic apparatus. This process is associated with the photo-transformation of protochlorophyll(ide)s into chlorophyll(ide)s (Boardman 1966, Ryberg and Sundqvist 1991, Skribanek *et al.* 2000), the accumulation of chlorophyll (Chl) *a* and *b* (see Boardman and Anderson 1979, and others), and the acceleration of synthesis and accumulation of carotenoids (Cars) (Więckowski and Waloszek 1993) and many proteins (Takabe 1986, Mullet 1988, Pauncz *et al.* 1992,

Hachtel and Friemann 1993). During the initial few hours of irradiation of etiolated plants, prolamellar bodies and prothylakoids are rapidly disintegrated and thylakoid formation takes place (Gunning and Jagoe 1967, Henningsen 1970, Myśliwa-Kurdziel *et al.* 1997). At the same time the efficiency of excitation energy transfer from accessory pigments into fluorescent Chl *a* increases almost twice (Więckowski and Waloszek 1993). However, the processes of excitation energy exchange between carotenoid (Car) and Chl molecules at various

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**Abbreviations:** BCP, bromocresol purple; Cars, carotenoids;  $^1\text{Cars}$ ,  $^3\text{Cars}$ , carotenoids in excited singlet and in triplet states; Chl, chlorophyll;  $^1\text{Chls}$ ,  $^3\text{Chls}$ , chlorophylls in excited singlet and in triplet states; ET, excitation energy transfer; LIOAS, laser induced optoacoustic spectroscopy; NR, non-radiative; PAS, photoacoustic spectra or photoacoustic signal; TD, thermal deactivation; 3-h sample, 6-h sample, or 24-h sample, thylakoid suspension prepared from cotyledons of dark-grown seedlings and irradiated for 3, 6, or 24 h, respectively.

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stages of greening as well as energy dissipation from excited states of pigments have not yet been elucidated.

Cars in photosynthetic assemblies work as an antennae system transferring their singlet state excitation to chlorophylls (Chls) (Goedheer 1969, Thrash *et al.* 1979, Naqvi 1980, Lichtenthaler 1987, Koyama 1991, Höfer *et al.* 1987, Koyama *et al.* 1996) and protect Chls against photodestruction by quenching  $^3\text{Chl}$  (Egorov *et al.* 1995, Frąckowiak *et al.* 1995, Frank and Cogdell 1996) and/or  $^1\text{O}_2$  (see Asada and Takahashi 1987). Most photodestructive reactions occur in the pigment excited to long-lived triplet states. Quenching of the  $^3\text{Chl}$  by Cars is more efficient than quenching by oxygen that leads to the formation of destructive  $^1\text{O}_2$  (Papageorgiou 1975, Höfer *et al.* 1987). The participation of Cars in excitation energy transfer (ET) or protection depends, apart from other factors, on their conformation: all-*trans* configuration is rather suitable for light-harvesting function, whereas the 15-*cis* configuration is suitable for quenching of  $^3\text{Chl}$  (Koyama 1991, Bialek-Bylka *et al.* 1995, 1996). The conformation and function of Cars depends also on their close surroundings, which should change during the development of photosynthetic apparatus. Frąckowiak *et al.* (1995, 1997) and Bialek-Bylka *et al.* (1982) showed that the ET from Cars to Chls occurs much more effectively in photosynthetic complexes than in model systems. Thus, the change in the efficiency of both processes occurring between Cars and Chls cannot be explained solely by the change in the concentration ratio of various pigments during the greening process (Frąckowiak *et al.* 1997).

In some cases Cars can also quench the fluorescence of excited Chl-type pigments as it follows from reports by Snyder *et al.* (1984) and Polivka *et al.* (1999). Transfer of excitation from Chls to Cars can happen even when  $^1\text{Cars}$  state is located above  $^1\text{Chls}$  state (Naqvi 1998). It is possible because the excited singlet states of both types of pigments can be mixed (van Amerongen and van Grondele 2001). In some cases on behalf of excitation energy transferred from Chls to the Cars the reactions undergoing in Cars were observed (Fiedor *et al.* 2001). But, usually, Cars dissipate the energy obtained from triplet states of Chls into heat (Frank and Cogdell 1996). The quantum yield of Cars triplet formation [ $\Phi_T(\text{Cars})$ ] is near to zero (Truscott 1990) and the lifetime of this triplet depends on the solvent and is reported from 10 to 14  $\mu\text{s}$  (Truscott 1990, Burke *et al.* 2000).

The interpretation of molecular processes occurring in thylakoids is complicated, because the photosynthetic apparatus contains several pigments and the amounts of these pigments as well as the structure of their surroundings are changed as a result of the greening process. All spectroscopic data (absorption, fluorescence, photothermal spectra) provide information about the fate of energy absorbed by photosynthetic apparatus, therefore they can indirectly inform also about other processes such as elec-

tron transport.

