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APPLICATION OF PHOTOTHERMAL METHODS IN BIOPHYSICS AND IN MEDICINE

DANUTA FRĄCKOWIAK

Institute of Physics, Poznań University of Technology, Nieszawska 13A, 60-965 Poznań, Poland

The application of steady state photoacoustic spectroscopy (PAS) as well as time-resolved photothermal method (Laser Induced Optoacoustic Spectroscopy-LIOAS) in the investigations carried out in our laboratory are described. The investigations concern following problems: (1) the possibility of the application of various dyes in photodynamic therapy (PTD) of cancer;(2)the process of excitation energy transfer between antenna complexes of photosynthetic organisms;(3)the conversion of light energy into the electrical energy in electrochemical cells containing the elements of photosynthetic organisms;(4)various paths of excitation deactivation in the bacteria producing hydrogen.

INTRODUCTION

The basic principle of Physics is the low of the conservation of the energy. On the grounds of this low, from measurements of the amount of thermal energy created in investigated system,we are able to drown the information concerning the other paths of the deactivation of excitation. These other paths of deexcitation of excited molecules are shown in Fig.1.They are following: the emission of fluorescence (F),delayed fluorescence (DF) and phosphorescence (Ph),the transfer of excitation energy (ET) to other molecules or use energy for various photochemical reactions. The sum of yields of deactivation processes undergoing on all possible paths is equal unity (Frąckowiak,Goc & Waszkowiak 2000a; Frąckowiak & Planner 2000). The yield of thermal deactivation (TD)is the ratio of the energy converted into heat to the energy absorbed by the investigated system.

In complex biological systems, containing several various chromophores located in the anisotropic complicated structure, the observation of processes of radiative and thermal deactivations delivers information about others molecular and intermolecular processes such the photoreactions undergoing in absorbing molecules and about the intermolecular transfer of excitation energy.

The thermal deactivation undergoing as a result of the transitions between energy levels of the same multiplicity (for ex. between $S_1 \rightarrow S_0$ in Fig.1) is usually faster than TD being the results of intersystem crossing (ISC)transitions (for ex. $T_1 \rightarrow S_0$ in Fig.1).In order to distinguish between the contributions to thermal energy created by deactivations at transitions between various energy levels the time resolved photothermal methods should be applied (Braslavsky & Heibel, 1992; Braslavsky, 1996; Planner & Frąckowiak, 2001; Frąckowiak, Planner, Waszkowiak, Boguta, Ion & Wiktorowicz, 2001b; Frąckowiak, Planner, Waloszek & Więckowski, 2001a).

The population of triplet states due to intersystem crossing transition $S_1 \rightarrow T_1$ (Fig.1) is very important in photochemistry because from triplet states are efficiently undergoing photoreactions: either directly by interaction of the molecules in triplet states with other molecules (type I of photoreaction) or by the generation the singlet oxygen $^1O_2$ (type II of photoreaction).The yields of delayed fluorescence and phosphorescence are usually low, therefore from the measurements of slow thermal deactivation one can evaluate the generation of triplet states through the $S_1 \rightarrow T_1$ transition.

The slow TD in μs range is usually related to the triplet deactivation. The heat produced as a result of reaction for ex. with singlet oxygen is usually emitted in longer time than decay time of triplet state (Frąckowiak *et al*., 2001a, b).

THE ARRANGEMENTS

Scheme of arrangement for the measurements of the time resolved photothermal spectra is shown in Fig.2. Used in our laboratory arrangement is typical for, elaborated by professor Braslavsky, LIOAS method (Braslavsky & Heibel 1992; Braslavsky, 1996). The sample is illuminated subnano seconds nitrogen-day laser fleshes. Energy of laser flash was measured by pirroelectric radiometer, and it was chosen always the same for the

