The chlorophyll fluorescence quenching and changes of absorbance in pea chloroplasts

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Abstract

Chlorophyll (Chl) fluorescence quenching parameters were measured in dark-adapted pea leaves and chloroplasts with the purpose to find the conditions of high and low non-photochemical quenching, that would be stable during a prolonged irradiation. A PAM fluorometer was used for measuring induction curves in the range of actinic radiation of 3-35 W m^{-2}, with an ordinary value of about 15 W m^{-2}. The effects of various mediators, *i.e.*, ascorbate, methyl viologen (MV), dithiothreitol (DTT) and nigericin, on the quenching process were tested. Simultaneously, the absorbance was measured during a 15-20 min period of irradiation and after the actinic radiation was turned off, *i.e.*, in the recovery period. The pH values of chloroplast suspensions were 5.5, 6.5 and 8.0, the largest non-photochemical quenching was observed at pH of 6.5. The irradiation of chloroplasts led to an absorption decrease within the entire photosynthetically active range, attaining saturation when the fluorescence reached F_s level, and to an absorption increase during the recovery period. Absorbance changes at the maximum of red band were 10-20 %. A decrease in Chl concentration (10 %) after irradiation was found only at pH of 5.5, when the recovery time was the longest, *i.e.*, about 60 min.

Additional key words: ascorbate; dithiothreitol; irradiation; methyl viologen; nigericin; pH; *Pisum sativum*.

Introduction

The phenomenon of Chl fluorescence induction has been known for more than 50 years (cf. Lichtenthaler 1992). Nowadays this process is used as a tool for studying many plant physiology aspects related to photosynthesis (Krause and Weis 1991). The induction curve is a dependence of photosynthetic objects' fluorescence yield on time under a continuous irradiation. Usually the fluorescence increases as a response to turning the light on, and then decreases with the time of irradiation to a stationary value (F_s). The lowest value of fluorescence (F_0) is observed in dark-adapted plants

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under a weak irradiation. In dark-adapted samples the maximal level is achieved at a high (saturating) irradiation (F_m). The fluorescence yield depends on the redox state of the first stable electron acceptor of photosystem 2 (PS2), Q_A (Duysens and Sweers 1963), and it oscillates between maximal and minimal values in dark-adapted intact systems during the irradiation, or with redox agents in different preparations of PS2 [for a hypothesis on the origin of variable fluorescence (F_m - F_0) from the charges' recombination of the state of reaction centres with reduced Q_A see Klimov *et al.* 1978]. Usually the processes connected with the reduction of Q_A are studied in a short term time scale, up to several seconds. Investigations of fluorescence dependence mostly *in vivo* in the long-term time scale [min] show that F_m, measured after saturating flashes, is also an unstable parameter of the system, usually declining during the irradiation. Thus the fluorescence quenching may be divided into two parts: photochemical quenching, dependent on the state of reaction centres, and non-photochemical quenching, the nature of which depends on other than primary photochemical processes (Briantais *et al.* 1979, Bilger and Schreiber 1986).

Changes in xanthophyll/Chl interactions that take place during irradiation play an important role in these processes (Demmig-Adams 1990, Gilmore and Yamamoto 1991, Horton *et al.* 1991, Krieger *et al.* 1992, Owens *et al.* 1992, Ruban *et al.* 1992). The nature of non-photochemical fluorescence quenching may be explained by an energy transfer from an excited Chl molecule to zeaxanthin, newly formed under the irradiation, with a further energy dissipation into heat (Demmig *et al.* 1987, Demmig-Adams 1990). The light-dependent accumulation of zeaxanthin relates to a higher fluorescence quenching (for a review see Demmig-Adams 1990). Both phenomena depend on pH or pH gradient of a thylakoid membrane (the enzyme deepoxidation converting violaxanthin to zeaxanthin has an activity optimum near pH of 5.0; an opposite reaction is provided by epoxidases at a pH near 8.0 - Hager 1980/1981).

The radiation-induced fluorescence quenching appears in isolated pigment-protein complexes (Jennings *et al.* 1991), and the aggregation state of light-harvesting pigment-protein complex 2 (LHC2) may determine the "high energy state" quenching *in vivo* (Horton *et al.* 1991, Ruban *et al.* 1992, Hagen *et al.* 1996) with a low probability of zeaxanthin participation (Mullineaux *et al.* 1993). The process of fluorescence decline under irradiation is a complex one; it may be a superposition of all processes influencing the state of both reaction centres and antenna.

