

Photoacoustic Spectroscopy Applied to Biological Systems*

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Abbreviations: ES, energy storage; f_{mod} , modulation frequency; PA, photoacoustic; PAS, PA spectroscopy; PSI, photosystem I; PSII, photosystem II, μ_{β} , optical absorption length; μ_s , thermal diffusion length

Photoacoustic spectroscopy detects sound waves induced by periodic heating of a thin layer of gas above a sample placed in a closed cell. This heat results from non-radiative transitions following periodic illumination of the sample. A brief overview of the photoacoustic theory in solids is presented. An advantage of photoacoustic spectroscopy over other spectrisopic techniques is that it permits the analysis of any opaque or highly diffusive material. Neither transmittted nor reflected light affect the signal, as photoacoustic methodology measures only heat. This is exemplified in dermatological studies of sunscreens *in vivo* and works in hematology field. Another interesting application of photoacoustic spectroscopy is the depth profile analysis, which allows to produce a chromophoric map of the sample simply by changing the modulation frequency of analyzing light. An example of such application is demonstrated for the posterior part of the eye, which shows stratification of the cell layers. Further, in photosynthetically active samples, photochemistry and heat emission are two competitive processes of deexcitations. Hence, it becomes possible to record photochemical activity with photoacoustic spectroscopy. The case of leaf's photochemistry is used to show such an application.

KEY WORDS: Photoacoustic spectroscopy / Dermatology / Hematology / Photochemistry / Photosynthesis / Theory / Vision

INTRODUCTION

From a semantic point of view, photoacoustic spectroscopy implies the conversion of light into acoustic waves. Photoacoustic spectroscopy detects sound waves created by periodic heating of a thin layer of gas above a sample placed in a closed cell. This heat results from non-radiative transitions following absorption of periodic illumination by the sample.

The photoacoustic principle was first discovered by A.G. Bell (1880), whom was doing at that time some research on communication apparatus. The spectrophone, one apparatus built by Bell, was using the sun as light source, a sewing machine to drive a mechanical chopper, and the ear to detect the sound created in the cell which was containing a strong light-absorbing substance. The work of Bell and his collaborator Tainter was followed shortly after by several European researchers (Rayleigh, 1881). But, principally due to the low

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sensitivity of the detector (the ear), this first boom of interest in the photoacoustic technique dropped rapidly. The applications of photoacoustic methodology was confined to study absorbing gases until 20 years ago. The achievement of powerful electronic devices allowed to detect and amplify efficiently the acoustic waves following light absorption by solids (Harschbarger and Robin, 1973). With the proposal of a theory explaining the photoacoustic effect in solids (Rosencwaig and Gersho, 1976), this methodology emerged as a powerful tool to study the properties of light-absorbing materials.

Because photoacoustic spectroscopy permits to measure heat emitted by a sample following absorption of a modulated light, both thermal and optical properties of the material are crucial, in comparison to absorption spectroscopy where only optical properties regulate the detection. The most important advantage of photoacoustic spectroscopy over absorption techniques is that it permits the measurement of optical properties of any opaque sample (Adams and Kirkbright, 1977). With biological material, which is known to be a highly dispersive medium, the photoacoustic methodology allows to obtain interesting information related to optical and photochemical properties (for a review, see Braslavsky, 1986).

The aim of the following pages is: i- to introduce briefly the theory of the photoacoustic effect in solids and, ii- to describe some applications of this relatively new technique to biology domain.

The photoacoustic spectrometer In the photoacoustic spectrometer, the sample is put in a photoacoustic cell (Figure 1). The cell consists of a hermetically closed chamber adjacent to a detector, usually a sensitive microphone or piezoelectric transducer (Cahen, 1981, Ducharme *et al.*, 1979, Marquezini, *et al.*, 1991). This chamber, usually a few tens of mm³ volume, has a frontal transparent quartz window from which the excitation light beam is directed towards the sample. A monochromatic light beam is chopped at an audio frequency (10 - 1000 Hz) to produce the excitation beam. Following light absorption by the sample, the energy can be converted into radiative emission (fluorescence or phosphorescence), non radiative emission (heat) or can induce photochemical processes (Buschmann *et al.*, 1984).

Modulated heat, resulting from radiationless decay, diffuses throughout the sample up to its surface. At that stage, heat waves induce a periodic heating of surrounding gas. The oscillations of gas temperature result in a periodic variation of the pressure inside the cell. These pressure changes induce sound waves, which are detected by a sensitive microphone. Then, electrical signals generated by the microphone are amplified and analyzed by a lock-in amplifier with regard to their phase and amplitude components. Finally, they are sent to a chart recorder for direct valuation, or to a computer for further treatments. When a photoacoustic spectrum is processed, the raw photoacoustic data must be divided by the spectrum of the excitation light beam (usually by PA spectrum of carbon black), in order to avoid any discrepancy related to differential light intensity throughout the lamp spectrum (Rosencwaig, 1975).

