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CHLOROPHYLL-CAROTENOIDS INTERACTIONS IN MODEL SYSTEMS AND IN ORGANISMS INVESTIGATED BY PHOTOACOUSTIC SPECTROSCOPY*

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Carotenoids in photosynthetic organisms are involved in the light harvesting process and in the protection of chlorophylls against photo destructions. The first process is realized by the transfer of singlet excitation from carotenoids to chlorophylls, the second, by the quenching of triplet excitation of chlorophylls by carotenoids. Both processes occur with various yields for different chlorophyll-protein complexes and for different carotenoids. In this paper are described the results of the spectral investigations of these processes in organisms and in model systems. It was shown that photoacoustic spectra and time-resolved, delayed-luminescence spectra can deliver new information about carotenoids and chlorophylls interactions.

INTRODUCTION

Carotenoids are involved in the photosynthetic light-harvesting process and in the protection of chlorophyll (Chl) against photobleaching (Krinsky, 1989; Koyama, 1991; Koyama,1993). The energy absorbed by carotenoids is transferred to chlorophylls by a singlet-singlet transition (Govindjee, 1975), whereas photo-protection is associated with a very efficient quenching of the chlorophyll triplet states by the carotenoids. This quenching prevents harmful photodynamic reactions (Kramer & Mathias, 1980). It is still unclear if these dual interactions concern all carotenoid species and all chlorophyll forms equally. There is some evidence that in thylakoids a small part of the chlorophyll triplet states is not quenched by carotenoids (Kramer & Mathias, 1980). Also, some pools of xanthophylls do not seem to be involved in the transfer of singlet excitation to the chlorophylls (Goedher, 1969; Pfündel, 1993). The scheme of the interactions between chlorophyll *a* and β-carotene is presented in Fig.1. This scheme can be used in more general discussion on interactions between various carotenoids and different chlorophyll forms. Energy absorbed by antenna pigment can be converted to heat, transferred to some other pigment molecule, emitted as luminescence or used for some photochemical reaction. The last process in native antenna has low efficiency, because the main role of the antenna is to deliver excitation energy to a photosynthetic reaction center. Other paths of deexcitation compete between themselves and have various yields depending on the surrounding of the absorbing molecule. In organisms, the thermal deactivation of carotenoids is low (Cegielski, Frąckowiak & Leblanc, 1992) due to the high yield of the transfer of the singlet excitation from carotenoids to chlorophylls. Carotenoids fluorescence is weak, (Gilbro & Cogdell, 1989) therefore, for an isolated molecule, its thermal efficiency yield is much higher than that for an isolated chlorophyll molecule which exhibits a higher yield of fluorescence. Therefore, the perturbation of the excitation transfer from carotenoids to chlorophylls located in organisms causes the change in the thermal deactivation (TD) spectrum. The change in the shape of the photoacoustic spectrum as a result of the perturbation of the excitation energy transfer between some donor and acceptor of excitation characterized by various yields of fluorescence or various yields of photochemical reactions are explained in Fig.2. Let us suppose that pigment A has a low yield of fluorescence, pigment B much higher and both pigments are photochemically

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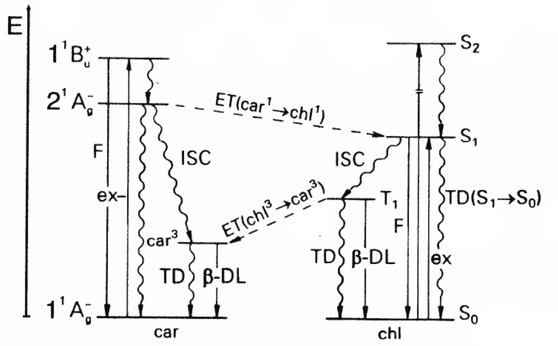

Fig. 1 Diagram of energy levels of Chl a and β-carotene.