In this work I present the results of simultaneously measured fluorescence quenching and absorption changes in pea chloroplast suspensions during irradiation and after it (recovery time).

Materials and methods

The chloroplasts were isolated from fresh pea leaves grown in a growth chamber (12 h, 22 °C, ≈ 20 W m^{-2}). The leaves were washed with distilled water and kept in the dark on ice overnight. Usual 10 g leaves were homogenized for 5 s in a *Waring* blender with 100 cm^3 grinding buffer, containing 50 mM $NaHPO_4$, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 25 mM NaCl, and 0.33 M α-(D)-glucose, pH 6.5. The grinding and

assay buffers were prepared according to Gilmore and Yamamoto (1992). Just before the tissue homogenization, 0.1 % bovine serum albumin and 0.1 % ascorbate were added into the grinding buffer. The homogenized material was filtered through four layers of cheese cloth, and centrifuged at 2 000 × *g* for 2 min in a *Beckman* centrifuge. The pellet was collected and resuspended in 1-2 cm^3 of grinding buffer, and kept on ice in the dark before measurements. For measurements, this fresh chloroplast suspension was diluted with the assay buffer to a proper Chl concentration, usually 30-50 g m^{-3}. Changed pH values were obtained by diluting with buffers (Tricine-NaOH or Tricine-HCl).

The Chl fluorescence induction parameters were measured as in Ting and Owens (1993) with a *PAM-101* Chl fluorometer (*H. Walz*, Effeltrich, Germany). Photochemical and non-photochemical fluorescence quenching coefficients, q_p and q_n, were calculated according to Bilger and Schreiber (1986) as

$$q_p = (F_m' - F_s)/(F_m' - F_0'); \quad q_n = 1 - (F_m' - F_0')/(F_m - F_0),$$

where F_0, F_m, and F_s are parameters of a dark-adapted sample, and $F_{(0,m,s)}'$ are fluorescence values measured in an irradiated sample. The fluorescence was excited with a modulated low irradiance, and detected by a selective technique. The saturating pulse irradiance for measuring the F_m was about 100 W m^{-2}. The continuous actinic irradiance was changed in the range of 3-35 W m^{-2}. Broad blue filters were used in both irradiations. In addition to the blue filter the saturated pulse radiation was passing through a heat absorption filter. The absorbance spectra were measured with a spectrophotometer *Cary 14* connected with a computer.

Results

PAM fluorescence measurements; relation between quenching parameters; influence of nigericin, ascorbate, MV, DTT, and pH: The ratio F_m/F_0 in all experiments was close to 5.0. Irradiance dependences of F_s, q_P, and q_N of leaves and chloroplasts with and without added mediators after 15 min of irradiation are shown in Fig. 1. During further irradiation, up to 60 min, the tendency of changes was the same: q_P did not change or it declined very slowly, and q_N usually rose during the irradiation (for example, from 0.32 at 15 min irradiation to 0.50 at 60 min). The actinic irradiance and time of irradiation led to an increase of q_N, the addition of ascorbate or MV also led to an increase of q_N. The contribution of q_P to the decrease of fluorescence under irradiation was much smaller in chloroplast suspensions than in leaves, where q_P was high. The DTT generally decreases q_N (Neubauer and Yamamoto 1992, Yamamoto and Kamite 1972). In my experimental conditions, an addition of DTT into chloroplast or thylakoid suspensions also made the quenching decrease. Usually during the first 10 min of irradiation both quenching processes developed in the DTT suspensions, but to a smaller degree than in control chloroplasts; during the next 20-30 min the maximal level F_m was reached, and q_P and q_N became zero.

The maximum effect of quenching was observed at pH of 6.5 (Table 1). Also the recovery period of F_m (time of reversibility or dark adaptation) was different at

different pH values. At pH of 8.0, the F_m returned to the value determined before the irradiation after 1-3 min in the dark already. At pH of 6.5 the recovery was longer, about 5-10 min, and at pH of 5.5 it was about 60 min. Also the effect of nigericin addition was dependent on pH: at pH values of 6.5 and 8.0 the addition of 10 μM nigericin to the irradiated suspension led to an increase of F_m to the initial value, *i.e.*, the recovery process was accelerated, but at pH of 5.5 no effect of nigericin on F_m was observed.