The primary processes within photosynthetic assemblies have been investigated, among others, by photo-acoustic spectroscopy (PAS) that can be applied also for the measurement of energy storage in photosynthetic apparatus (Nitsch *et al.* 1988, Carpentier *et al.* 1989). For photosynthetic organisms three sources of PAS are possible: those generated by photothermal effects (Brumfeld *et al.* 1999), by photobaric effects, due to gas evolution, and some small signal due to possible changes in volume of some complexes (Braslavsky and Heibel 1992, Nagy *et al.* 2001). A photobaric effect is not probable for our wet thylakoids, because no electron acceptor was added and therefore no oxygen evolution took place. Our time-resolved photothermal results suggest that also volume changes are not the crucial effects. Therefore the main contributions to measured signals are due to thermal deactivation (TD) of excitation. Ratios of the contributions from various types of effects depend on the sample irradiation and heat treatment (Bukhov *et al.* 2000). Steady state PAS measured at various frequencies of modulation of actinic radiation in thylakoid suspensions show the occurrence of thermal deactivation processes with different time constants (Frąckowiak *et al.* 1996). Very slow (in ms time range) TD decays can not be measured by our time-resolved photothermal arrangement, which analyses only effects occurring in time shorter than 5  $\mu\text{s}$ . From the analysis of time resolved photothermal data the yield of triplet states generation by intersystem crossing (ISC) (from excited singlet to the triplet state) and the pigment triplet states decay time can be established (Braslavsky and Heibel 1992). The source of such slow (in  $\mu\text{s}$  range) signal of TD of thylakoids has not been exactly established till now. Yield of Cars triplet generation [ $\Phi_T(\text{Cars})$ ] is low (Truscott 1990), therefore the observed slow component of TD is probably due mainly to a decay of the Chl triplet states. This supposition should be experimentally confirmed.

In such case the decrease in the yield and lifetime of slow component of TD would be due to the quenching of Chls triplets by Cars. The efficiency of such quenching depends not only on the ratio of pigment contents but also on their mutual orientations and environment. In thylakoids the protecting action of Car can be observed by the quenching of Chl triplet states.

We investigated the interactions between Cars and Chls in suspensions of thylakoids isolated from cotyledons of dark-grown cucumber seedlings exposed to continuous irradiation, using steady state and time-resolved photothermal methods. The yield of generation and the decay time of pigment triplet states were established. To our knowledge, this is the first report on energy dissipation in the form of heat at various phases of chloroplast development.

## Materials and methods

Experiments were performed with thylakoid membranes isolated from cotyledons of 6-d-old etiolated cucumber (*Cucumis sativus* L. cv. Wisconsin) seedlings, that were continuously irradiated (ca. 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation) for 3, 6, or 24 h. The technique of seedling cultivation was described by Więckowski and Majewska (1990). The cotyledons were homogenised for 3×15 s in a 66 mM phosphate buffer (pH 7.2) with addition of 10 mM KCl and 400 mM sucrose. The homogenate was filtered off through nylon and the filtrate was centrifuged for 5 min at 600×g. The supernatant was centrifuged again at 2 400×g for 10 min and the pellet was re-suspended in the same medium deprived of sucrose. After 15-min incubation on ice with continuous stirring, the suspension was centrifuged at 21 000×g for 15 min, and the pellet containing thylakoid membranes was re-suspended and then washed twice with buffer deprived of sucrose. All operations were carried out at +4 °C.

The absorption spectra were measured using a *Specord M40* recording spectrophotometer (C. Zeiss, Jena, Germany). The fluorescence spectra were recorded by means of a home made device. With the same apparatus the yield of fluorescence was established using as reference *Rhodamine 6G* dissolved in the same buffer as thylakoids applying the method of Lakowicz (1999).

The steady state PAS were measured with a single beam photoacoustic spectrometer (Ducharme *et al.* 1979, Frąckowiak *et al.* 1997) equipped with an *MTEC* (model 300) photoacoustic cell produced by *MTEC Photoacoustic* (Ames, Iowa, USA). All PAS were corrected for spectral distribution of the radiation source using the division by carbon black photoacoustic signals.