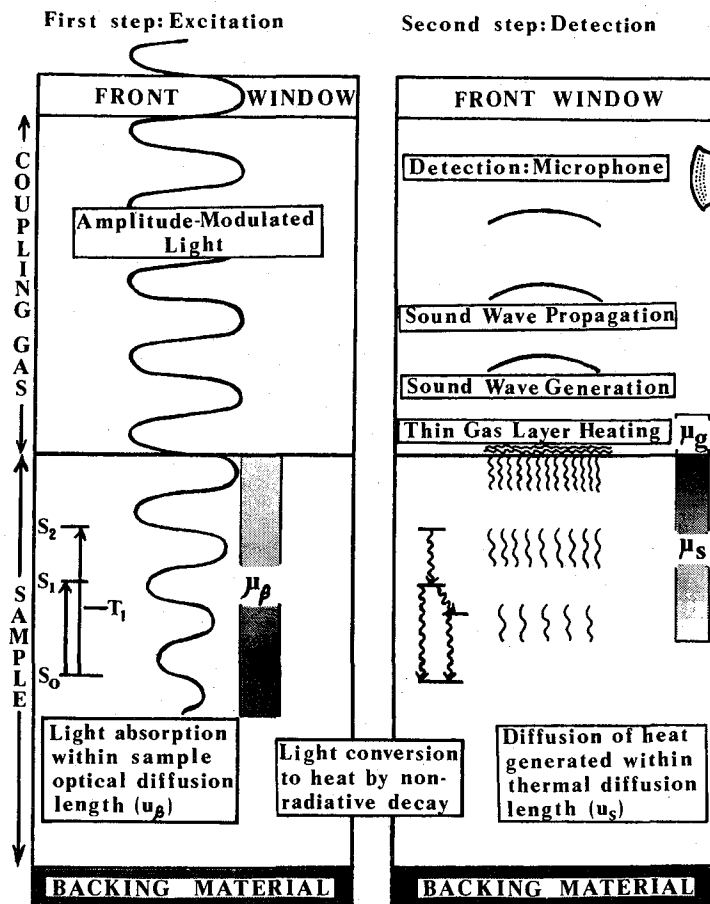


FIGURE1: PRINCIPLE OF PHOTOACOUSTIC DETECTION: COUPLING GAS-MICROPHONE

Excitation: The sample is put in a closed, air-tight photoacoustic cell. The excitation beam is modulated at an audio frequency (10 - 1000 Hz) and is directed towards the sample. Periodic light absorption occurs in a thin layer of the (opaque) sample, the absorption length (μ_β , [m]). This length is independent of modulation frequency, and is only related to optical properties of the sample.

Detection: Non-radiative deexcitation process leads to periodic heat emission by pigments located inside the absorption length. Heat waves diffuse throughout the sample up to the surface, where they induce periodic heating of the surrounding gas. The heated gas layer then acts as a piston in the cell and generates sound waves. This phenomena is related to thermal properties of the sample and gas, and to modulation frequency used, since only a known heated layer (μ_s) can transmit generated heat to the interface during a period of illumination. The lowest is modulation frequency, the deepest is the layer sensed by photoacoustics. Acoustic waves generated in the gas layer are detected by a microphone. The discrimination between signal and noise is achieved by the use of a lock-in amplifier.

Photoacoustic effect in solids

The photoacoustic effect in gases is directly related to Boyle's law:

$$P.V = n.R.T \quad (1)$$

For an ideal gas, the product of the pressure (P [atm]) and the volume (V [l]) is equal to the product of the amount of moles of gas (n [mol]) times the ideal gas constant (R [atm l mol⁻¹ K⁻¹]) times the absolute temperature (T [K]) of the system. From this basic equation, it is possible to link the pressure and the temperature by:

$$P = \frac{n.R}{V} . T \quad (2)$$

The photoacoustic cell that contains the sample being a closed hermetic chamber; thus n , R and V are held constant. Then, any variation of the temperature of the gas leads to a change of the pressure inside the closed chamber. The principle of photoacoustic spectroscopy is related to that pressure variation. Following modulated light absorption by the sample, non-radiative decay produces modulated heat. These heat waves diffuse throughout the sample up to the surface where they warm the surrounding gas. The gas close to the sample's surface, following successive heating and cooling, acts as a piston and creates periodic oscillations of the pressure. The periodic variations of pressure are sound waves, which are detected by a sensitive detector. It is then possible to state that photoacoustic spectroscopy measure the conversion of light into sound.