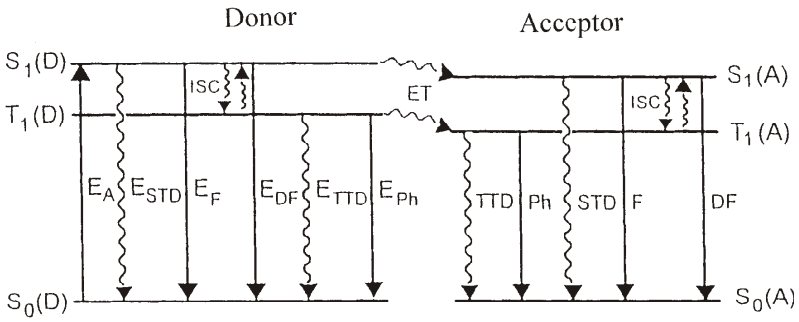


Fig.1 Jabłoński diagram for two molecules: donor and acceptor of excitation energy S-singlet states, T-triplet states,waveform lines-nonradiative transitions. TD-thermal deactivation, F-fluorescence, DF-delayed fluorescence, Ph-phosphorescence

sample and for reference. The sample in cuvette, to which was attached piezoelectric transducer, was located in thermostated container. The width of laser beam was regulated by the diaphragm *a* (Fig.2).

The wave-form signal reach the piezoelectric transducer and is memorized in numerical oscilloscope. The reference is chosen such that whole excitation energy in it is converted into heat in time shorter than time resolution (τ_a) of the arrangement. The shape of reference signal depends on thermoelastic properties of medium, impedance of used electronic systems, apparatus geometry etc.

The shape of sample signal is different, because in investigated sample part of excitation energy is exchanged into heat in time longer than singlet state fast deactivation, therefore the first maximum (H_{max} in Fig.3) is lower than for reference, the other maxima, related with longer times after laser flash, are also changed. The reference and the sample have to absorb the same number of quanta.

The apparatus delivers the opportunity to distinguish between the TD occurring in time shorter than its time-resolution and the slower processes of TD.

In order to do signals analysis it is possible to use two methods: first one was elaborated by group researchers from Portugal (Marti, Jurgens, Cuenca, Casals & Nonell, 1996; Marti, Nonell & Nicolaus, 2000), the second one it is deconvolution program giving opportunity to describe the decay time of slow TD components (Rudzki-Small, Libertini & Small, 1992).In both cases as reference a dye converting its excitation into heat in very short time should be applied.

The laser flashes are short, the impedance of electronic introduces bigger contribution to time-resolution of apparatus but most important is the time of propagation of generated signal to transducer, which is different for differently distant edged of light beam (maximal difference is *a* in Fig.2). This time has the influence on the time-dependence of signal and its time-resolution :
$\tau_a = 2R/V_a$ (where R — radius of the slit *a* in Fig.2; V_a — the velocity of sound in the medium) (Braslavsky & Heibel, 1992).