The scheme of the arrangement for the measurements of the time resolved photothermal signal is typical for all laser induced optoacoustic spectroscopy (LIOAS) measurements (Braslavsky and Heibel 1992). The sample was irradiated by flash from dye-nitrogen laser from *Photon Technology International* (Canada) model *GL-3300/GL301*. The pulse duration was 0.2 ns. Laser radiation energy was registered by a *Laser Probe* (USA) energy radiometer model *RJ-7620* with an *RJP-736* pyroelectric probe. The sample was placed in a temperature-controlled cuvette holder (produced by *Quantum Northwest Comp.*, USA). The piezoelectric transducer (*Panametric*, model *V1030*) with 1 MHz frequency resolution was attached to the cuvette wall. The signal from the transducer was memorised using a *Gold Star* model *OS3060* (60 MHz) digital oscilloscope. The waveform LIOAS signals for sample as well as for reference dye-bromocresol purple (BCP) in the same medium were measured. According to Braslavsky and Heibel (1992) BCP reference dye deactivates all excitation promptly in time shorter than the time resolution of a usually applied

arrangement (about 0.4  $\mu\text{s}$ ). The time resolution of the arrangement used depends on three factors: (1) the time width of laser pulse (in our case 0.2 ns), (2) the time response of electronic equipment (in our apparatus about 0.05  $\mu\text{s}$ ), and (3) geometrical arrangement of the equipment. In our case the third factor was critical. It depends on the effective acoustic transit time  $\tau_a = 2R/v_a$ , where  $R$  is the radius of laser beam and  $v_a$  is sound velocity in the medium used. It was supposed that for thylakoids it is water with  $v_a = 1.48 \text{ m s}^{-1}$ . In fact, the samples consist of lipid membranes and proteins but water (buffer) is the medium in which thylakoids are suspended. Two widths of laser beam were used:  $2R_1 = 5 \times 10^{-4} \text{ m}$  and  $2R_2 = 8 \times 10^{-4} \text{ m}$ . The following transit times  $\tau_1 = 0.34 \mu\text{s}$  and  $\tau_2 = 0.54 \mu\text{s}$  were obtained. Both times are much longer than those described in points (1) and (2). The time of the non-radiative (NR) excitation deactivation ( $\tau_{\text{NR}}$ ) influences also the position and width of the wave-shaped LIOAS signal.

The LIOAS signals of our samples exhibit, beside the first main maximum, a small peak at shorter time range. These short-time maxima are due to the effect of radiation scattering reported previously for LIOAS measurements by Nitsch *et al.* (1988) and Strassburger *et al.* (1997). Such effects of scattered radiation, which generate heat in region closer to piezoelectric transducer than quanta from regular laser beam, were always observed for cell suspensions (Nitch *et al.* 1988). We subtracted these scattering effects before the LIOAS signal analysis and its presentation in figures. The scattering was higher for sample at shorter time of greening because to reach the same absorption at wavelength of laser beam, at lower pigment contents in thylakoids, a greater amount of cells had been used. The modification of cell constituents that occurs during greening (Papageorgiou 1975) can also have some influence on the scattering effects. The possibility that the generation of scattering can be related to some Cars clusters occurring in different amounts in the thylakoids at various phases of greening was excluded by the measurements of LIOAS signals for cyclohexane solutions at high ( $10^{-2}$ – $10^{-4}$  M)  $\beta$ -carotene concentrations. The shapes of these LIOAS signals depended on Cars concentration, which is in agreement with literature (Burke *et al.* 2000), but an additional LIOAS peak was not observed.

The magnitude of the first main maximum of LIOAS signal ( $H_{\text{max}}$ ) of the reference and the sample provide the opportunity to evaluate a part of energy thermally deactivated promptly or slowly (this means in time longer than time resolution of apparatus) or used in photoreactions. The procedure proposed by Marti *et al.* (1996, 2000) was applied for analysis of the results. The lifetimes of triplet state were obtained by the deconvolution of the LIOAS signals of the sample and reference using a program

elaborated by Rudzki-Small *et al.* (1992). The results obtained by both methods are complementary. The comparison of results taking into account other literature

(Lebedev *et al.* 1991, Myśliva-Kurdziel *et al.* 1997) enables to decide which group of pigments is responsible for the slow component of thermal deactivation.

## Results

**Absorption and fluorescence spectra:** The normalised absorption and fluorescence spectra of thylakoids isolated from cucumber cotyledons at three phases of greening (3, 6, and 24 h samples) of dark-grown seedlings are presented in Fig. 1A,B. As one could expect, the main absorption bands occurred at about 680 and 438 nm, independent of the investigated phase of greening (Fig. 1A). However, the ratio of absorbances at 680/438 nm increased from about 0.46 (in the 3-h sample) to about 0.72 (in the 24-h sample). It is the result of a more intensive accumulation of Chls when compared with Cars in cotyledons during the greening. Fig. 1A also implies that in 6-h and 24-h samples enhanced absorbance at about 473 nm can be due to the appearance of Chl *b*.

