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SIMULTANEOUS DETECTION OF PHOTOSYNTHETIC ENERGY STORAGE AND OXYGEN EVOLUTION IN LEAVES BY PHOTOTHERMAL RADIOMETRY AND PHOTOACOUSTICS

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We have measured simultaneously the photothermal radiometry and the photoacoustic signals from intact leaves. We have confirmed that while the former senses that part of the modulated absorbed radiation not used in photosynthesis, but converted into heat, the latter, at low modulation frequencies, senses not only this heat but also the modulated oxygen evolution resulting from photosynthesis in the leaf. When photosynthetic activity is saturated upon additional excitation with strong non-modulated light, the photothermal radiometry signal increases (virtually all absorbed modulated light being converted into heat), while at the same time the photoacoustic signal decreases, because virtually no modulated oxygen evolution occurs any more. At higher modulation frequencies the behaviour of the photoacoustic signal closely follows that of the photothermal radiometry signal. We have used combined photothermal radiometry/photoacoustic measurements to estimate directly the yield of chemical energy storage in various plant species which applies for different times after excitation. Measurement of light saturation curves for wheat and Siberian pea bush leaves and of action spectra for the latter confirm the similarity between photothermal radiometry and high-frequency photoacoustic signals, and their difference from the low-frequency photoacoustic signal. Combined use of photothermal radiometry (or high-frequency photoacoustics) and low-frequency photoacoustics can thus provide more information than any one method alone. Experiments on intact chloroplasts and on a blue-green alga demonstrate that photothermal radiometry and photoacoustic methodologies can also be used for these tissues.

Introduction

Photoacoustic and photothermal measurements on photosynthetic systems can be used to estimate the fraction of the absorbed photon energy that is utilized in photosynthesis and stored on its reaction intermediates [1–6]. Such measurements are based on thermal conversion of intensity-modulated light that is absorbed by a sample. Absorption of such illumination gives rise to temperature variations in the sample, periodic in time and space, but exponentially damped with increasing distance from the locus of absorption. The amplitude of the temperature modulation is related to the extent of heat generation following light absorption [7] and can thus give information for spectroscopy (amount of light absorbed) and/or, indirectly, on photochemical energy-conversion processes (amount of light absorbed that is not degraded to heat during a modulation period) [2]. This temperature modulation (actually the surface temperature modulation for our case, viz., a sample in a condensed phase) can be monitored in a

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closed system through the photoacoustic effect, by measuring, with a microphone, the amplitude of the resulting pressure modulation in the gas (air) phase that surrounds the sample [7]. Alternatively, we can monitor the extent of the temperature modulation in an open system by measuring the modulated part of the infrared radiation that is emitted from the sample surface or from layers close to it. This latter approach has been termed 'photothermal radiometry' [8].

The above measurements can be applied to photosynthetic systems with relative ease, because a reference measurement, in which no modulated photochemical conversion occurs and all the absorbed modulated light is converted to modulated heat, can be obtained simply by illuminating the sample with an additional intense and non-modulated light beam, which saturates photosynthesis. For suspensions of chloroplasts [3], algae (unpublished data) and photosynthetic bacteria [9] the photoacoustic signal increased upon application of such a saturating background light. This increment was termed 'photochemical loss' because its ratio to the increased signal (with background light) equals the fraction of the absorbed photon energy that is stored chemically. For clarity's sake we will use the term 'positive' effect here for this signal increase.

When, in the photoacoustic measurements, leaves were used as samples we found a different behaviour though [4,5]. At low modulation frequencies (much less than 200 Hz), a large decrease in the photoacoustic signal was observed upon application of additional continuous saturating light ('negative' effect). We explained this phenomenon by assuming that in this case the photoacoustic signal contains a second, non-thermal, contribution and that this contribution is eliminated by applying the background light. From indirect evidence we concluded that this additional contribution is due to modulated photosynthetic oxygen evolution. This negative effect is not observed in suspensions mainly because the modulation in oxygen concentration is damped out practically completely over the long diffusion paths from the loci of O_2 formation to the nearest gaseous phase. In leaves these diffusion paths are relatively short (about 1 μm) and at low modulation frequencies, such damping becomes small. However, when the modulation frequency is increased, the characteristic diffusion length (i.e., the distance over which the modulation amplitude is attenuated by e^{-1}; cf. Ref. 5, Appendix) will decrease, so that, at sufficiently high modulation frequencies, the negative effect disappears. This leaves only the positive effect, which at lower frequencies was masked by the negative effect.