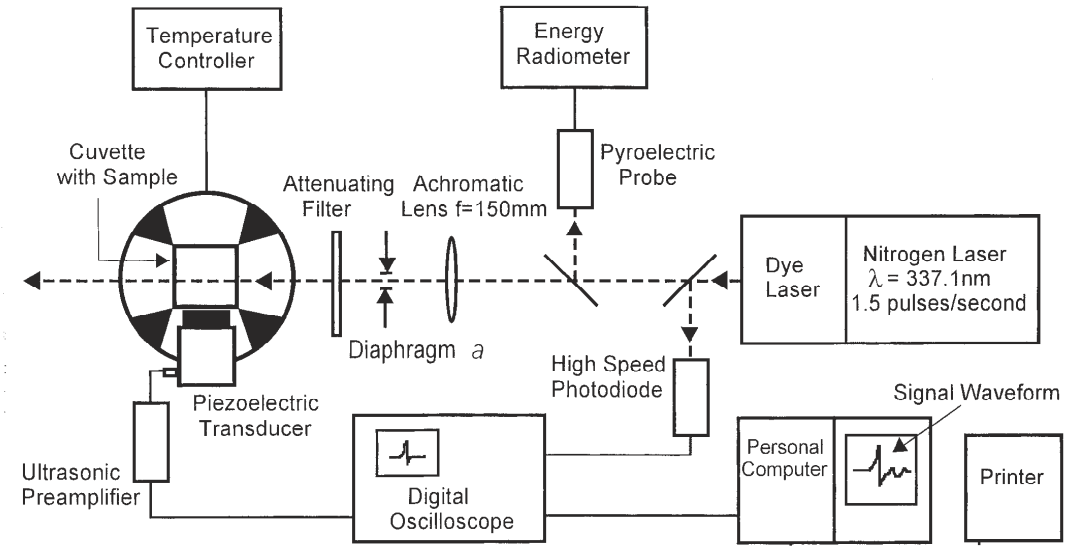
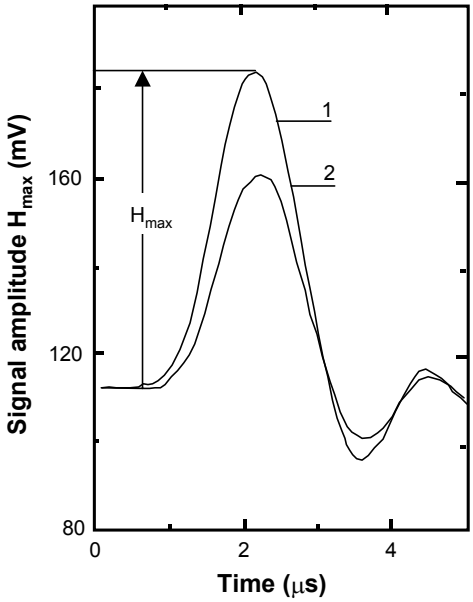


Fig.2 Scheme of apparatus for the measurements of the time resolved photothermal signals (LIOAS).



Rys.3 Signals of LIOAS,1-reference,2-measured sample

In "Portuguese" method (Marti *et al*. 1996, 2000), described in detailed previously (Frąckowiak & Planner, 2000), one can obtain information which part of excitation is converted into heat in time longer than time resolution of apparatus (which is in our arrangement from 0.3 μs to 0.8 μs depending on slit *a* from Fig.2).In many cases it is the efficiency of triplet state generation, but one has to take into account that in this part of TD can be also included other very slow processes of TD due to photochemical reactions.

In this method the high of first maximum H_{max} (Fig.3) for the sample and for reference are compared. This maximum is described by the formula:

$$H_{max} = k\alpha E_{las}(1\text{-}10^{-A}) \qquad (1)$$

where k — the coefficient the same for the sample and for reference, related to geometry of arrangement, solution properties and type of electronic;α - part of excitation energy converted into heat "promptly" (it means in time shorter than time-resolution τ_a); A — absorbance of the sample and of the reference for the wavelength of light emitted by laser ; E_{las} — energy of laser light.

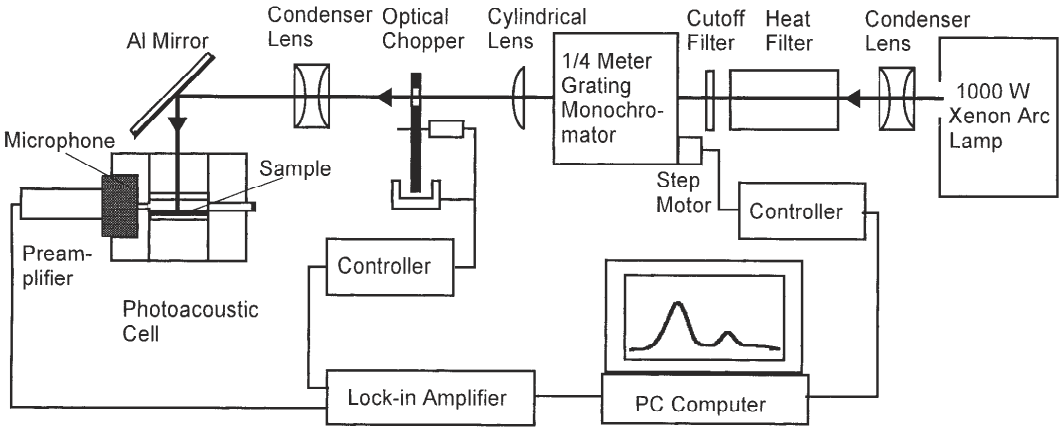
The H_{max} of sample and reference are measured for several values of the laser energy, then taken into account that for the reference $\alpha_o = 1$, it is possible (from the slope of the lines giving the dependence of H_{max} on laser energy) to establish α, it means to evaluate which part of absorbed by our sample energy was "promptly" exchanged into heat.