stable. Under the supposition that excitation energy transfer from A to B is very high (yield of the transfer about 1) the energy transfer (ET) to B is much more effective than TD in A and as a result of illumination with the same amount of energy of the various spectral regions is obtained PAS with the same shape as absorption and with and amplitude dependent on TD in B. Thermal deactivation will have a constant value in the region of both pigments' absorption. When the ET is blocked, the PAS will have higher amplitude in the region of A absorption and lower in the region of B. Thermal deactivation will change the value, as is shown in Fig.2. It is thus possible to evaluate the yield ef excitation energy transfer in highly scattering biologiocal samples. This method using phycobilins and chlorophyll mixtures (Frąckowiak, Hotchandani & Leblanc, 1985; Frąckowiak, Erokhina, Balter, Lorrain, Szurkowski & Szych, 1986) was described in detail by Frąckowiak (1990).

For chlorophyll molecules strongly interacting with the carotenoids, the direct decay of excitation from the triplet state (T_1) to ground state (S_0) can be neglected with respect to their triplet-quenching by carotenoids. For chlorophyll molecules not interacting with carotenoid, the decay (T_1-S_0) is in a range of hundreds of μs (Shepanski, Williams & Kalisky,1984; Lavanon & Norris, 1978; Gurinovich, Sevchenko & Soloviev, 1968; Gurinovich, Slyk & Zenkevitch, 1974). The shorter triplet lifetimes observed in chloroplasts (Neshusthtai, Thornber, Petterson, Fessenden & Lavanon, 1988; Lavanon & Norris, 1978; Shepanski *et al.*, 1984) can be due, at least partially, to the interactions with carotenoids. Carotenoids separated from chlorophyll have a non-radiative decay time of about 5μs-10μs, whereas, for molecules strongly interacting with chlorophyll, intersystem crossing from excited singlet to triplet can be neglected because of the very effective transfer of singlet excitation to chlorophyll (Shepanski *et*

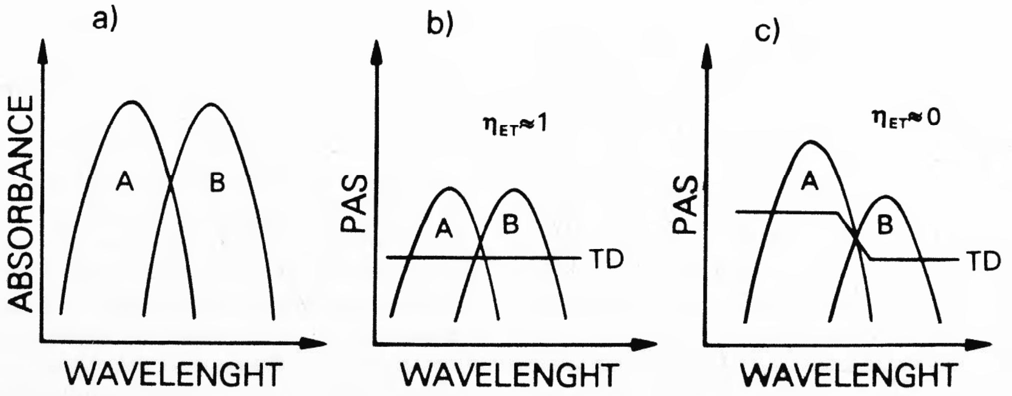

Fig.2 The schematic explanation of the change in PAS and TD spectra of two pigments A (low yield of fluorescence, high TD yield) and B (high yield of fluorescence, low TD yield) as a result of the perturbation of excitation energy transfer (ET) between pigments. (a) Absorption of dye with high yield of thermal deactivation (η_{TD}) and dye with lower η_{TD}; (b) photoacoustic at high energy transfer ($\eta_{ET} \approx 1$); (c) photoacoustic at low energy transfer ($\eta_{ET} \approx 0$).