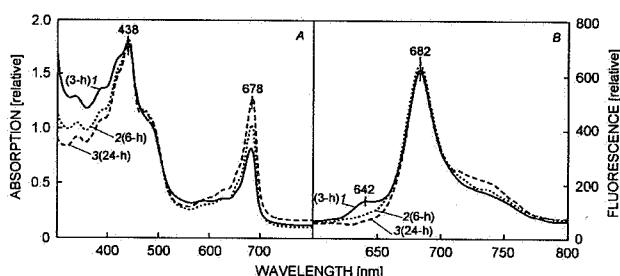


Fig. 1. Normalised absorption (A) and fluorescence (B) spectra of thylakoid suspensions prepared from 3-h, 6-h, or 24-h irradiated dark-grown cucumber seedlings. Absorption at 460 nm was normalised to 1.2. Fluorescence spectra were normalised to equal absorption of exciting laser radiation  $A = 1.2$  at 460 nm.  $\lambda_{ex} = 410$  nm.

Radiation scattering to some extent perturbs absorption spectra of thylakoids. This effect depends on the amount of membranes in sample volume and on the pig-

ment contents in cells (Merzlyak and Naqvi 2000). But the positions of main absorption maxima (Fig. 1A) are similar in all three samples.

The main peak in the room temperature emission fluorescence spectra of the samples investigated (Fig. 1B) was located at about 682 nm and its position did not depend on the phase of greening, whereas the intensity ratio of this peak to the shoulder at 720-730 nm increased during de-etiolation. In the 3-h sample a fluorescence band at about 642 nm appeared which was absent in the fluorescence spectra of 6-h and 24-h samples. This peak is associated with the presence of protochlorophyll(ide) (Więckowski and Waloszek 1993). As follows from Table 1, the fluorescence yield ( $\Phi_F$ ) of the 3-h sample was very low, much lower than that for model systems (Sauer 1975). The  $\Phi_F$  value increased twice (to about 15 %) during 24-h irradiation of the dark-grown seedlings. All fluorescence yields were established at 460-nm excitation. Various pools of Chls and Cars contributed to the absorption at 460 nm. Fluorescence emission is a result of direct excitation of Chl *a* and its excitation by singlet excitation transfer from Chl *b* and Cars. The increase in  $\Phi_F$  with time of greening is due to the increase of the amount of fluorescent forms of Chls as well as to increase in the yield of excitation energy transfer from other pigments to these forms (Więckowski and Waloszek 1993) during greening of dark-grown seedlings.

Snyder *et al.* (1984), Polivka *et al.* (1999), and Naqvi (1998) state that in some cases Cars can quench the singlet excited state of Chl-like pigments but usually they transfer their excitation to Chls (Goedheer 1969). The increase in Chl fluorescence yield can be therefore also related with the decrease of quenching due to the decrease in Cars/Chls concentration ratio.

Table 1. Spectral properties of thylakoid membranes isolated in various time of greening.  $t$  – time of irradiation,  $R$  – ratio of Chls (*a+b*) to Cars (according to Więckowski and Waloszek 1993),  $\Phi_F$  – yield of fluorescence average for all pigments absorbing at 460 nm (accuracy 0.02),  $\alpha$  – part of excitation converted promptly into heat in whole sample,  $\Phi_T(\text{Chls})$  – yield of triplet formation at supposition that are observed  $^3\text{Chls}$ ,  $\Phi_T(\text{Cars})$  – yield of triplet formation at supposition that are observed  $^3\text{Cars}$ ,  $A(\text{Cars})$ ,  $A(\text{Chl } a)$ ,  $A(\text{Chl } b)$  – contributions to absorption at 460 nm given by various pigments (method of calculation is described in the text).

$t$ [h]	$R$	$\Phi_F$	$\alpha$	$\Phi_T(\text{Chls})$	$\Phi_T(\text{Cars})$	$A(\text{Cars})$	$A(\text{Chl } a)$	$A(\text{Chl } b)$
3	1.2	0.07	0.73	0.46	0.63	88	12	0
6	3.2	0.07	0.78	0.36	0.49	19	17	64
24	6.5	0.15	0.81	0.18	0.25	26	19	55

The steady state PAS for two types of sample (6-h and 24-h) are shown in Fig. 2. The 6-h sample was measured at various frequencies of radiation modulation. All spectra

were normalised at 670 nm. As follows from the comparison of Figs. 1A and 2, the PAS of the suspension of thylakoids differ from the absorption spectra. The

maxima of PAS of the 6-h sample were shifted by 3 nm (in Soret band) or 10 nm (in red region) towards shorter wavelengths when compared with the absorption maxima (Fig. 1A). Hence various forms of pigments created by their interactions with environment exhibit different yields of TD. To ensure absorption of the same pigments at laser line wavelength (460 nm), necessary for LIOAS measurements, different amounts of thylakoid membranes for various phases of greening have to be used. Therefore, the PAS signal was formed in slightly different medium for different samples. The radiation scattering effects were highest for the 3-h sample, lower for longer times of greening. The PAS were perturbed by radiation scattering to a lower degree than absorption spectra. Similar shapes of absorption spectra of all samples, changed predominantly by the increase in Chls content, suggest that the positions of absorption maxima are not strongly perturbed by scattering and that the differences between absorption and PAS spectra are due to different yields of TD of various forms of pigments. Because of strong overlapping of the spectra of various forms, the results of TD obtained as a ratio of PAS signal to absorbed energy are approximated. Such crude evaluation shows that the TD value increases with greening.