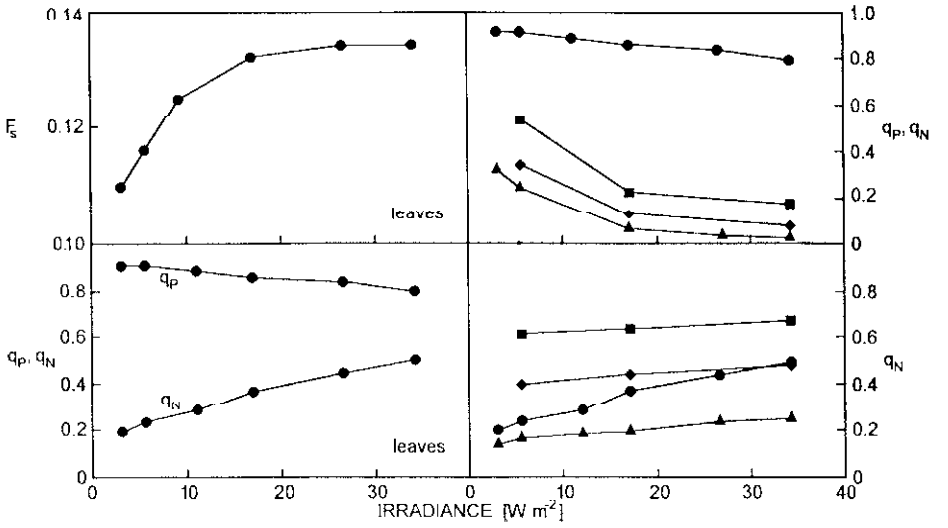

Fig. 1. Dependences of steady state fluorescence F_s [V] and coefficients of photochemical (q_P) and non-photochemical (q_N) quenching on intensity of actinic irradiance measured in dark adapted leaves (●) and chloroplasts (▼ control; ∇ +ascorbate; ❑ +methyl viologen) after 15 min irradiation.

So, in these experiments with pea chloroplasts, an addition of ascorbate or MV, and increase in time of irradiation led to an increase in q_N in accordance with the results of other researchers. The DTT reduced the q_N almost to zero during 20-30 min of irradiation. Nigericin did not affect the quenching at pH of 5.5.

Absorption spectroscopy measurements: The irradiation of chloroplast suspension decreased the absorption simultaneously with the decrease of fluorescence.

At pH of 6.5 during irradiation through the blue filter an about 20 % drop of absorbance was observed in the whole measured range of 550-750 nm. The time of reaching the "saturated" value of these changes was the same as that of the fluorescence decline to the steady state, *i.e.*, about 20 min.

After 20 min of irradiation the thylakoid suspension of pH 5.5 was darkened and difference spectra in the range of 400-800 nm were measured after 2-65 min in the dark (Fig. 2*B*) without adding the ascorbate (ascorbate was present during the isolation procedure). The measurement of one spectrum took 1.5 min. The changes at the red maximum were about 10 %. Their absolute value continuously decreased with time (spectra *1* and *2*), and after 30 min in dark the whole differential spectrum was

above zero line (spectrum *3*). The spectra *4* and *5* (Fig. 2*B,C*) were measured 50 and 65 min after irradiation, respectively. The time 65 min corresponded to the time of reversibility of F_m. A further increase of F_m was not observed. The absorbance changes measured 65 min after irradiation were in shape close to the initial chloroplast spectrum; absolute values of these positive changes were about 10 % of the initial maximum absorption. Fig. 3 compares the spectra *1* and *2* of Fig. 2. The shape of the main bands was similar with the exception of the regions of 500-600 nm and beyond 700 nm. The shape of differential spectra is shown in Fig. 3*D*.