Introducing α into formula:

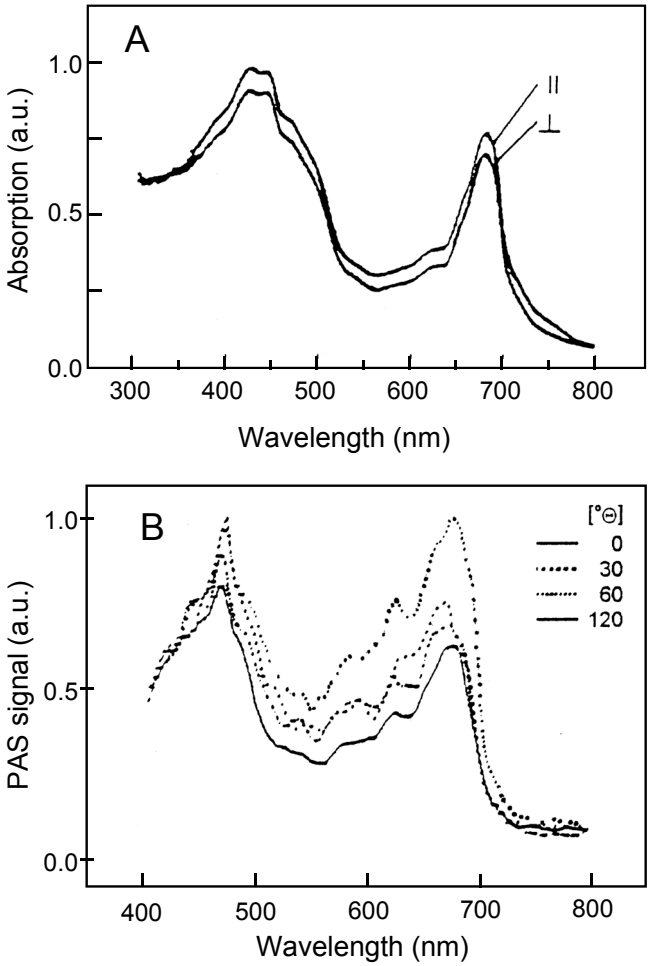
$$\Phi_T E_T = (1 - \alpha)E_{las} - \Phi_F E_F \qquad (2)$$

(where Φ_T and Φ_F — the yield of triplet state generation and fluorescence yield, E_T and E_F — energies of triplet and fluorescence) one can calculate the yield of triplet generation in a case when other quantities are known from literature or independently measured (Frąckowiak & Planner, 2000; Planner & Frąckowiak, 2001; Frąckowiak *et al*. 2001a, b).

The deconvolution of signals according the program elaborated by Rudzki-Small *et al*. (1992) gives us not only the decay times of TD undergoing in time range measured by LIOAS (usually from τ_a to some μs) but also their contributions in whole TD. It gives opportunity to evaluate the contributions from the processes faster than τ_a, as well as from processes slower than can be measured by used apparatus.



Rys.4 The arrangement for the measurements of steady state photoacoustic spectra.