In fact, the photoacoustic methodology is dependent on three important processes: the absorption of light, its conversion into heat and finally, the diffusion of heat through the sample. This relation can be summarized as:

$$\text{Intensity of PA Signal} = f \left(\begin{array}{ccc} \text{Absorbed} & \text{Non-radiative} & \text{Thermal} \\ \text{Energy} & \text{Conversion} & \text{Transfer} \\ & \text{Efficiency} & \text{Efficiency} \end{array} \right) \quad (3)$$

In an opaque or semi-transparent sample, the light energy is absorbed inside a layer called the optical absorption length:

$$\mu_{\beta} = \frac{1}{\beta} = \frac{1}{2.3\epsilon O_1} \quad (4)$$

where the optical absorption length (μ_{β} [m]) is the reciprocal of the absorption coefficient, which is related, following Beer-Lambert's law, to the molar absorptivity (ϵ [l mol⁻¹ m⁻¹]) times the molar concentration (C_i [mol l⁻¹]). The optical absorption length represents the depth in the material where the light intensity is 1/e of the incident light intensity.

The general theory of photoacoustic effect in solids (Rosencwaig and Gersho, 1976) is particularly related to the thermal transfer efficiency. We will describe here some of its im-

portant features.

This theory is related to an unidirectional heat flow in a material which thermal properties are independent of position and temperature (Adams, 1982). Thus, the equation of linear heat flow is expressed by:

$$\frac{\delta^2 T}{\delta x^2} - \frac{1}{\alpha} \cdot \frac{\delta T}{\delta t} = 0 \quad (5)$$

where temperature (T [K]) is given as a function of position (x) and time (t). The thermal diffusivity of the sample (α [$\text{m}^2 \text{s}^{-1}$]) is defined as:

$$\alpha = \frac{k}{\rho \cdot c} \quad (6)$$

where thermal conductivity (k [$\text{J s}^{-1} \text{m}^{-1} \text{K}^{-1}$]), density (ρ [kg m^{-3}]) and specific heat (C [$\text{J kg}^{-1} \text{K}^{-1}$]) of the material could be found in appropriate physical tables.

The boundary conditions govern the appropriate solution of equation (5). If the surface temperature, at $x = 0$, is a harmonic function of time expressed by:

$$T = T_0 \cos(\omega \cdot t) \quad (7)$$

where ω is the angular frequency ($\omega = 2\pi f$, [s^{-1}]) of periodic temperature variations. The solution of equation (5) is then:

$$T = T_0 \exp \left[-x \left(\frac{\omega}{2 \cdot \alpha} \right)^{1/2} \right] \cdot \left[\cos \left((\omega \cdot t) - x \left(\frac{\omega}{2 \cdot \alpha} \right)^{1/2} \right) \right] - A \quad (8)$$

where A is a transient disturbance caused by starting the oscillation at time $t = 0$. As time increases, this disturbance becomes negligible. From this equation, it is possible to conclude that the amplitude of the temperature wave is linked to:

$$\exp \left[-x \left(\frac{\omega}{2 \cdot \alpha} \right)^{1/2} \right] \quad (9)$$

Then, an increase of x and ω provoke its decrease. The factor $(\omega / 2 \alpha)^{1/2}$ is defined as the thermal diffusion coefficient (a [m^{-1}]). From this factor, Rosencwaig and Gersho described a very useful parameter, the thermal diffusion length as:

$$\mu_s = \frac{1}{a} = \left(\frac{2 \cdot \alpha}{\omega} \right)^{1/2} \quad (10)$$

The thermal diffusion length (μ_s [m]) represents the active thermal length responsible for the heat waves that reach the surface of the sample. This value is between those reported by Rosencwaig and Gersho (1976) - $2\pi\mu$ and Malkin and Cahen (1981) - $\mu/\sqrt{2}$. From equation (10), it is possible to conclude that μ_s is inversely proportional to the modulation frequency (ω). It is then possible to scan throughout a sample, simply by changing the modulation frequency of excitation light. This permits the depth profile analysis of chromophores in a sample, a very interesting characteristic of photoacoustic technique that will be discussed later.

In an opaque or semi-transparent sample, μ_β is smaller than the width of the sample; the resulting optical saturation that occurs can distort the PA spectrum. But, the selection of a suitable modulation frequency, where μ_s becomes smaller than μ_β , permits the establishment of a reliable PA spectrum. This represents the main advantage of PA spectroscopy over absorption spectroscopies: the possibility to record spectra of opaque or highly diffuse material. We will present in the next section some results drawn from photoacoustic studies on biological materials.

Applications in biomedical field

a. Dermatology

Many applications of photoacoustic spectroscopy can be found in the field of dermatology, which demonstrates the high sensitivity of this technique (Pines, 1978, Kanstad *et al.*, 1981, Deffond *et al.*, 1985, Giese and Kolmel, 1983). The melanin, one of the most important epithelial skin pigments, was studied in view of its non-radiative process characteristics (Crippa and Viappiani, 1990). *In vivo* studies of topical creams on skin were made, using a special PA cell (Poulet and Chambron, 1985), which allows the use of living skin and avoids the noise generated by blood pulsations.