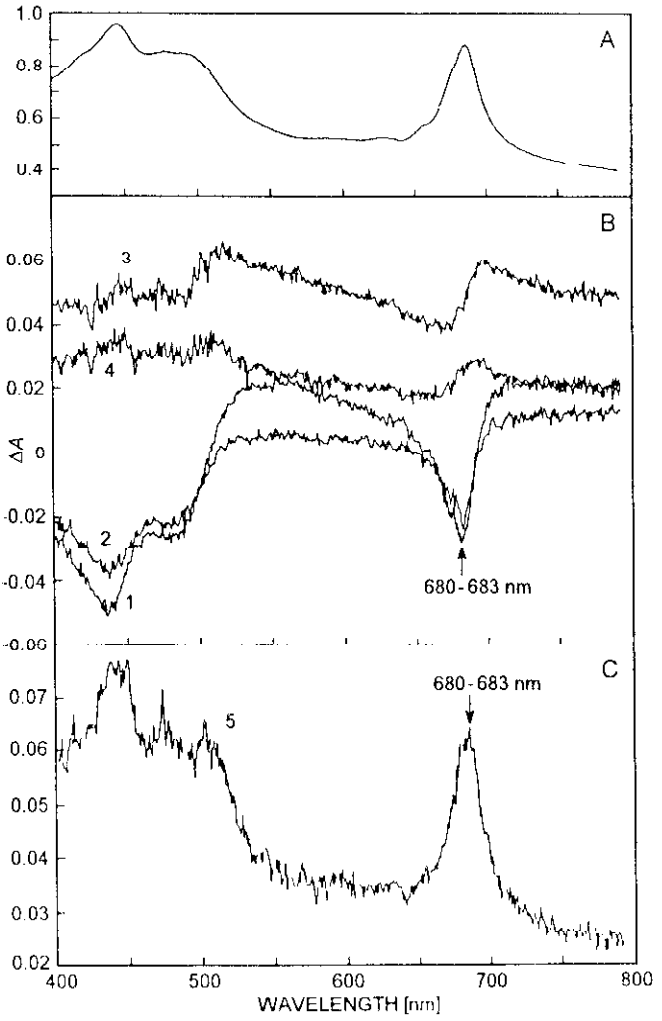

Fig. 2. Absorption spectrum of dark adapted thylakoid suspension (*A*) and difference spectra of irradiated and dark adapted samples (*B, C*). Spectra *1, 2, 3, 4,* and *5* correspond to the times of 2, 4, 30, 50, and 65 min after irradiation in case of reversible fluorescence. Spectra were measured in a 2 mm cuvette; pH 5.5.

Similar measurements were made at pH values of 6.5 and 8.0 (Fig. 4*A,B*), the respective fluorescence parameters are in Table 1. The absorption changes at pH of 6.5 measured just after the irradiation were negative (bleaching) along the whole spectrum, but they were smaller than those of curves *1* and *2* in Fig. 2. Two positive

differential spectra shown in Fig. 3 were measured 30-35 and 55-60 min after irradiation. These spectra resemble those in Fig. 2. Fig. 4*A* clearly shows the bands at 500-600 nm and near 700 nm. At pH of 8.0, the differential spectrum recorded 60 min after irradiation (Fig. 4*B*, *lower curve*) also has a character of the chloroplast

Table 1. Dependence of fluorescence quenching characteristics in pea thylakoids on pH. F_m1 fluorescence at saturating pulse of dark adapted sample (control) and F_m2 after irradiation and next dark period of increasing (time of reversibility).

pH	q_P	q_N	Time of reversal to F_m2 [min]	F_m2/F_m1
8.0	0.09	0.35	1-3	0.98
6.5	0.21	0.46	5-10	0.90
5.5	0.15	0.34	60	0.87

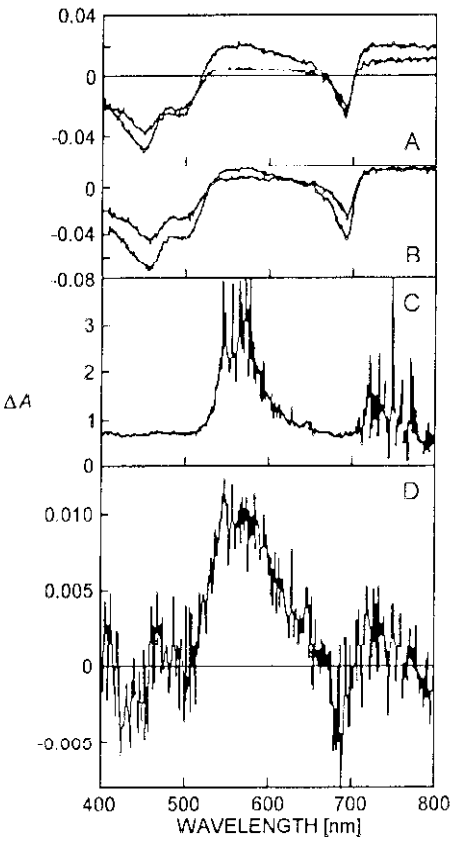

Fig. 3. Analysis of two spectra from Fig. 2*B*, *1* and *2*. *A*: spectra as recorded, *B*: spectra shifted to zero line in the red region; *C*: the ratio of these spectra; *D*: the difference between spectra normalized in the 400-500 nm region.

spectrum, but it is shifted along the vertical axis in negative direction. Similar changes with time appeared in almost all differential spectra: they looked like shifts

of axis along the Y axis (Figs. 2 and 4). The value of these changes was about 0.01-0.05 with the absorbance at 680-682 nm being about 1.0.