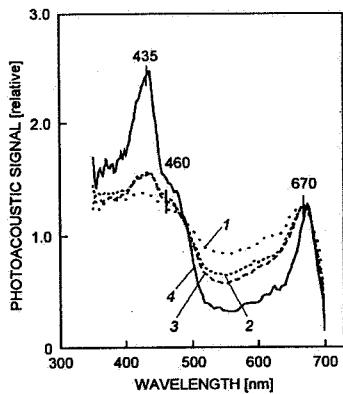


Fig. 2. Steady state photoacoustic spectra of thylakoid suspension, measured at various frequency modulation of incident radiation. Curves 1 to 3 for 6-h sample. Modulation: curve 1 – 11 Hz, 2 – 33 Hz, 3 – 66 Hz. Curve 4 for 24-h sample, 33 Hz. All spectra are normalised at 670 nm.

The dependence of the shape of PAS on frequency of radiation modulation (Fig. 2) shows the spectral regions involved in the very slow (in ms time range) processes of TD (Ouzafe *et al.* 1992). The shape of PAS changed with the frequency of radiation modulation (Fig. 2). The differences between the 33 and 66 Hz spectra were insignificant. On the contrary, the 11 Hz PAS differed considerably from that monitored at 33 or 66 Hz. In the 11 Hz PAS the main peak in the red region was at about 665 nm whereas it was located at 670 nm in the 33 (or 66) Hz PAS. Differences in the intensity of the PAS signals were also observable in the region of Cars absorption.

The shape of PAS for the 24-h sample was different than that for the 6-h sample (Fig. 2, curves 4 and 2). As a result of prolongation of time of greening, the ratio of maxima of the red band to the Soret band increased in absorption but decreased in PAS.

The dependence of steady-state PAS on the frequency of radiation modulation confirms the supposition that under the conditions applied the change in pressure in the photoacoustic cell is predominantly due to thermal effects and not to oxygen evolution which can be predominant at low frequencies of radiation modulation (Canaani *et al.* 1982, Brumfeld *et al.* 1999).

**The time resolved photothermal effects:** The LIOAS signals were measured for the samples and the PCB reference exhibiting the same absorption at the wavelength of laser flash (460 nm). Sample irradiation causes the LIOAS signal due to thermal effects and to volume changes of macromolecules (Gensch and Braslavsky 1997). These two effects, in our samples containing different amounts of pigment-protein complexes, have to exhibit various kinetics and amplitudes of LIOAS (Strassburger *et al.* 1997). Similar positions of  $H_{\max}$  in Fig. 3 suggest that the influence of volume changes on measured photothermal signals is low. The oxygen evolution effects occur in longer time range (Canaani *et al.* 1982, Brumfeld *et al.* 1999) than the presented signals.

Fig. 3 presents examples of LIOAS wave-form signals generated by laser flashes at 1.7  $\mu$ J intensity for 3-, 6-, and 24-h samples and for BCP as a reference. The main maximum ( $H_{\max}$ ) of LIOAS was located in time range from 0.65 to 0.70  $\mu$ s and it was always higher for the reference than for the three samples. The reason is that the quick TD in the reference is more effective than in thylakoids, which proves that contributions from slow TD in thylakoids is higher.

At earlier phases of greening, the content of Cars in relation to Chls is much higher than in the developed chloroplasts (Więckowski and Waloszek 1993) and about 88 % of the 460 nm radiation is absorbed by Cars. Based on the results of Więckowski and Waloszek (1993) as well as on absorption spectra of Chl *a* and Chl *b* pigment-protein complexes (Murata *et al.* 1971) and molar absorption coefficients in organic solvents (Lichtenthaler 1987), the contributions of both Chls to absorption at 460 nm were evaluated. By the subtraction of Chls contributions from experimental absorption of samples at 460 nm the contributions from Cars were calculated. We assumed that in the 3-h sample only Cars and Chl *a* occur, whereas at 6 and 24 h Cars and both Chl *a* and Chl *b* are present. The evaluation is shown in Table 1. Such procedure is, of course, only a crude approximation but it gives the opportunity to evaluate the contents of various pigments in the samples.