Photoacoustic technique, because of its unique feature of allowing depth profile analysis of pigments in a sample, was utilized to locate pigments in the skin. Anjo and Moore (1984) analyzed the depth profile of β -carotene in the skin by employing the following procedure: Heat emission from pigments located at different depths in a sample reaches the surface with a different phase angle. By using phase analysis throughout the spectrum, it becomes possible to separate the contribution of the surface from the one arising from the deeper parts of the sample. It is then possible to follow the differential penetration of some compounds in the skin; Anjo and Moore (1984) report that methylene blue dye stayed at the surface of the skin, thus the β -carotene penetrated up to the epithelium cell layer.

The rate of penetration of topical creams, which act as sunscreen in the skin can also be estimated with PAS (Giese *et al.*, 1986). For a given frequency, μ_s can be evaluated, using equation (10) (for example, μ_s is about 4 μm at 1200 Hz and about 10 μm at 180 Hz). Thus, during the diffusion of a sunscreen into skin, the level of pigments in a superficial layer is decreasing, which leads to a loss of PA signal from this layer. From such measurements, it is possible to know the sunscreen penetration rate and its time of residence in different skin layers. These parameters are important to characterize the usefulness of these topical creams to protect efficiently the skin against UV radiations (see figure 2 for more details and examples).

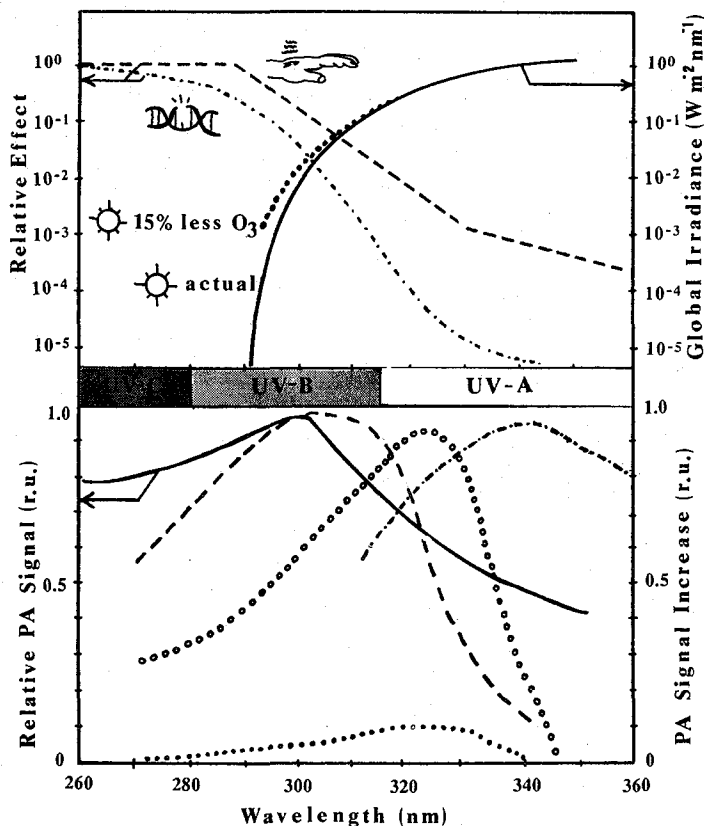


FIGURE 2: PAS APPLICATIONS IN DERMATOLOGY: SUNSCREENS.

A. Sunlight in the UV region could provoke some biological phenomena, as shown by the relative responses curves of sunburns (---) (Parrish *et al.*, 1982) and DNA absorption (---) (Green, 1983). Ultimately, UV absorption by DNA results in breakage of DNA strands, which can induce physiological disorders such as cancer. In comparison, full curve (—) is a plot of UV-global irradiance which attains Earth at sea level on a sunny day at noon at the latitude of Florida (Green, 1983). The companion dotted line (···) is the amount of solar UV reaching the ground if a 15% depletion in stratospheric ozone is assumed. It can be easily inferred from these curves that a decrease of O_3 layer will increase sunburns and DNA strands breaks.