al., 1984; Breton, Gacintov & Swenberg, 1979; Nechusthtai *et al.*, 1988). As follows from this short discussion, it is possible to distinguish between strongly and weakly mutually-interacting pools of chlorophylls and carotenoids molecules on the basis of photo-thermal spectra compared with fluorescence excitation, delayed luminescence and absorption spectra. Because of the various kinetics of irradiative deactivation of separated and mutually interacting molecules, the most suitable would be the application of time-resolved photothermal spectroscopy. But, even using steady state apparatus, one can obtain some information about kinetics of thermal deactivation on the basis of a method proposed by Moore (1983) and developed in Poulet's laboratory (Ouzafe, Poulet & Chambron, 1992). From the spectra measured at various frequencies of light modulation and different phase shifts between a measured photoacoustic signal and modulated light, the mean decay time of the "slow" component of thermal deactivation can be obtained and the intensity ratio of the slow to fast components can be evaluated (Ouzafe *et al.*, 1992; Frąckowiak, Zelent, Malak, Cegielski, Goc, Niedbalska & Ptak, 1995; Frąckowiak, Cegielski, Niedbalska, Waloszek & Więckowski, in press). The slow component which can be investigated by such a procedure can be located in μs or in ms time range, depending on the light modulation frequencies used. The fast component contains the contributions from all the fast deactivation processes occurring in ns, ps and fs times.

There is evidence (Pfündel, 1993; Więckowski & Majewska, 1990; Więckowski & Waloszek, 1993) that, during the illumination of etiolated seedlings, the photo-resistance of chlorophyll increases, whereas, at the same time, the concentration ratio of carotenoids to chlorophylls decreases. This suggests that the efficiency of the quenching of the triplet state of chlorophyll by carotenoids in an early stage of greening is lower than after greening.

In order to establish the molecular mechanisms responsible for the interactions between carotenoids and chlorophylls, we investigated three types of samples: (i) chlorophyll a and β-carotene in liquid crystal (LC) solution (Frąckowiak *et al.*, 1995); (ii) thylakoids from etiolated seedlings greened during 24h (Frąckowiak *et al.*, in press), and (iii) thylakoids from etiolated seedlings after 6 h of illumination. The oriented layer of nematic LC containing photosynthetic pigments can be a model of biological membrane. It is anisotropic

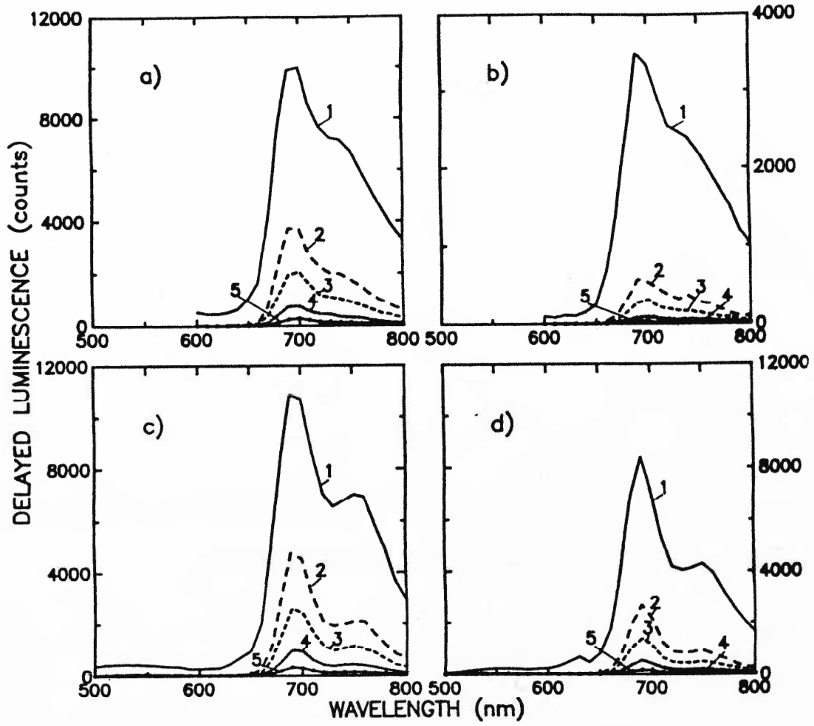

Fig.3 Time resolved delayed luminescence of Chl a and β-carotene in LC. (a), (c) Chl a; (b), (d) Chl a with β-carotene; (a), (b) higher pigment concentration; (c), (d)-lower concentration. λ_{exc}=420nm. Time windows: 1) 0.2-5.2 μs; 2) 5.2-10.2 μs; 3) 10.2-15.2 μs; 4) 40.2-45.2 μs; 5) 55.2-60.2 μs.