The greatest maximum related to thermal effect was observed for various samples at only slightly different positions (Fig. 3). Thus, *e.g.* in the 24-h sample the con-

tributions from quick NR deactivation occurred in slightly longer time than that for the reference.

In the analysis of wave-form signal, done according

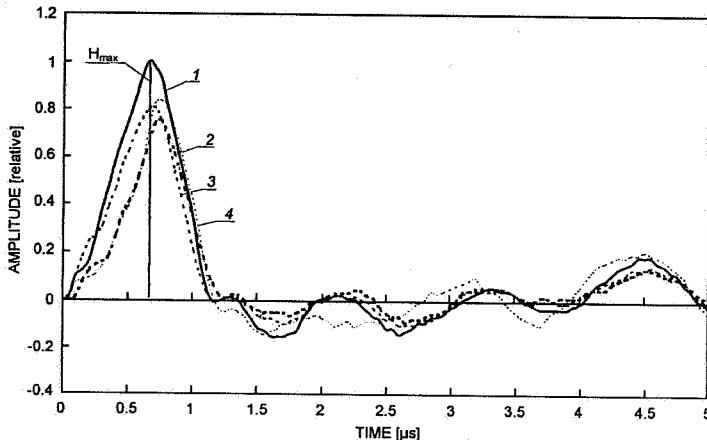


Fig. 3. The examples of waveform photo-thermal (LIOAS) signals. Curves: 1 – reference BPC; 2 – 3-h sample, 3 – 6-h sample, 4 – 24-h sample.

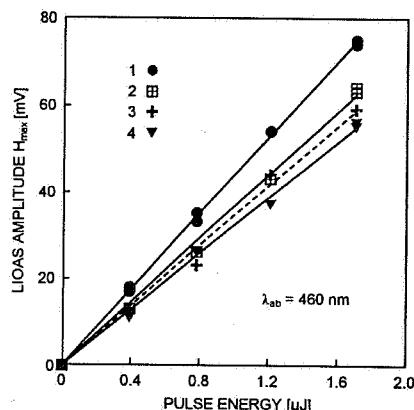


Fig. 4. The dependence of the first thermal maximum  $H_{\max}$  (Fig. 3) on the energy of laser radiation for the reference (BCP) and samples located in the same medium. Curves: 1 – BCP with  $A = 1.17$  at 460 nm; 2 – thylakoids 24-h with  $A = 1.20$  at 460 nm; 3 – thylakoids 6-h with  $A = 1.18$  at 460 nm; 4 – thylakoids 3-h with  $A = 1.23$  at 460 nm.

to methods proposed by Marti *et al.* (1996, 2000), the dependence of  $H_{\max}$  on laser energy for the samples and BCP shown in Fig. 4 was used. Laser energy was changed to a known degree by grey filters. On the basis of the set of lines presented in Fig. 4, the dependence of  $dH_{\max}/dE_{\text{las}}$  on the energy absorbed ( $1 - 10^{-A}$ , where  $A$  is the sample absorbance) was established and the part of energy converted promptly into heat was calculated (Marti *et al.* 1996, 2000). This part is denoted as  $\alpha$  and it is supposed to be equal to one for the reference. As a result of cotyledon greening, the value of  $\alpha$  increased from 0.73 (3-h sample) to 0.81 (24-h sample) (Table 1) showing that the average amount of slow TD measured at the same absorption of the Cars and Chls mixture decreases. The ratio of the amount of Cars/Chls in this mixture decreased

during the greening process. For the same amount of absorbed photons, the contribution from Cars to absorption also decreased.

Using the procedure of Marti *et al.* (1996, 2000), on the basis of LIOAS data the yield of triplet-state formation ( $\Phi_T$ ) was calculated from the formula:

$$\Phi_T E_T = (1 - \alpha) E_{\text{las}} - \Phi_F E_F \quad (1)$$

where  $\Phi_T$  and  $\Phi_F$  are the yields of triplet state formation and of fluorescence, respectively,  $E_T$  and  $E_F$  are the energies of triplet state and fluorescence, respectively, and  $\alpha$  is the part of energy converted promptly into heat (see Table 1). Two different hypotheses were made for the calculations: in the first hypothesis the observed slow TD decay is supposed to arise predominantly from Chl triplet, in the second, less probable hypothesis, it is supposed to arise from Car triplets.