An additional proof for the contribution from modulated gas evolution to the photoacoustic signal in leaves was obtained from photothermal radiometry. This method can respond to photothermal conversions only, and as such should be insensitive to modulated gas evolution, to a first approximation. Preliminary photothermal radiometry experiments indeed showed that, at any modulation frequency, only positive effects were obtained [6].

In this report we complete this direct evidence by giving data for simultaneous photoacoustic and photothermal radiometry experiments on leaves. Such additional evidence was deemed necessary because under certain conditions (e.g., immediately after a long period of dark adaption) positive photoacoustic effects were observed also at low modulation frequencies, indicating a temporary absence of oxygen evolution. In addition, the use of photothermal radiometry cum photoacoustic measurements at various modulation frequencies can allow us to separate the thermal from the oxygen evolution component to the photoacoustic signal, i.e., evaluation of photochemical loss also in low-frequency photoacoustic work alone. Also, because our previous photoacoustic measurements were limited to tobacco leaves, and the preliminary photothermal radiometry measurements were done on Siberian pea bush leaves only, it was necessary to carry out the combined, and where possible, simultaneous measurements on both species. Finally, here we used leaves of several other plant species to explore the general applicability of the methods, and we verified the possibility of using photothermal radiometry for suspensions of photosynthetic material.

Experimental Procedure

The basic set-up used illumination optics similar to those described in Ref. 5, including two quartz-iodine 250 W projectors as light sources for modulated and continuous excitation. Both projectors were operated on d.c. stabilized power supplies and delivered their light, through different optical trains including lenses and optical filters, to either of two branches of a light guide having a common end terminating in front of the sample. Modulation was achieved in one of the optical trains by adding a mechanical chopper (Laser Precision) which covered a wide frequency range from about 2 to 1000 Hz. The photoacoustic and photothermal signals were analyzed each by separate lock-in amplifiers (Brookdeal) and then recorded separately or simultaneously on a dual-channel chart recorder.

For separate photoacoustic measurements we used a locally constructed cell [10]. For photothermal radiometry measurements a liquid nitrogen cooled, infrared detector (Plessey PbSnTe, optimized for room-temperature black body radiation at wavelengths around 10 μm), collecting emission through a wide-angle $f/1$ infrared lens was used [6,8]. The most useful cell, however, was the thermophone, i.e., a photoacoustic cell covered with a window (BaF_2) transparent to the thermal (infrared) emission at around 10 μm, which allowed both photoacoustic and photothermal measurements in a true simultaneous manner (Kanstad and Nordal, unpublished observations). Both photoacoustic cells used Knowles Electronics microphones and the same infrared detector was used for thermophone and separate photothermal radiometry measurements.

Leaves from different plant species growing in the greenhouse were collected at random and put in water till the experiment. Growth conditions were not specified. Species collected were: Siberian pea bush (*Caragana arborescens*), wheat (*Triticum aestivum*), beans (*Phaseolus vulgaris*), tobacco (*Nicotiana tabacum*) and spinach (*Spinacea oleracea*).