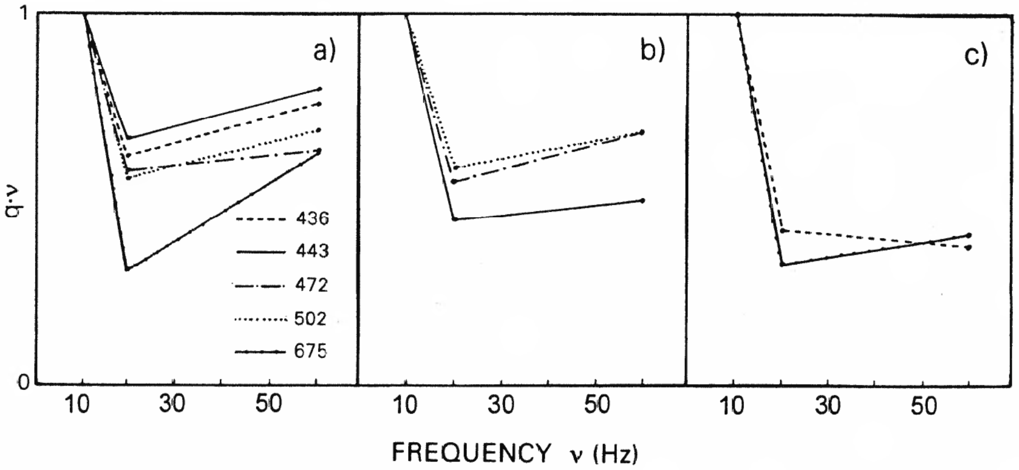

Fig.4 Dependence $q \cdot \nu$ versus ν (q — PAS amplitude; ν — frequency of light modulation). (a) Chl *a* with β-carotene; (b) β-carotene; (c) Chl *a*.