In both cases it is assumed that all slow processes of deactivation are due to triplet states. In the calculation it was supposed that only Chls are fluorescent with  $E_F = 176 \text{ kJ mol}^{-1}$  (it means that the emission is supposed to be at about 680 nm),  $E_{\text{las}} = 260 \text{ kJ mol}^{-1}$  (emission at 460 nm),  $E_T(\text{Car}) = 92 \text{ kJ mol}^{-1}$ , at 1300 nm (Krasnovskii *et al.* 1973),  $E_T(\text{Chl } \alpha) = 126 \text{ kJ mol}^{-1}$  at 940 nm (Hanson 1991, p. 1009),  $\alpha$  values as in Table 1. Various wavelengths are reported for Chls phosphorescence (Goedheer 1966, Sauer 1975). We took for the calculation a triplet state energy of Chl  $\alpha$  reported by Hanson (1991, p. 1009). Results of such calculations of  $\Phi_T(\text{Chls})$  and  $\Phi_T(\text{Cars})$  are in Table 1. For LIOAS measurements the amounts of cells were always such that the same amounts of laser quanta were absorbed by the sample. For samples at various time of greening, the ratio of Chls/Cars contribution to absorption was different (Table 1). The  $\Phi_T$  values were averaged for all absorbing pigments. When the  $\Phi_T$  value

decreases with greening of cotyledons, the pigments are better shielded against photo-destruction. The yield of thermal deactivation of the triplet states of both types of pigment mixture was always higher in the 3-h sample than in the 24-h sample, but the exact value depended on the group of pigments (Chls or Cars) responsible for the slow TD component (Table 1).

To solve this problem, the LIOAS signals (Rudzki-Small *et al.* 1992) were deconvoluted (Table 2). The pre-exponential factor  $\chi_1$  describes all processes occurring in

Table 2. Results of deconvolution of LIOAS signal of thylakoid samples.  $k$  – pre-exponential factor,  $\tau$  – lifetime of triplet state.

$t$ [h]	$k_1$	$\tau_1$ [ $\mu$ s]	$k_2$	$\tau_2$ [ $\mu$ s]
3	0.694	$\leq 0.4$	0.245	6.168
6	0.783	$\leq 0.4$	0.182	2.351
24	0.837	$\leq 0.4$	0.171	1.483

## Discussion

Irradiation-induced gradual development of pigment-protein complexes (Sigrist and Staehelin 1994) should influence the efficiency of excitation energy exchange within the photosynthetic assemblies which can be evidenced in the changes of some spectroscopic properties of Chls and Cars in thylakoid membranes. Our previous results (Waloszek *et al.* 1992, Więckowski and Waloszek 1993) show that full photosynthetic assembly is reached after the first 6–8 h of irradiation of dark-grown cucumber seedlings. During that period of time, the photoresistance of Chls as well as the efficiency of excitation transfer from accessory pigments to fluorescent Chl increased nearly 2-fold. This suggests that at a relative greater content of Cars compared to that of Chls in etioplasts the Car molecules are not functionally associated with photosynthetically active pigment-protein complexes.

The presented time-resolved photothermal data for thylakoids after 3 h of irradiation indicate that Cars quench  $^3$ Chls less than at later phases of greening. Cars dissipate quickly the energy obtained from  $^3$ Chls energy, exchanging it into heat. As a result of the less efficient quenching by Cars, the Chls are less protected against photodestruction. In other words, a great part of Cars is de-coupled from Chls at the earlier phases of greening and can not protect them. When more triplet excitation is quenched by Cars, the steady state TD, as well as deactivation in short time, increase. In accordance with previous reports (Boardman 1966, Więckowski and Waloszek 1993, Schoefs *et al.* 2000, and others) we also found that in the 3-h sample some amount of fluorescent form of protochlorophyll(ide) could be detected. Protochlorophyllide was not detected in the 6-h and 24-h samples. This may be associated either with its transformation into

time shorter or equal to the time resolution of the apparatus. The values were, within limits of accuracy of both methods, similar to  $\alpha$  values from Table 1. The  $\chi_2$  values were closer to  $\Phi_T$ (Chls) than to  $\Phi_T$ (Cars). The sum of both pre-exponential factors was close to one for all samples. This shows that the participation of eventual processes, slower than those measured by our apparatus, is weak. Such processes are nevertheless occurring, as follows from the frequency dependence of steady state PAS. On the basis of deconvolution of LIOAS signals we conclude that  $^3$ Chls are responsible for the slow component of TD observed, and that they are quenched by Cars more efficiently at later phases of greening. Their TD decay time decreases during greening from about 6.2 till 1.5  $\mu$ s. Thus at later phases of thylakoid greening Cars efficiently quench the triplet states of large number of Chls molecules, whereas the Cars in etioplasts are less effective.

chlorophyllide or with its photodestruction because this pigment is extremely photosusceptible (Więckowski and Waloszek 1993). The enhancement of fluorescence intensity in the region of 720–730 nm during the greening time may be associated with the development of photosystem 1 (PS1) assembly (for review see Šesták and Šiffel 1997) and/or with increasing yield of excitation energy transfer from LHC2 complex into fluorescent Chl associated with PS1 as a result of increasing capability of specific protein phosphorylation (Bredenkamp and Baker 1988, 1990). The increase in  $\Phi_F$  is probably related, among others, with the content of specific forms of Chls. At 460 nm (wavelength of excitation) various pools of Chls and Cars contribute to photon absorption. Thus, fluorescence is a result of direct Chls excitation and Chls excitation by singlet excitation transfer from Cars. Earlier findings (Więckowski and Waloszek 1993) indicate that the yield of excitation energy transfer from accessory pigments to fluorescent forms of Chls increases with cotyledon greening.