B. The figure A emphasizes the importance of sunscreen creams utilization. Then, the determination of properties of some sunscreen compounds under *in vivo* conditions becomes crucial. The full curve (—) represents PA spectra of skin (Poulet and Chambron, 1985) while the other curves are differential PA, spectra of different compounds applied on skin *in vivo*. We normalized all these spectra in regard to their maximum effect wavelength. Previous studies used both UV-B (--- Eusolex 6300, Giese *et al.*, 1986) and UV-A (--- Eusolex 8020, Giese *et al.*, 1986) absorbing substances. A compound absorbing in both regions (PABA, Pines, 1978) was tested in relation to its capacity to stay on skin following immersion in water (ooo: before immersion, ····: after immersion). Nevertheless, PA spectra of a related chemical (Padimate-O, not showed) before and after immersion were about similar. Thus, PA spectroscopy shows its efficiency to characterize sunscreens, *in vivo* conditions, which represents an appreciable gain of time and an easy way to study their effectiveness under different realistic conditions.

b. Hematology

The hemoglobin, the protein responsible of O_2 - CO_2 exchanges between blood and tissues, has a tetraporphyrin cycle bound to its amino acids skeleton, which gives its reddish color (Alter, 1983). Photoacoustic spectroscopy was used in the determination of haem protein content in tissues (Bernini *et al.*, 1991). Moreover, due to their similar structure, many hematoporphyrin derivatives are now used in cancer research to induce selective photosensitization of tumoral tissues (Pottier *et al.*, 1988). Their detection in target-tissues and in the body is crucial to insure a complete treatment and to avoid any side effects due to their potential toxicity.

Typical PA spectra of hemoglobin present three major bands (415, 540 and 580 nm), then oxyhemoglobin spectra show two bands at 470 and 555 nm (Poulet *et al.*, 1988). These authors also used a property of PAS to follow sedimentation process of a blood sample, following this principle: For a given modulation frequency, the thermal diffusion depth stays constant in a given material; then, any change of the amount of chromophores in this region should induce a change of PA signal. Thus, in a liquid, such as plasma, the sedimentation process induces a movement of erythrocytes (red cells) to the bottom. This in turn decreases the amount of pigments in the upper layers of the sample. As a result both amplitude and phase angle of PA signal decrease; thus allowing the measurement of the erythrocytes sedimentation rate, which was found to be in good agreement with other techniques.

The presence of Photofrin II (Andreoni *et al.*, 1990) and manganese III hematoporphyrin (Ouzafe *et al.*, 1988) two photosensitizers used in photodynamic therapy, was determined in liver and kidney, respectively, with photoacoustic spectroscopy. The photobleaching of an other photosensitizer, namely the hematoporphyrin IX, was followed in a highly scattering medium, a study difficult with any other absorbance technique (Lachaine *et al.*, 1990).

The advantages of PAS in this field of research are: i- its applicability to detect accurately some compounds present in highly diffusive media, such as the blood, or in non-uniform and opaque material, such as the surface of organs, ii- its avoidance of any complicated chemical extraction and purification processes, limiting manipulations and iii- it requires only small amount of substance and is non-destructive, permitting further investigations by other techniques.

c. Vision

The vertebrate ocular tissues present a complete stratification of their structure and their pigmentation (Figure 3a). In Figure 3 b and c, the data show PA spectra of the retinal pigment epithelium, which is a single layer of cells underlying the photoreceptor outer segments and supported by the highly vascularized choroid. The amelanotic area (Figure 3b) of epithelium presents absorption bands of hemoglobin (415, 540 and 580 nm) while the amelanotic area (Figure 3c) shows, in addition to these bands, the presence of the spectrally non-selective pigment melanin, which absorbs almost uniformly throughout 320 to 600 nm region of the photoacoustic depth profile can also provide important informations on the location of pigments in the complex structure. With the depth profile analysis, it becomes possible to observe that the outer region of the epithelium is in contact with the choroid (which irrigate the eye with blood), since the hemoglobin bands appears at deeper levels inside the epithelium (Boucher *et al.*, 1986).