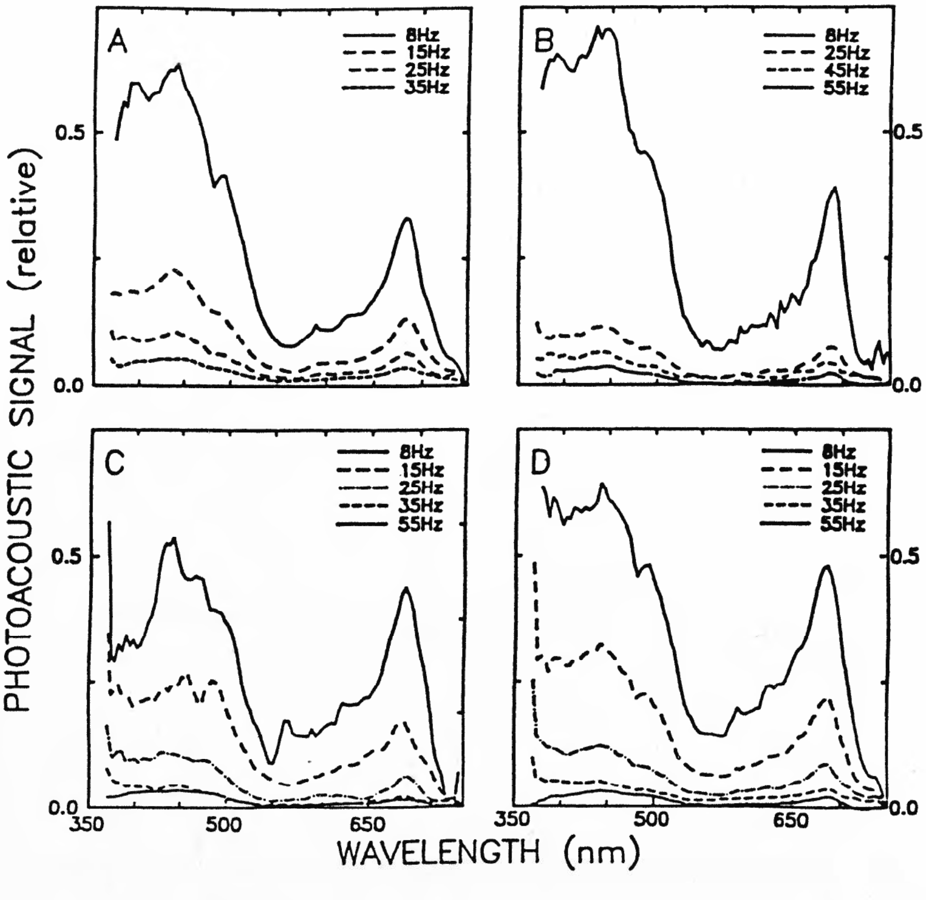

Fig.5 .Photoacoustic spectra of thylakoids (24 h of light) after one day of storage. (a) in air without DCMU; (b) in air with DCMU; (c) in helium without DCMU; (D) in helium with DCMU. Phase shift: (a) and (b) $f = -96$deg; (c) and (d) $f = -117$deg. Frequencies of light modulation (ν) marked in figure

and fluid. The introduced pigments are oriented in LC by "guest-host" effect. The very strong interactions between the chlorophyll and the LC molecules diminish the yield of the formation of Chl aggregates. Therefore, it is possible to introduce a high concentration of the monomeric form of pigment.

As a result, molecules of both pigments can be

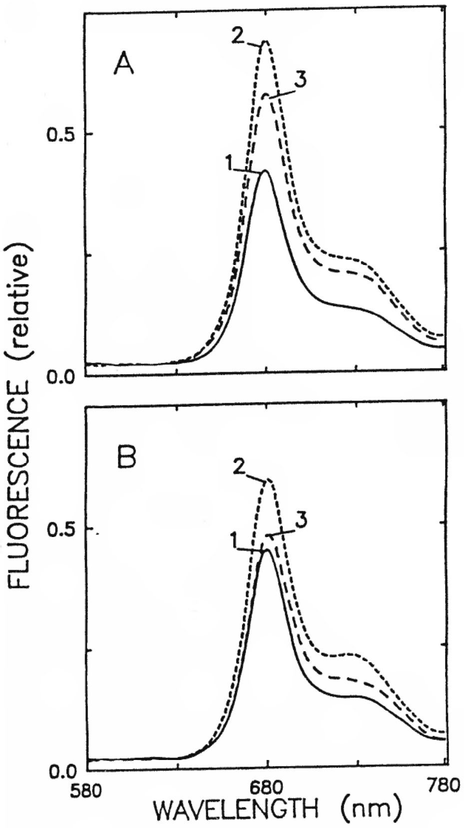

Fig.6 Fluorescence spectra of thylakoids suspension. (A)without DCMU; (B)with DCMU; excitation wavelengths: curves (1) 437nm; (2) 472nm; (3) 486.

located close together with definite mutual orientation. For the LC solution of Chl, β-carotene and their mixture several spectral properties such as polarized absorption, fluorescence, delayed luminescence and photoacoustic spectra were measured (Frąckowiak *et al.*, 1995). The fluorescence lifetime of Chl with carotene addition is longer than that of Chl alone, which shows that carotene molecules present in a sample prevent the formation of the "special pair" of strongly interacting Chl which act as a quencher (Beddar & Porter, 1967; van Zandvoort, Wróbel, Schlten, de Jager, van Ginkel & Levine, 1993). The lifetime of fluorescence was analyzed, using the sum of exponential decay components and a fractal model, in which it was supposed that every Chl molecule is in the presence of some fluorescence quenchers (Kowalczyk, Zelent, Malak, Planner, Sanocka & Frąckowiak, in press). At the same number of adjustable parameters, the last model better fits experimental data in the case of Chl *a* alone, whereas in the case of pigment mixture, both models describe measured decay with similar adequacy.