Results and Discussion

Fig. 1 shows results from simultaneous photothermal radiometry and photoacoustic experiments at low modulation frequency, in the steady state. It is obvious that while negative effects of saturating background light are sensed by the microphone, the infrared emission shows a positive effect only. Such simultaneous experiments were repeated with other species and the results are summarized in Table I. Comparison between these photothermal radiometry and photoacoustic signals confirms that at these frequencies the microphone signal contains an additional contribution, not sensed by the infrared detector, which is eliminated by the background light (cf. Introduction). The occurrence of this additional component in the photoacoustic signal from leaves makes it impossible to evaluate the photochemical loss from such photoacoustic data alone. If, however, photothermal radiometry data are available as well, both the photochemical loss (from photothermal radiometry alone) and the contribution from oxygen evolution (= extent of negative effect plus photochemical loss, cf. Ref. 5) can be obtained. Because the nature of the two contributions to the photo-

TABLE I

COMPARISON OF BACKGROUND LIGHT EFFECT IN PHOTOTHERMAL RADIOMETRY (ΔPTR) AND PHOTOACOUSTIC (ΔPA) EXPERIMENTS WITH DIFFERENT SPECIES, AT LOW FREQUENCY (23 Hz)

Data are from traces similar to those shown in Fig. 1. Values are relative. The signal obtained in the presence of background light, which is due to total thermal conversion of the absorbed light, without any contribution from oxygen evolution, is taken as 100% in both methods. Oxygen evolution is the sum of the absolute values of the positive and negative effects (cf. Ref. 5), and may exceed 100%, as the photoacoustic signal can increase more than 2-fold, upon removal of background light.

	Siberian pea	Bean	Tobacco	Wheat	Spinach
ΔPTR (%)	+22	+12	+29	+33	+28
ΔPA (%)	−28	−14	−60	−44	−75
O_2 evolution, calculated (%)	50	26	89	77	103

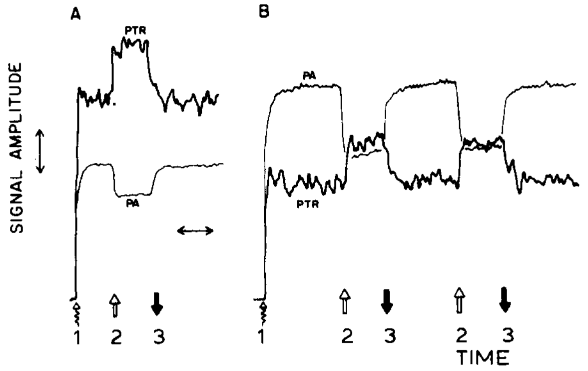

Fig. 1. Typical simultaneous photoacoustic (PA, thin curve) and photothermal radiometry (PTR, thick curve) traces (at low frequency, 23 Hz), obtained with modulated light on (indicated by arrow 1) and with alternately switching on (arrow 2) and switching off (arrow 3) of the saturating continuous background light. (A) Siberian pea. (B) Wheat leaves. Time scale arrow, 2 min; signal scale arrow, 20 μV for photothermal radiometry, 50 μV for photoacoustic signal in A and 25 μV for photoacoustic signal in B. Modulated light, $\lambda = 570-660$ nm, 25 W/m^2. Background light, white, $\lambda < 720$ nm, 225 W/m^2.

acoustic signal is different, elimination of one of them (by applying strong background light) will affect the phase of the photoacoustic signal (Poulet, Cahen and Malkin, unpublished observations). On the other hand, no such phase shift is expected in photothermal radiometry, and, indeed, none was observed.

Because at high modulation frequency the photoacoustic signal contains only thermal contributions, as the oxygen contribution is damped out, it was of interest to compare such high-frequency data with photothermal radiometry data. Photothermal radiometry measurements are generally done at low frequencies because of increasingly poor signal-to-noise ratios with increasing frequencies. There is, however, a clear rationale for comparing high-frequency photoacoustic data with low-frequency photothermal radiometry ones: If the photochemical loss does not vary significantly between the low and high frequencies used and usable in photoacoustics, then photoacoustic measurements alone can suffice to separate the thermal and oxygen evolution contributions to the leaf photoacoustic signal. Table II shows that in several cases the photochemical loss (photothermal radiometry) agreed with the high-frequency photoacoustic measurements, and did not vary significantly with the modulation frequency.

However, we also found cases where no such constancy of the photochemical loss was observed, notably the tobacco example in Table II and a sample of bean.