As follows from a comparison of the absorption spectrum (Fig. 1) with PAS (Fig. 2), their shapes as well as peak positions are different. The differences for 6-h sample suggest that the short-wavelength absorbing forms of Chl, localised more peripherally towards the reaction centre in photosynthetic assemblies, exhibit higher yields of TD than the long-wavelength forms that are located near the reaction centres. Besides that, longer-wavelength absorbing forms of Chl are more sensitive to photo-bleaching due to generation of  $^1\text{O}_2$  (Carpentier *et al.* 1986). This may suggest that energy dissipation in the form of heat and the  $^1\text{O}_2$  generation leading to Chl bleaching dominate in two different pools of Chls

within the thylakoid membranes.

Both methods of LIOAS signal analysis showed that the slow component of TD with the decay time in  $\mu$ s range decreases with the greening process. It is important to which group of pigments (Chls or Cars) this component belongs. The yields of Cars triplet formation by ISC is very low (Truscott 1990) and the decay time  $\tau_T$  is reported from 10  $\mu$ s (Truscott 1990) till 15  $\mu$ s (Burke *et al.* 2000). The Chls triplets were investigated by several methods in model systems and in organisms (Sauer 1975, Litvin and Sineschchekov 1975, Angerhofer 1991, Hanson 1991). Different values of  $\Phi_T$  and  $\tau_T$  were reported. For ether solutions,  $\Phi_T$  values were 0.64 and 0.88 for Chl *a* and Chl *b*, respectively (Sauer 1975). The  $\Phi_T$  values depend of the course on pigment interactions with the surrounding, therefore they should be different in thylakoids. Under supposition that Chl triplets predominantly contribute to slow TD, the value  $\Phi_T = 0.46$  was obtained for the 3-h sample. Under supposition of Car triplets this value was higher (Table 1) but in both cases  $\Phi_T$  decreased with thylakoid greening. Also the lifetime of triplet ( $\tau_T$ ) decreased during the greening process (Table 2). Observed lifetime values can suggest that LIOAS is related to the Chl triplet decay, which was reported in the 10  $\mu$ s (Litvin and Sineschchekov 1975, Sauer 1975) or much longer range (Angenhofer 1991). The decay of Car triplets with similar lifetimes was observed (Truscott 1990) but the yield of Cars triplets formation by intersystem crossing transition is usually low whereas the  $\Phi_T$  obtained by both suppositions (Table 1) was high. The values of  $\Phi_T$  (Table 1) and  $\chi_2$  (Table 2), both related to slow components of TD, suggest that the supposition of Chls participation seems to be more suitable.

Such conclusion is strongly supported by the literature

data: Lebedev *et al.* (1991) report that the intensity of Chls phosphorescence decreases as a result of thylakoid greening. This also suggests that Chl triplets are quenched more efficiently at later phase of greening. The results obtained by LIOAS deconvolution are less approximated than those using the Marti *et al.* (1996, 2000) method, but both methods have to give similar results.

The supposition that Chls forms weakly interacting with Cars occur in thylakoids at prior phase of greening is strongly supported by results obtained by Myśliwa-Kurdziel *et al.* (1997) showing that the contribution to fluorescence of such short-wavelength Chls form decreases as a result of thylakoid greening. Therefore we supposed that slow TD proceeded predominantly from the  $^3$ Chls which are efficiently quenched by Cars.

The triplet states of Chls can be also quenched by oxygen (Pineiro *et al.* 1998). This leads to singlet oxygen formation and as a consequence to Chls degradation. But the last process is less efficient than quenching by Cars assuming that Chls and Cars are in close contact (Frąckowiak *et al.* 1995). We also suppose that this contact becomes closer during greening and that the efficiency of Chl triplet state quenching by Cars also increases. These statements are in agreement with our previous results (Waloszek *et al.* 1992, Więckowski and Waloszek 1993) which indicate that Chls in earlier phases of greening are highly susceptible for photobleaching, and with the findings indicating that  $^1\text{O}_2$  production is greater in the initial phase of greening (Caspi *et al.* 2000). This supposition is also confirmed by the decrease in average yield of triplet formation ( $\Phi_T$ ). It is not possible to exclude the influence of photosynthetic energy storage on the data presented, but the increase in TD with chloroplast greening suggests that it is not a crucial factor.

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