From polarized spectra, it follows that carotene perturbs Chl orientation. It changes mutual Chl molecules interactions and introduces strong interaction between carotene and Chl. Instead of a "special pair" of Chl molecules, such as was proposed by Beddard and Porter (1976), some closely-located systems are formed consisting of carotene and Chl. Such systems seems to be unfluorescent, because the total intensity of fluorescence decreases. The increase of lifetime suggests that only Chl molecules not interacting with carotene contribute to emission. The arrangements of such molecules can be, because of the perturbation in system orientation, less favorable for special pair formation.

The Chl in LC emits the DL located in the prompt fluorescence spectral region. It is probably β-type DL with the participation of the triplet state and activation from triplet to the excited singlet state of Chl. The carotene quenched the DL of Chl (Fig.3) what can suggest the quenching of the Chl triplet states by carotene. In the model system investigated, the transfer of singlet excitation from carotene to Chl was not observed even at high pigment concentrations. The frequency and phase-shift dependences of photoacoustic spectra suggest that part of the excitation is converted into heat in a slow process with the decay time of the order of µs. Fig.4 shows the $q \cdot \nu$ *versus* ν dependence for several wavelengths for pigment mixture (a), β-carotene alone (b) and for Chl alone (c). These dependences are different which shows that the participation of the slow component of TD decay is changed as a result of the addition of carotene to the Chl solution.

The addition of β-carotene to Chl changes the shape of the thermal deactivation (TD) spectrum. In the case of the efficient energy transfer from carotene to Chl the shape of TD for pigment mixture and Chl alone should be similar and the TD has to have constant value dependent on the yield of non-radiative deactivation of Chl. It shows once again that our model is much less efficient than the natural system in ET from carotenoids to Chl. But the triplet states of Chl are quenched, as follows from DL spectra. The whole set of data suggests that Chl molecules interact strongly with the β-carotene, forming some nonfluorescent or weakly fluorescent aggregates. Even such a simple model shows how complex is the problem of carotene-Chl interaction.

The second type of sample, greened thylakoids, exhibit some similarities and some differences with respect to the LC model. The PAS of such samples is much more sensitive to the sample