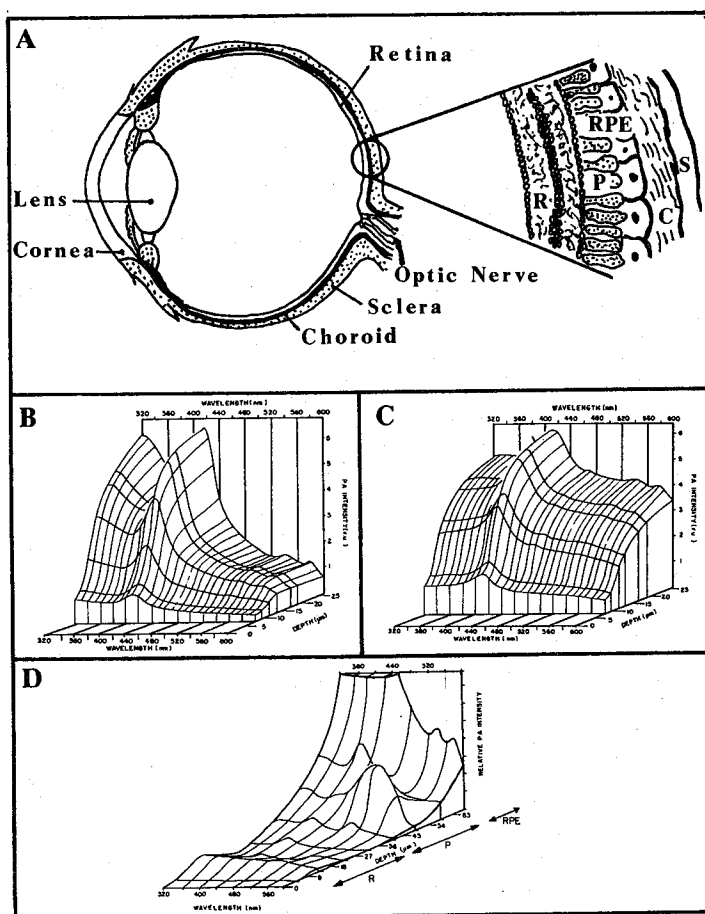


FIGURE 3: PHOTOACOUSTIC SPECTROSCOPY AND VISION: DEPTH PROFILE OF THE EYE

- A. Posterior ocular tissues present a structural arrangement of different cells. The retina (R) contains the photoreceptor cells which can be divided into the pigmented photoreceptor cells (P) and the nervous ones which are responsible for the electrical excitation of the optical nerve. The visual pigment, the rhodopsin, once excited becomes lumi-rhodopsin, which presents absorption bands at around 350 and 520 nm (Boucher and Leblanc, 1981). The retina is backed by the retinal pigment epithelial cells layer (RPE) which permits the degradation of old photoreceptors. The RPE cells appear as an area of different colors ranging from pale green (amelanotic) to dark blue (melanotic). It contains melanin (which absorbs almost uniformly throughout the spectrum) and presents characteristic bands of hemoglobin (415, 540 and 580 nm). The choroid (C), which carry blood to the cells of the eye, and the sclera (S) constitute the latest two cellular layers of the eye.
- B and C. The depth-profile analysis allows to study a known layer of material by choosing a suitable modulation frequency (see theory). It is then possible to measure a chromophoric map of the RPE, and to appreciate the pigment differences between amelanotic (B) and melanotic (C) area of the RPE (Boucher *et al.*, 1986). It was concluded from these spectra that the range of colors observed in RPE was due to interference phenomena, since only hemoglobin (415, 540 and 580 nm) and melanin pigments can be detected by PAS.
- D. Sketch of the depth distribution of chromophores in the neural (R) and photoreceptor (P) sides of the retina and the retinal pigment epithelium (RPE). It is then possible to localize lumi-rhodopsin (520 nm) in the photoreceptor area of retina. Spectra were obtained by calculation of the difference PA spectrum between each 9- μ m thermal depth layer of R, P and RPE. (From Boucher *et al.*, 1986).

The chromophoric map of ocular tissue can be obtained by using differential analysis of PA spectra taken at different depths (under different modulation frequencies). It is then possible to sketch the depth distribution of pigments (Figure 3d).

Photoacoustic measurements at low temperature (77K) enables to detect some intermediates of visual pigments (Boucher *et al.*, 1981, Yoon *et al.*, 1988). The effects of some damages (aging: cataract and UV radiation) to the eye lenses can also be followed by PAS (Lerman *et al.*, 1978, Andjus *et al.*, 1982). The utilization of photoacoustic methodology might find some applications in the estimation of some eye diseases *in vitro*, as photodynamic damages, retinal dystrophy or retinitis pigmentosa.

d. Other applications

Photoacoustic spectroscopy was also used to characterize or to detect molecules of biological interest in different media using different spectral regions. The nucleic acids, or their isolated nucleotides, were examined in the UV region (Inagaki *et al.*, 1986, Sugitani *et al.*, 1988) and PAS permitted the detection of 5-methylcytosine in DNA with high accuracy (Achwal *et al.*, 1984).

In food sciences, PAS was successfully used in the determination, in the near infrared region, of moisture content of starch (Belton and Tanner, 1983), and of esterification degree of pectin, which is used as gelling agent in various foods (Haas and Jager, 1986). Feasibility of PAS utilization as analytical technique to control protein content in milk (UV-vis, Martel *et al.*, 1987), water content in condensed milk (NIR, Martel *et al.*, 1990) or iron content in milk (UV, Doka *et al.*, 1991) was also tested. Fungal growth on cellulose support was evaluated using FTIR-PAS (Greene *et al.*, 1988, Gordon *et al.*, 1990). When FTIR spectroscopy is applied to living material, a major concern is related to the presence of water bands interference on spectra. Gordon (1987) described a method to exclude these bands.