Rys.5 Polarized spectra of cyanobacterium *Synechococcus* cells located in anisotropic PVA film. A)absorption spectra,B)photoacoustic spectra,Θ-angle between electric vector of light PVA. [according Planner *et al.* 2000]

For the measurements of such very slow processes the apparatus in which the declination of probe light beam going near to the surface of sample illuminated by modulated acting light beam (Frąckowiak & Planner, 2000) can be used. It is so called "mirage effect" apparatus.

Steady state photoacoustic (PAS) spectrometer (Fig.4) (Rosencweig, 1980; Ducharme, Tessier & Leblanc, 1979) can measured together fast and slow components of thermal signals, but the ratio of contributions from slow and fast processes depends on frequency of acting light modulation (Ouzafe, Poulet & Chambron, 1991). Changing the frequency of light modulation we are able to establish, from the change in a shape of spectra, the spectral regions in which the contributions from slow processes of TD are large. As it is seen from Fig. 4 it is one beam apparatus. The sample is located in the photoacoustic cell filled by acting gas and connected with microphone. The heat created in the sample, as a result of absorption of modulated light, after diffusion to the boundary between sample surface and gas heats the thin layer of gas which expends and acts on the volume of gas forming standing wave measured by microphone. In order to eliminate the influence of spectral distribution of light source the measurement is repeated for black body and the spectrum of sample is divided by this spectrum of black body. Results are obtained in arbitrary unit, but the same for whole set of samples.

APPLICATION IN PHOTODYNAMIC THERAPY OF CANCER

In our laboratory, in collaboration with Karol Marcinkowski University of Medical Sciences, we have curried out the investigations of possibility of the application of various dyes in photodynamic therapy (PDT). The suitable sensitizer has to be selectively incorporated into tissue, it means be introduced more efficiently into malignant than into healthy cells, has to be also possibly not toxic for healthy cells and being quickly expended from organism, but has to destroy efficiently the cancerous cells as a result of illumination. It has to posses also the intensive absorption in the spectral region in which tissue is possibly transparent (600 nm–900 nm). Our investigations are carried out on the human peripheral blood cells (predominantly on leukocytes). Cells are resting or so called stimulated (being the model of cancerous cells). We tried to apply various activators for the stimulation of the cells (Frąckowiak, Niedbalska & Wiktorowicz,1996; Frąckowiak, Planner & Wiktorowicz, 2001c), we applied the incubation of cells in different group of dyes: stilbazolium me-

rocyanines (Frąckowiak, Wiktorowicz, Cofta, Niedbalska & Latosińska, 1995; Planner & Frąckowiak, 2001), porphyrins (Ion, Planner, Wiktorowicz & Frąckowiak, 1998), phthalocyanines (Frąckowiak *et al.*, 2001b, d). We have to compare spectral properties of the dyes in incubation solvents and in the cells. Usually the yield of dye fluorescence in the cells is much lower than in the incubation solvent. Therefore it is very often not easy to establish the efficiency and selectivity of dye incorporation into cells only on the basis of fluorescence measurements of stained cells. In some cases it is anyway possible evaluate the incorporation using fluorescence emission. The photographs of stained cells done under fluorescence microscope enable to establish in which parts of cells dye molecules are gathered (Waszkowiak *et al.*, 2001). The spectra of endogenous emission of cell material are giving opportunity to investigate the influence of dye on biological macromolecules (Waszkowiak *et al*., 2001). In same cases the fluorescence yield of the same dye can be different in healthy and cancerous cells. For such samples the evaluated on the basis of fluorescence intensities selectivity of incorporation can be wrong. The absorption spectra of the cells are in a great extend perturbed by scattering of light (Waszkowiak *et al.*, 2001; Frąckowiak *et al.*, 1998). In a such situation very useful can be the investigations of the steady state photoacoustic spectra (PAS).The PAS can be measured also for highly scattering samples. The amplitudes of PAS signals, when the saturation effect is not occurring, are proportional to the dye concentration in the cells (Rosencweig, 1980).