TABLE II

POSITIVE EFFECTS, FROM PHOTOTHERMAL RADIOMETRY MEASUREMENTS AT VARIOUS FREQUENCIES, AND FROM PHOTOACOUSTIC MEASUREMENTS AT HIGH FREQUENCY (EXPRESSED AS PERCENTAGE OF THE SIGNALS OBTAINED IN THE PRESENCE OF SATURATING BACKGROUND LIGHT)

Signal-to-noise ratio usually limits photothermal measurements to low frequencies (less than approx. 50 Hz). The higher frequency measurements were possible by sacrificing resolution time and averaging over long periods. Accuracy of the photothermal radiometry results is estimated to be about ±10% and about ±15% of the values indicated in the table, at low (below 50 Hz) and high frequencies, respectively. For photoacoustic measurements the accuracy is about ±7%. These accuracy figures do not include biological variations. Experimental details: modulated light, 570–660 nm, 32 W/m^2; background light, white, $\lambda < 720$ nm, 250 W/m^2.

Expt. No	Species	Photothermal radiometry Frequency (Hz):												Photoacoustics	
		2	3	7	14	21	23	39	67	90	185	280	311	311	622
1	Siberian pea	26	26	27	–	26	23	–	–	26	24	29	–	–	–
2	Siberian pea	–	–	–	21	–	25	–	24	–	–	–	23	21	22
3	Wheat I	–	–	–	–	25	–	20	24	–	–	27	–	–	–
4	Wheat II	–	–	–	28	–	32	–	–	–	–	–	30	33	–
5	Bean	–	–	–	17	–	–	–	20	–	–	–	20	–	–
6	Bean	–	–	–	–	–	16	–	–	–	–	–	–	22	–
7	Tobacco	–	–	–	–	–	–	19	–	–	–	–	–	40	–

The above observations are of interest because they allow a comparison of the extent of energy storage on early and late intermediates in the photosynthetic reaction chain (cf. Refs. 2, 3 or 9). The experiments with Siberian pea show that photosynthetic intermediates with short lifetimes, below the rate-limiting step time (10–30 ms, here represented by the 'high' frequency range, much greater than 15 Hz), store essentially the same amount of energy as the longer-lived intermediates (here represented by the frequency range 2–15 Hz), which presumably follow the first ones consecutively. Thus, only little energy loss must occur in the photosynthetic reaction chain between about 0.5 and 100 ms after photoexcitation *. The value of energy storage obtained for Siberian pea is about 26% (of the absorbed radiation), corresponding to 11.5 kcal/einstein (taking the energy of 1 einstein to be 45 kcal on the average, for the wavelength range and spectral distribution used). The final products of photosynthesis (e.g., glucose) store about 110 kcal/fixed CO_2 [11]. With an obligatory maximal theoretical quantum yield of 1/8 per CO_2 [12] the maximal theoretical amount of energy storage should be 13.75 kcal/einstein. Thus, the value of 11.5 kcal/einstein obtained in this experiment indicates an efficiency of approx. 85% of the maximum theoretically possible. With the other species, the lowest frequencies tried were between 14 and 23 Hz, corresponding to decay times of intermediates around 10 ms. There the energy storage could be above the CO_2 fixation products. The maximal value at these frequencies was obtained with wheat (33%, i.e., approx. 15 kcal/einstein). Also, in at least two cases, tobacco and perhaps bean, our results indicate the occurrence of reaction steps with decay times between about 0.5 and 10 ms, during which appreciable energy is lost.

These differences between various species (or growth conditions) may well warrant further investigations in the future.