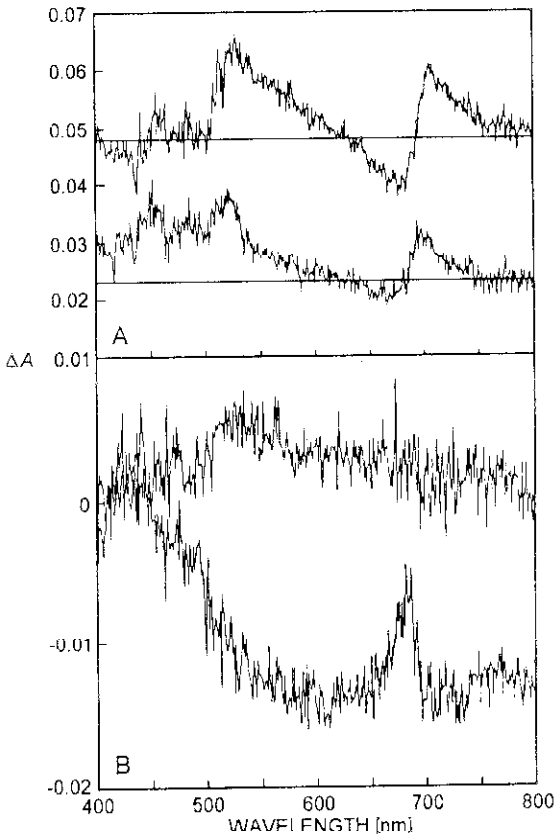

Fig. 4. Differences in absorption caused by irradiation of chloroplast suspension with actinic radiation, pH 6.5 (*A*) and 8.0 (*B*) (compare with Fig. 2), measured in first 2 min (*upper curves*) and 60 min (*lower curves*) after irradiation.

Discussion

The observed decrease in chloroplast absorption could not entirely explain the decrease in fluorescence yield during irradiation. Absorption in the main bands was 10-20 % lower, but the fluorescence non-photochemical quenching measured here was in the range of 30-50 %. This decrease in chloroplast absorption under irradiation followed by changes in the dark period can be explained as changes in concentrations of pigments or as absorption coefficient changes or as both of them. Concentration of Chl molecules was measured before and after the irradiation and also during the recovery period. The 10 % drop of Chl concentration was registered in chloroplast suspension at pH 5.5 during the 20 min irradiation. Under other conditions, the reduction in concentration was not obvious, perhaps because of the faster recovery period. It is difficult to measure Chl concentration during this period because of increased absorption of acetone extract in the 700-800 nm region

(Figs. 2*B*, 3, 4*A*). These pigments may also contribute to fluorescence quenching processes. I did not study the mechanism of Chl degradation. Sometimes the increase in Chl concentration was registered after an irradiation at pH of 8.0 (5-12 %). Without additional irradiation (under a very weak spectrophotometer irradiance) the small increase of absorption (6-8 %) was observed (see Fig. 5) in the chloroplast suspension at pH of 5.5.

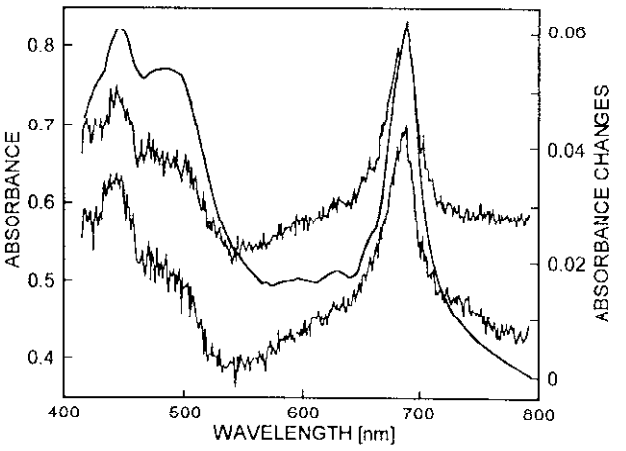

Fig. 5. Absorption spectrum, measured in dark adapted chloroplasts, and changes in absorbance in time without irradiation: *upper* in 35 min, and *lower* in 55 min after the measuring of absorption spectrum; pH 5.5.