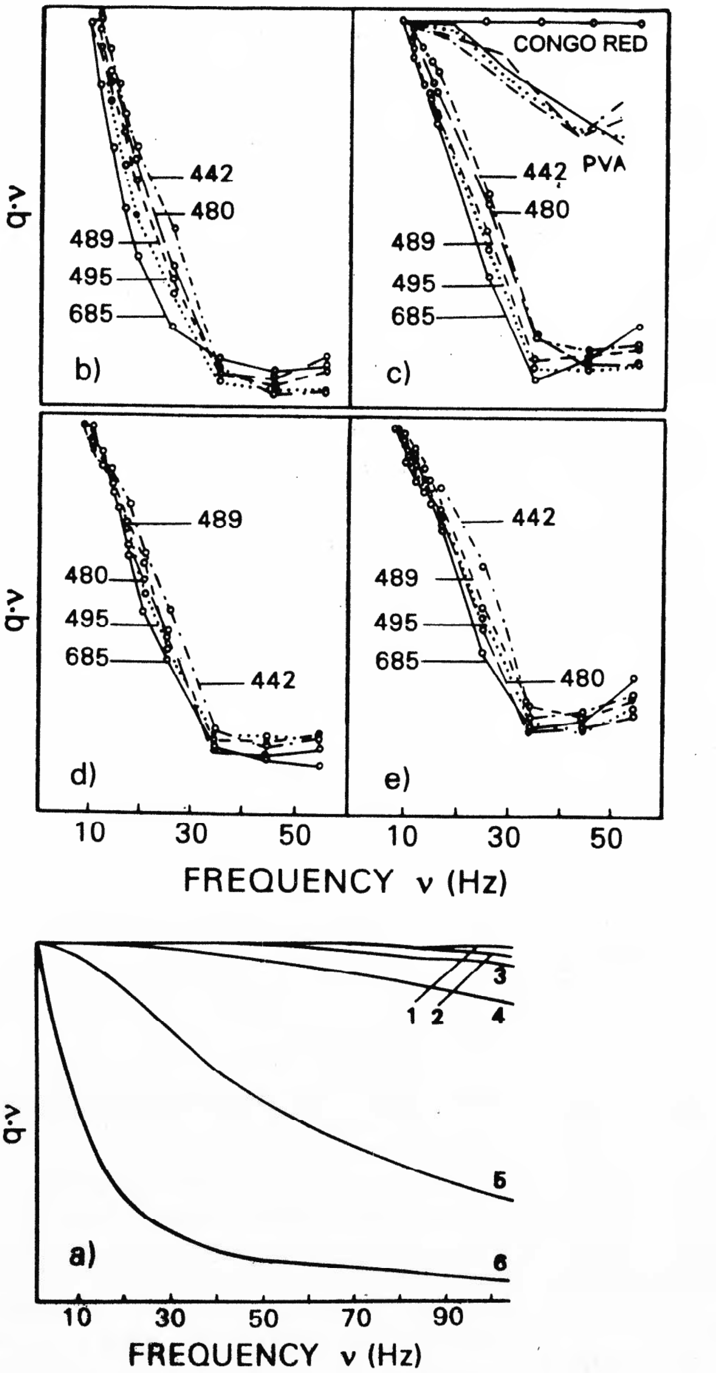

Fig.7 The dependence of $q·\nu$ versus ν for thylakoid (24h greening) compared with mathematical simulation results. (a) mathematical simulation done under the supposition that decay is mono-exponential for the following decay times: curve (1) 10μs; (2) 300μs; (3) 500μs; (4) 1000μs; (5) 5000μs; (6) 50 000μs. (b)-(e) experimental results for freshly-separated thylakoids (24h greening): (b) in He; (c) in He with DCMU addition; (d) in air; (e) in air with DCMU. In (c) the data for Congo red and Chl *a* in PVA film are also shown. Wavelengths for Chl *a* in PVA are marked as for thylakoids, for Congo red all wavelengths located inside of the absorption band give the same dependence.

storage than absorption spectra (Fig. 5). It shows that during storage the content of pigments and their locations are not dramatically changed, but energy storage in photoproducts is higher in the more active sample and/or the sample denaturation perturbs the efficiency of energy transfer between various antenna molecules having different yields of thermal deactivation. Fig.6 shows that the DCMU addition changes the fluorescence intensity ratio of the spectra excited with various λ_{exc}. DCMU blocked the linear electron transport. The change in the fluorescence intensities, different at various wavelengths of excitation, are in agreement with various and differently perturbed by DCMU yields of TD in different spectral regions. The change in the frequency of light modulation and/or phase between light and photoacoustic waves changed the shape of PAS which suggests the occurrence of a slow component of TD. The observed changes are dependent on the gas in the acoustic cell, DCMU addition to the sample, sample storage etc. The $q·\nu$ *versus* ν plots (Fig.7c-e) are compared with the mathematical simulation (Fig.7a) due under the supposition that only a slow component of TD exists and that it is changed from 10 μs (curve 1) till 5000 μs (curve 6). Experimental curves have a slope similar to those simulated for long decay time. The PVA film with photochemically stable dye Congo red, exhibits only a fast component of TD, Chl *a* in PVA has faster decay than Chl *a* in LC, and the slopes of experimental curves in a

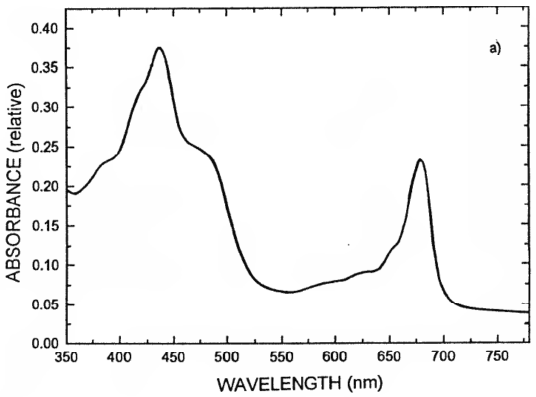

Fig.8 Absorption spectra of thylakoids (6h greening).