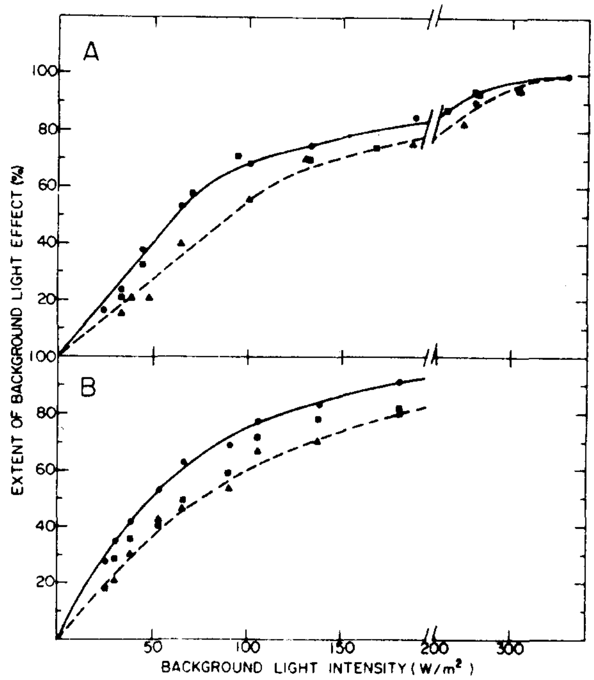

Fig. 2. Saturation curves for the positive (photoacoustic at 314 Hz (▲) and photothermal radiometry at 38 Hz (■)) and negative (photoacoustic at 38 Hz (●)) effects. (A) Wheat. (B) Siberian pea. All changes were normalized in terms of percent of the maximum extent, and were obtained by varying the background light intensity from zero to the maximal possible level. Account was also taken of the average continuous intensity of the modulated light: The raw data were plotted first, adding this average continuous intensity to that applied by use of the background light. The true level of the changes was found by extrapolating to true zero light level, after which the data were normalized. In the case where saturation was not complete, even at the highest background light intensity used, the maximal extent was estimated by plotting the data vs. 1/(light intensity) and extrapolating to 1/(light intensity) = 0. Modulated light, $\lambda = 570–660$ nm; intensity, 60 W/m^2 for Siberian pea, 43 W/m^2 for wheat photothermal radiometry data, 66 W/m^2 for wheat photoacoustic data; photoacoustic and photothermal radiometry data for Siberian pea were obtained simultaneously, using the thermophone.

If high-frequency photoacoustic and low-frequency photothermal radiometry data represent the same phenomenon (with the above noted caveats), and the low-frequency photoacoustic data a different one, we may expect the light saturation behaviour of the positive and negative effects to reflect this. Fig. 2 shows these light saturation curves for Siberian pea and for wheat leaves. From it we see that, to a first approximation, all effects have similar saturation profiles. A closer look shows that the negative effect (low-frequency photoacoustic measurements) tends to saturate somewhat faster than the positive effect (from high-

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* These times correspond to the reciprocal of the angular frequency (2π times the frequency in Hz); cf. Ref. 2.

frequency photoacoustic measurements). The accuracy of the photothermal radiometry measurement makes it difficult to determine if the positive effect measured in this way saturates in the same way as the positive effect measured by high-frequency photoacoustics (seemingly the case for Siberian pea) or as the negative effect measured by low-frequency photoacoustics (seemingly the case for wheat). The above-mentioned frequency independence of the photochemical loss for Siberian pea makes the result for this species a reasonable one, however. One possibility to explain the differences in saturation behaviour is to invoke the existence, at low modulation frequency, of a small, partial modulation of the concentration of open reaction centres, out of phase with respect to the modulation of oxygen evolution. Such a modulation is expected to be damped out completely at higher frequencies. Theoretical considerations (unpublished data) show that there is an additional modulation term in the thermal or acoustic output signal. This term, due to the modulated component of the concentration of reaction centres, has to be multiplied by the intensity of the continuous background light, and thus is zero for zero background light intensity and for completely saturating background light (no modulation of concentration of reaction centres). It can be shown that, at intermediate background light intensities, this term will tend to increase the background light effect, because it decreases the modulation amplitude of oxygen evolution. Then the change of the signal will be closer to its saturation value.