Photoacoustic analysis of photochemical processes

Up to now, we have interpreted heat production as the only deactivation process that occurs following light absorption by pigments. As a matter of fact, the energy absorbed by the pigment systems is released by several mechanisms; the resulting equation which resumes energy transformation is:

$$E_{\text{absorbed}} = E_{\text{heat}} + E_{\text{luminescence}} + E_{\text{photochemistry}} \quad (11)$$

In biological samples such as a leaf (Figure 4a), the luminescence processes are mostly due to fluorescence (emission of a photon of lesser energy than the one which was absorbed). Fluorescence represents in a leaf 3 to 5 % of all deactivations (Krause and Weis, 1991) then, it is considered as negligible. Thus, the energy transformed into heat can be expressed as:

$$E_{\text{heat}} = E_{\text{absorbed}} - E_{\text{photochemistry}} \quad (12)$$

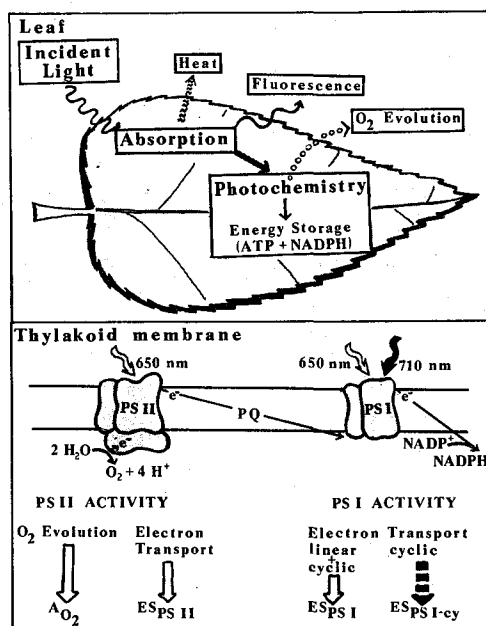


FIGURE 4: PHOTOACOUSTICS IN PHOTOSYNTHESIS RESEARCH: RECORDING ACTIVITY

- A. The leaf is a complex pigments system where, following light absorption by pigments, four major physical processes can happen: i- energy transfer, ii- fluorescence, iii- heat emission and iv- photochemistry. Generally, all these four processes are competitive in a leaf then, an increase in the quantum yield of any processes should produce a decrease of the yield of the other three other. Photochemistry results in energy storage by chemical intermediates of the electron transport chain and in O₂ evolution.
- B. From a molecular point of view, the physico-chemical reactions of photosynthesis occur in the thylakoid membrane of the chloroplast, which are specific to the plant cell. Pigment-protein complexes of photosystems I and II (PSI and PSII), present in the thylakoid membranes, are responsible for light absorption, transfer of this energy up to their reaction center and charge separation (photochemistry). This photochemical activity results in the reduction of various intermediates of the electron transport chain (ultimately to the reduction of NADP to NADPH), in the build-up of a proton gradient (from which energy is used to synthesize ATP) and H₂O oxidation (which provokes O₂ evolution). The photosynthetic reactions can be followed by photoacoustic methodology. The result of photosystems' photochemistry is the photosynthetic energy storage (ES) which represents the part of the absorbed energy stored in the electron transport chain intermediates. The determination of activities of PSI (ES_{PSI}) and PSII (ES_{PSII}) is achieved by utilizing the method of Veeranjanyulu *et al.* (1990) under 650 modulated light. Cyclic electron transport around PSI is estimated under 705 nm modulated light (Herbert *et al.*, 1990). Moreover, PSII photochemistry produces O₂ which diffuses through the plant cells up to the leaf surface (Bults *et al.*, 1982). It is possible to separate the O₂ signal from the thermal signal by following Poulet's vectorial analysis (Poulet *et al.*, 1983).

where N is Avogadro's number, h is Planck's constant, ν is the frequency of incident light, Φ_i is the quantum yield of the photochemical reaction i , ΔE_{pi} is the internal energy change per mole of product formation in the photochemical reaction, and I is the absorbed light intensity in Einstein per unit of volume and per unit of time. a is an instrumental proportionality constant which is related to optical and thermal properties of the sample, and to the answer efficiency of the apparatus (Malkin and Cahen, 1979).

It is possible to determine the value of $\Sigma (\Phi_i \Delta E_{pi})$ by using the effect produced by the addition of a continuous saturating light beam (Bults *et al.*, 1982). Under such conditions, the modulated photochemistry is damped, since all modulated light absorbed is transformed into heat. Then, the photoacoustic signal generated by the use of a weak modulated and a saturating continuous lights (Q_{ms}) becomes:

$$Q_{ms} = a(N \cdot h \cdot \nu) I \quad (15)$$

Thus, the energy storage (ES), which represents the part of absorbed photon energy stored into chemical intermediates, can be evaluated by:

$$ES = \frac{\Sigma (\Phi_i \Delta E_{pi})}{h \cdot \nu} = \frac{Q_{ms} - Q_m}{Q_{ms}} \quad (16)$$

Beyond the methodology of a saturating, continuous light, an inhibited or a inactivated (by freeze-drying) sample can be used as the reference sample, where maximal thermal deactivation occurs, following modulated light absorption. This methodology was found to be useful in studies with chloroplasts (inhibited with DCMU, Cahen *et al.*, 1978 a) and purple membranes of halobacteria (Cahen *et al.*, 1978 b).