If the dye molecules, incorporated into cells, have efficiently destroy tissue as a result of their illumination, they have to have strong population of triplet states (Frąckowiak *et al*., 2001b, c). For the set of investigated phthalocyanines the sequence of the yields of triplet states generation were established (Frąckowiak *et al*., 2001c).

On the grounds of the steady state and LIOAS measurements we have establish, that between investigated compounds most suitable for PDT is phtalocyanine complex with Zn, because it exhibits high selectivity and efficiency of incorporation into cells as well as high yield of triplet state generation.

THE INVESTIGATIONS OF PHOTOSYNTHESIS PROCESS

Steady state photoacoustic (PAS) is from many years used in photosynthesis process investigations (Buschmann & Prehn, 1990; Fork & Herbert, 1993). In our laboratory PAS is used predominantly in order to investigate the perturbation of the process of excitation energy transfer between antennae complexes which are responsible for the light absorption and for the delivery of excitation to reaction centers. In a case when from some antenna chromophore less energy is transferred to the next participants in the donor-acceptor chain then both-the yield of this chromophore fluorescence as well as the yield of its TD should increase (Cegielski *et al.*, 1992).

Especially useful is the application of PAS for the investigation of excitation energy transfer from weakly fluorescent molecules e.g. from carotenoids (Frąckowiak, Cegielski & Abdurakhmanov, 1991). The excitation energy transfer towards the reaction centers is facilitated by proper distribution of antenna complexes :molecules absorbing in short wavelengths spectral region are located far away from centers, whereas antenna absorbing in longer wavelengths range closer to the centers. Acceptors and donors of excitation energy have also in such a way directed theirs absorption and emission transition moments that the process of excitation energy transfer (ET) between them is efficient. It is therefore close relation between the structure and the function of photosynthetic apparatus. In a such situation in eightieth years of last century we have elaborate together with our colleagues from Canada photoacoustic with the application of linearly polarized light (Frąckowiak *et al.*, 1986a). This method, was used later in many investigations (Frąckowiak *et al.*, 1986b; 1990; Frąckowiak & Dudkowiak, 1992; Frąckowiak & Ptak, 1994; Białek-Bylka *et al.*, 2000), what approved that it is very practical approach.

The PAS of the several oriented in stretched polymer samples such as cyanobacteria cells (Planner *et al.*, 2000) and green bacteria (Klaczyńska, Dudkowiak, Frąckowiak, Planner, Hara & Miyake, 2000) were investigated using the light with electric vector forming various angles (0º,30º,90º) with the sample orientation axis. It was shown that polarized light PAS enable in more convenient way than polarized absorption establish the average angles between TMs of absorption of various photosynthetic antenna.

Figs 5a and 5b show polarized spectra of absorption and PAS taken for the same sample (*Synechococcus* cyanobacterium cells in anisotropic PVA film). As one can see the changes in light polarization have stronger influence on PAS than on absorption spectra. It is, because various "forms" of pigments, created by pigment molecules interactions with their close surroundings, are better characterized by their strongly different

yields of TD than by their more similar absorbances (Frąckowiak & Ptak, 1994; Goc, Dudkowiak, Gryczyński, Zelent & Frąckowiak, 2001a).

In last time we applied the polarized light PAS to the investigation of immobilized in polymer matrix film photosynthetic bacteria with eliminated or only perturbed some groups of chromophores of given orientation of their absorption TMs. This bleaching or perturbation of chromophores was reached by the illumination of the sample by the strong,polarized light (Goc & Klecha, 2001; Goc, Klecha, Waszkowiak, Miyake & Frąckowiak 2001b). Using, beside the polarized PAS also the polarized fluorescence spectra excited by various wavelengths of light, as well as polarized absorption we have established, that carotenoids are transferring their excitation energy predominantly to long wavelengths forms of chlorophylls (from so called photosystem I-PSI), whereas the giant biliprotein complexes phycobilisomes prefer as the receivers of their excitation shortwave chlorophyll forms (from photosystem II- PSII). Cyanobacteria,similarly as higher plants posses two types of reaction centers located in PSI and PSII respectively. The photoreactions undergoing in both RCI and RCII are necessary for the running of the photosynthesis process, it means for the synthesis of the carbohydrates.