An additional consideration is that oxygen evolution follows the fraction of open reaction centres in Photosystem II (low-frequency photoacoustic measurements) while the photothermal radiometry follows essentially contributions from both photosystems separately and will depend on both types of reaction centres. Thus, one does not have to expect completely matched saturation curves, for the three cases, as in Fig. 2.

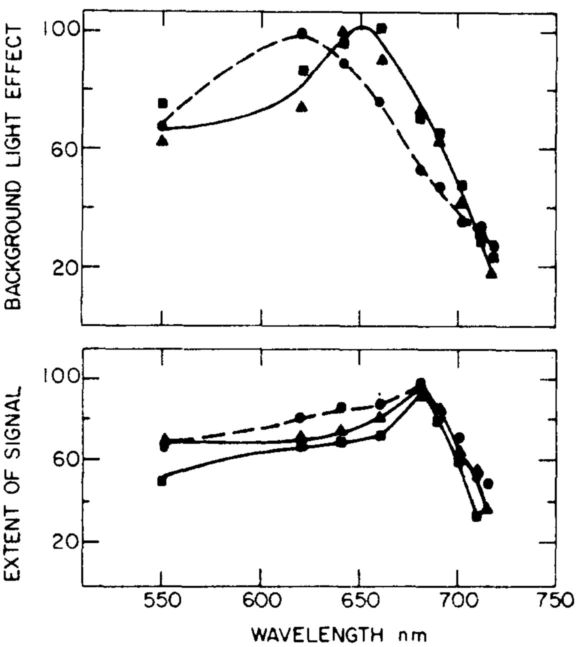

Fig. 3. (Top) Quantum yield spectra of the positive and negative effects in samples of Siberian pea leaves. The relative changes of the photoacoustic and photothermal radiometry signals were divided by the wavelength λ in order to obtain the relative value of the quantum yield vs. λ (cf. text). (■) From photothermal radiometry data, positive effect at 38 Hz. (▲) From photoacoustic data, positive effect at 309 Hz. (●) From photoacoustic data, negative effect at 38 Hz. The various wavelengths were obtained by use of interference filters (Ditric optic -3 cavity type). Care was taken to be in a range of modulated light intensities that does not affect the response to the background light intensity used (225 W/m^2, $\lambda < 720$ nm). (Bottom) Photoacoustic and photothermal radiometry absorption spectra, obtained in the presence of saturating background light (to eliminate photochemical effects). Each point was calculated by dividing the signal by the light intensity in power units. The symbols have the same meaning as above.

An additional feature of photoacoustic measurements, that should be available also from photothermal radiometry measurements, is the possibility of obtaining directly quantum yield spectra (wavelength dependence of quantum yield) [3,5]. Again, because low-frequency photoacoustic measurements on the one hand, compared to high-frequency photoacoustic and (any frequency) photothermal radiometry measurements on the other reflect different aspects of photosynthesis, complete similarity is not expected and differences have indeed been observed in photoacoustic experiments alone [5]. Here we had the possibility, using the thermophone, to measure simultaneously the low-frequency photoacoustic and photothermal radiometry signals, thus making a comparison more strictly valid. Fig. 3 shows that we do observe differences in the wavelength dependence of the negative effect (photoacoustic) and the positive

TABLE III

FEASIBILITY STUDIES OF PHOTOTHERMAL RADIOMETRY ON CELL AND CHLOROPLAST SUSPENSIONS

Measurement conditions were similar to those described in the previous figures and tables. Medium: for *Nostoc* see Ref. 14; for spinach: 0.35 M sorbitol, 40 mM Tricine (pH 8), 0.25 mM phosphate. PGA, phosphoglycerate.