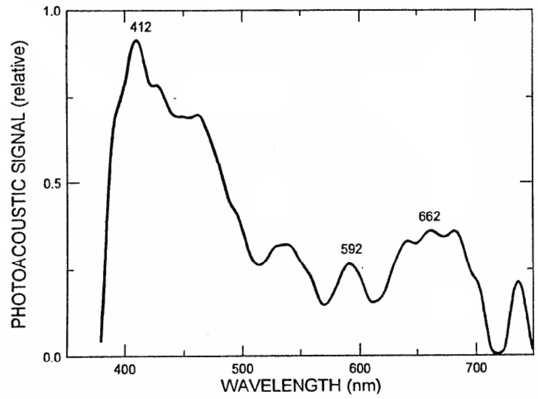

Fig.9 PAS of thylakoids (6h greening).

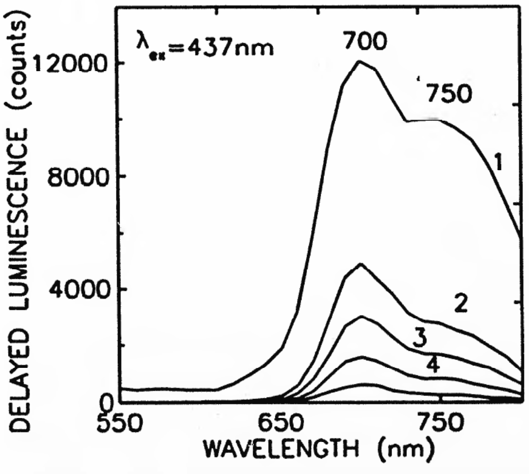

Fig.10 Delayed luminescence spectra of thylakoids (6h greening). λ_{exc}=437nm. Time windows: (1) 0.2-5.2 μs; (2) 5.2-10.2 μs; (3) 15.2 -20.2 μs; (4) 25.2-30.2 μs; (5) 50.2-55.2 μs.

case of Chl *a* in LC and Chl *a* and carotenoids in thylakoids are similar. Such curves for thylakoid, for various spectral regions, and different gas in the photoacoustic cell are not identical but the differences are not dramatic. Taking into account that all samples were measured using the same apparatus it is possible to draw qualitative conclusions concerning the decay of TD. The differences between TD values calculated for various spectral regions (after the Gaussian analysis of absorption and PAS) suggest that there are some pools of chlorophyll pigments interacting with different efficiency with various carotenoids, and that various carotenoids are differently engaged in interactions with given pool of Chl. The absorption and PAS spectra of various pigments are strongly overlapped which makes it difficult to evaluate quantitatively. The most promising sample is that obtained from etiolated seedlings in the early stage of greening (6h of illumination). Investigations of such samples are not easy, because of their instability during illumination and low chlorophyll concentration.

Up to now, we have only preliminary results, not yet elaborated, but they seem to us interesting. Comparing absorption spectrum (Fig.8) with PAS (Fig.9) one can see that in PAS, the red band is wider than in absorption, and it is resolved on three components. The PAS exhibits a lower red to Soret ratio than the absorption spectrum. It seems that contributions from several forms of Chl-like pigments with high TD yield are located in the edge of the red band. At this stage of investigation we do not want to speculate about the character of these forms.

Fig.10 shows time resolved DL spectra of thylakoids in the early stage of greening. The position of maximum is similar to that for Chl in LC, the decay much closer to that for Chl *a* in LC alone than for Chl *a* with carotenoids mixture. It may suggest, that there are carotenoids present in thylakoids which are not efficiently quenching the Chl triplets and are therefore not effective in the protection of Chl against photodamage. It seems that, using the same set of various spectral methods to model systems and to natural systems, we can, by the comparison of the results, draw conclusions concerning the complex processes taking place in living cells.

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