The methodology of the adjunction of saturating light to evaluate ES is now widely spread as shown by applications with membrane thylakoids (Carpentier *et al.*, 1985), chloroplasts (Lasser-Ross *et al.*, 1980), photosynthetic bacteria (Carpentier *et al.*, 1984), lichens (O'Hara *et al.*, 1983) and entire leaves (Bults *et al.*, 1982). It is interesting to note that such effect of continuous light was also found with other photothermal techniques, namely photothermal radiometry (Bults *et al.*, 1982 b, Malkin *et al.*, 1991) and photothermal beam deflection spectroscopy (mirage effect, Havaux *et al.*, 1989). In leaves, with a 650 nm modulated light, ES, as determined with the illumination of a saturating continuous white light, represents the energy stored by chemical intermediates from both photosystems I and II (Bults *et al.*, 1982 a). It was recently shown that the illumination with continuous far-red light gives the energy stored by PSI in leaves (Veeranjaneyulu *et al.*, 1991). Moreover, when the leaf is shined with a 705 nm modulated light, the use of continuous white light allows the determination of energy stored by cyclic electron transport (Herbert *et al.*, 1990). Then, PA methodology becomes the unique technique that permits the determination of photochemical activities of both photosystems *in vivo*, alone or together, with the same physiological and technical parameters.

In a leaf (Figure 4a and b), the PA signal contains a third component in addition to the photothermal one and the ES: it is the photobaric one, which is linked to the oxygen ex-

changes which occur in the leaf (Bults *et al.*, 1982 a). This component is principally due to O_2 evolution following light activation of PSII. Some experimental works showed that both photochemical (Malkin, 1987) and non-photochemical (Charland *et al.*, 1992) O_2 -uptake processes decrease the O_2 -evolution signal. It has to be noted that the O_2 component is only detected at low modulation frequency (usually less than 200 Hz (Bults *et al.*, 1982 a)). This is due to the low diffusion rate of O_2 in water, in comparison to thermal diffusion rate. Then, in a leaf, it is possible to record the energy storage component at high modulation frequency ($f_{mod} > 300$ Hz). This value of ES is used in the calculation of the O_2 signal at low modulation frequency, as demonstrated by Poulet *et al.* (1983).

Photoacoustic evaluation of photosynthetic activity has been extensively used in studies on effect of environmental stresses on plant physiology. PA methodology allowed to determine detrimental effects of water stress (Havaux *et al.*, 1986a,b, 1987a), high (Havaux *et al.*, 1987b,c) or low temperature constraints (Yakir *et al.*, 1985, 1986) and high-light (Buschmann, 1987, Canaani *et al.*, 1989, Havaux, 1989) or low-light (Canaani and Malkin, 1984, Havaux, 1990) treatments, on leaves or entire algal cells. Moreover, the effects of air-borne pollutants (Nagel *et al.*, 1987, Veeranjanyulu *et al.*, 1990) and herbicides (Szigeti *et al.*, 1989) were also analyzed.

The usefulness of PA methodology was also showed in basic studies on pigments presence in organisms (Veeranjanyulu and Das, 1982, Canaani *et al.*, 1985), light energy transfer between pigments (Buschmann and Prehn, 1982, Boucher *et al.*, 1983, Malkin *et al.*, 1990) and light energy distribution between photosystems (Canaani *et al.*, 1982, 1984, Fork *et al.*, 1991, Veeranjanyulu *et al.*, 1991b,c) of leaves or algae.

Since it is possible with PA technique to record photochemical activity of PSI and PSII alone (*via* ES_{PSI} or ES_{PSII} and O_2 signal, respectively) or together, its usefulness becomes undoubted in the field of plant physiology.

Concluding remarks

Because of its ability to perform studies of opaque or highly diffusive material, PAS has the potential to become a more widely used technique in the bio-medical field. Its non-destructiveness represents an important advantage, since this permits its use in addition to other techniques. We introduced PAS applications to dermatology, hematology or vision, but some studies of the effect of many drugs on the skin, the blood or the eye may be done. This work will help in the elucidation and understanding of *in vivo* mechanisms of some diseases, and their therapy.

Since most biological compounds have distinct absorption patterns in the IR region, the FTIR-PAS, coupled with other techniques, may provide powerful tools to study compounds of biological interest. With the emergence of signal deconvolution methods, the use of FTIR-PAS becomes accessible.

One of the most promising tool to study photochemical reactions is surely the pulsed photothermal methodology. It will be then possible to follow very fast reaction rates, and products formation. The energetics of such reactions is also followed with such methodology. Pulsed PAS will greatly help in the knowledge of very important biological processes, such as photosynthesis and vision.

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