Sample	Frequency (Hz)	Positive effect (% of signal with background light)
Nostoc muscorum 7119	23	17 ± 4
	67	40 ± 8
Whole chloroplasts (spinach) + KNO_2 (5 mM)	7	15 + 3
	23	17 ± 4
	67	12 ± 3
+ PGA (3 mM)	23	15 ± 3
+ PGA + NH_4Cl	23	12 ± 3

effects (photoacoustic; photothermal radiometry). The positive effect, expressed as the fraction of change relative to the signal with background light, is actually equal to $\phi \Delta E/h\nu$ where ϕ is the quantum yield, ΔE the energy stored per turnover, and $h\nu$ the photon energy. Thus, ϕ is proportional to the relative positive effect divided by the wavelength λ (assuming that ΔE is wavelength independent) and this is what is plotted in Fig. 3. The negative effect relative to the signal with background light is proportional to $\phi'/h\nu$ (ϕ' is proportional to the negative effect divided by λ). There ϕ' represents an average yield for the combined and opposite effects of oxygen evolution, the major contribution, and photochemical conversion. In Fig. 3 both quantum yields were normalized to 1 at their maximal value. The positive effects, from low-frequency photothermal radiometry and high-frequency photoacoustics, gave essentially similar quantum yield profiles. The basic feature is the well known 'red drop' in the quantum yield for $\lambda > 650$–660 nm. At 715 nm ϕ is about 20% of the maximum; ϕ' shows a peak at 620 nm, a moderate drop to 660 nm and a more noticeable drop for $\lambda > 660$ nm. Such differences between energy storage and oxygen evolution were found previously [5]. One expects indeed higher quantum yields for energy storage than for oxygen evolution in the red drop region, if Photosystem I alone contributes significantly to such energy storage (e.g., as in cyclic photophosphorylation, cf. Ref. 3).

It was of interest to see whether photothermal measurements are also feasible on suspensions. We prepared whole chloroplasts according to Ref. [13], and made a thick suspension in various media and loaded such a suspension on a round cut filter paper, which served as the sample. Similar studies were done with the blue-green alga *Nostoc muscorum*. Clear positive effects were obtained which are summarized in Table III. Thus, in suspensions, photothermal radiometry and photoacoustic measurements [3,9] agree with respect to the direction in which the signal changes upon application of background light.

Conclusions

We have demonstrated that the changes in low-frequency photothermal and high-frequency photoacoustic signals from leaves upon saturation of photosynthesis, are strongly correlated, not only qualitatively, but often also quantitatively. This correlation exists for all the various plant species investigated here. An important conclusion from this experimental finding is that it supports our interpretation of the decrease in the low-frequency photoacoustic signal upon saturation of photosynthesis, viz., that this photoacoustic signal contains a non-thermal contribution, namely modulated oxygen evolution. Another corollary of this correlation between photoacoustic and photothermal radiometry signal changes is that it allows the evaluation of the extent of photochemical energy conversion at low frequencies from photoacoustic data alone, if the correlation is a quantitative one, and an estimate of the extent of oxygen evolution.

While the present investigation was mainly qualitative in character, we did obtain quantitative results for the amount of energy stored in the latter stages of photosynthesis, by leaves of several plant species. These results approach the maximal theoretical amount for CO_2 fixation products. Such data show us only a glimpse of what we may hope to achieve by further investigations, both in terms of quantitative knowledge as well as in terms of further understanding of the interaction between the two photosystems in leaf photosynthesis, to name just one example.

It should be borne in mind that the information provided by both the photoacoustic and the photothermal radiometry methods is related to gross photosynthesis, because any contributions from dark or photorespiration will not be modulated at the frequencies normally used.

If we compare photoacoustic and photothermal radiometry methods we see that each has its own merits and that, when both are used in combination, photothermal radiometry measurements on leaves provide a valuable addition to photoacoustic ones. The photoacoustic technique is still by far the more sensitive one, but photothermal radiometry is particularly suitable for very low frequency (e.g., below 10 Hz) work and has the advantage that the sample can be open to the ambient. Because our collection efficiency of the re-emitted infrared radiation was far from optimum, we can be confident that the sensitivity of photothermal radiometry measurements can be improved so that it may approach that of photoacoustic ones. As combined photoacoustic/photothermal radiometry experiments seem to be a powerful tool in the study of photosynthesis, attempts to improve the signal-to-noise ratio in photothermal radiometry seem to be well worthwhile.

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