During the irradiation and first minutes of recovery, absorption changes of the main bands were similar (Fig. 3*C*); the only differences were observed within 500-600 nm and beyond 700 nm. In the following time, distinct changes developed also in the main absorption bands, where absorption in the 500-600 nm region outstripped the one at 400-500 nm.

The region of 500-600 nm is often used for absorbance measurements: of chloroplast electrochromic shift (Witt 1979), of light-dependent zeaxanthin formation at 500-520 nm usually measured against 540 nm (Demmig-Adams 1990, Gilmore and Yamamoto 1992, Pfündel and Dilley 1993), for finding maximum radiation scattering effect (Bilger *et al.* 1989, Bilger and Björkman 1994), and for determining the activity of light-induced electron transport in thylakoid membranes (Chylla *et al.* 1987). The absorbance changes I observed in the 500-600 nm region and near 700 nm may belong to the zeaxanthin molecules accumulated during irradiation due to the violaxanthin-zeaxanthin transformation. These regions coincide with the proposed excited states of zeaxanthin molecule on the basis of investigation of spectral properties of polyenes (Cosgrove *et al.* 1990): the evaluated transitions were around 500 nm (1^1B_u-1^1A_g) and 700 nm (2^1A_g-1^1A_g). Thus the formation of zeaxanthin under irradiation serves as an additional pathway of heat energy dissipation, lowering the fluorescence yield from singlet Chl state, and contributing to non-photochemical quenching (Owens *et al.* 1992). These xanthophylls were found in minor antenna pigment-protein complexes and LHC2 (Dainese *et al.* 1992). The pH of 5.5 I used was favourable for the formation of zeaxanthin: the de-epoxidizing enzyme has a maximal activity at pH of 5.5 (Hager 1980/1981).

In studies of photodependent events in chloroplasts of higher plants and in cells of green algae and cyanobacteria, especially in the developmental stages during photomorphogenesis, the absorption changes in the region of 500-600 nm and in the red region are explained as the activation of receptor molecules, such as rhodopsin, phycoerythrin, and phytochrome. The broad band 500-600 nm in Fig. 3*D* resembles the light-sensor property of such molecules that act in dark-light adaptation processes, leading to a partial elimination of photosynthetic capacity, in photoinhibitory effect, acting not only on the D1, D2 protein destruction, but also affecting the decrease in Chl content. The process shown here may be the "turnover" of pigments in and after prolonged irradiation, similar to protein turnover during photoinhibition. Zeaxanthin formation may be involved in the regulation of absorption, suitable for the used irradiance.

Absorbance spectra measured here revealed also small changes in the "zero line" position, clearly seen at 750-850 nm. During 60 min, the absorption at 800 nm was changing in 0.05 intervals, and these changes resembled passing through discrete levels of absorption (or scattering?), like oscillation changes.

Fluorescence and absorption properties are also related to the conformation state of membrane. Primarily it is a light-dependent (reversible) phosphorylation of the LHC2 complexes (Bennett 1983, Staehelin and Arntzen 1983), and their migration between two photosystems (Barber 1986). The aggregation state of LHC2 may also be changed during irradiation; it influences absorption and fluorescence properties (Horton *et al.* 1991, Ruban *et al.* 1992). There are many light-stimulated processes in chloroplasts that depend on each other. Here it is more correct to speak about the changes of pigment concentration during a prolonged irradiation. The *ca.* 10 % drop of absorption correlates with the 10 % decrease in Chl concentration. A drop along the entire spectrum with the following increase of absorption in the dark resembles a process regulating absorption of radiation, possibly by oxygen (during irradiation of Chl-pigment complexes, Chl is oxidatively destructed - Telfer *et al.* 1990, Purcell and Carpentier 1994).

Routine measurements similar to those described above confirmed always bleaching of the main Chl bands. Reversibility of absorption was faster and better reproducible in the presence of ascorbate.

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