Both steady state PAS and time resolved photothermal method (LIOAS) are applied in investigation of photosynthesis (Fork & Herbert, 1993; Buschmann & Prehn, 1990). In our laboratory we have investigated the interactions between carotenoids and chlorophylls in thylakoids isolated from cucumber cotyledons at various stages of greening (Frąckowiak *et al*., 2001a). The measurements of the absorption, fluorescence, PAS and LIOAS were done. It was found that the yield of chlorophyll fluorescence increases during greening. It shows that the yield of excitation energy transfer from various pigments to fluorescent forms of chlorophyll increases. It is known that carotenoids can prevent chlorophylls photodestruction. The ratio of carotenoids to chlorophylls contents in the thylakoids decreases during greening process approximately twice, because the content of chlorophylls strongly increases. One could therefore predict that in earlier stages of greening the chlorophylls would be better shielded against photobleaching, but from experiments follows that situation is opposite: in earlier stages of greening chlorophylls exhibit higher susceptibility for photobleaching than in later stage of greening. We had analyzed the LIOAS signal of thylakoids using both methods :proposed by Marti *et al.* (2000; 1996) and Rudzki-Small *et al*. (1992). The results obtained by both methods of analysis are in the limit of accuracy similar. The yield of chlorophylls triplet generation decreases as a result of greening. Also the decay time of chlorophyll triplet state is decreasing from 6.2μs for 3h of greening to 1.5μ s for 24h of greening process. It shows that the carotenoids more efficiently quench the chlorophyll triplet states when photosynthetic apparatus is better developed. The developed structure causes such location of carotenoids and chlorophylls molecules which is enabling their strong mutual interactions.

THE CONVERSION OF LIGHT ENERGY INTO ELECTRICAL ENERGY AND THE HYDROGEN PRODUCTION

This problem will be described shortly, because it was already presented previously (Frąckowiak *et al*., 2000a).

In semibiological systems for example in Langmuir-Blodgett monolayers containing reaction center complexes and artificially added antenna pigments, which when can be located between semitransparent metal and semiconducting electrodes as a result of illumination can generated electromotoric force the possible paths of deexcitation by PAS were established (Goc, Hara, Tateishi, Miyake, Planner & Frąckowiak, 1997; Hara, Miyake, Goc & Frąckowiak, 1999).

For the generation of photopotentials and photocurrents it is possible to use also adsorbed pigment layers or monolayers of pigments, diluted solutions of dyes in nematics (Ptak, Chrzumnicka, Dudkowiak & Frąckowiak, 1996),stained polymer solutions or layers of pigment-protein complexes (Frąckowiak *et al.*, 2000a; Ptak, Dudkowiak & Frąckowiak, 1998; Ptak, Der, Toth-Boconadi, Nase & Frąckowiak, 1997). It is crucial condition that in every one of such system the main part of absorbed light is used for pigment excitation. Excited pigments transfer electrons to conducting band of semiconducting electrode and afterwards the ion pigments recombine with electrons coming from metal electrode. The thermal deactivation of excitation competes with this useful process of electron transfer. Therefore using photothermal methods one has to establish conditions in which the TD is possibly low (Frąckowiak & Ptal, 1994).

Some photosynthetic bacteria mutants efficiently produce hydrogen, which is ecological fuel. Selecting type of especially effective mutants one has to check all other paths of pigment deexcitation in order to diminish them. In such work the PAS

experiments are also very useful (Goc, Planner, Frąckowiak, Vasilyeva, Hara & Miyake, 1999).

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