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Pulsed-laser induced optoacoustic spectroscopy of intact leaves

Merten Jabben and Kurt Schaffner *

Max-Planck-Institut für Strahlenchemie, D-4330 Mülheim a.d. Ruhr (F.R.G.)

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Pulsed-laser induced optoacoustic responses of intact leaves of several different plants were recorded as a function of wavelength (590–730 nm) and energy of the 15 ns laser pulse. After appropriate correction for the scattered-light-induced signals at the transducer (cuvette arrangement) 'normal' optoacoustic signals were obtained. The resulting action spectra resemble the corresponding overall absorption spectra of the leaves, and the wavelength-dependence of the correction factors resembles the diffuse reflectance spectra. Illumination with strong continuous background white light was used to 'close' the photosynthetic reaction centers to yield a suitable reference for optoacoustic signals in the study of the primary photoprocesses in photosynthesis. Under these conditions, the optoacoustic signal increases by a factor of approximately 2 over the signal measured without background illumination. Together with fluorescence spectroscopy, the method provides a basis for the quantitative evaluation of the combined primary photophysical and photochemical processes in the photosynthetic system.

Introduction

In photosynthetic systems the excitation energy absorbed by chlorophyll is transferred to the reaction centers. It is then available for effecting charge separation (i.e., photochemistry), and a small fraction is lost by fluorescence and radiationless processes. Chlorophyll fluorescence has been used as an intrinsic probe of reaction center photochemistry and has provided considerable insight into pigment protein interactions in reaction centers [1,2].

Recently conventional photoacoustic spectroscopy which is based on the modulation of a continuous light source, has also been used to analyze the photochemical energy storage in photosynthetic organisms including intact leaves [3–6]. Scanning the photoacoustic response as a function of the modulation frequency can provide information on photochemical events [7] in a time domain (millisecond to second range) which is determined by the relatively low frequency (usually under 1 kHz) of the light chopper [8]. The use of higher frequencies (shorter time domains) is prohibited by the low signal-to-noise ratio.

In pulsed-laser-induced optoacoustic spectroscopy, heat emitted after a single light pulse of 15 ns duration is monitored. The time resolution of heat detection is restricted by the transit time, τ_t, required by the sound pulse to cross the diameter of the laser beam (= prompt heat dissipation [9,10]). In experiments in which the laser beam is focussed to 0.8 mm, for example, the time window for the optoacoustic measurement is narrowed to about 0.5 μs. This technique thus covers a time range differing from that of the conventional photoacoustic technique. It provides more specific information on the very early intermediate(s). In particular, the chances to gain selective informa-

* To whom correspondence should be addressed.

tion on the primary photoproduct(s) are significantly greater.

Laser-induced optoacoustic spectroscopy has been used to study the in vitro primary photochemistry of biliverdin [9], phytochrome [10] and chlorophyll [11]. However, it has not yet been applied to intact plant tissue, mainly due to the highly scattering nature of this material. The present publication introduces a simple way to cancel the scattered-light-induced signals, and it demonstrates that laser-induced optoacoustic spectroscopy can be used to study the primary steps of photosynthesis in intact leaves.

Materials and Methods

Plant material

Maize (*Zea mays* L., Hybrid Inrakorn 1, Nungesser, Bad Krozingen, F.R.G.) seedlings were grown on moist vermiculite for 9 d under continuous white light. White control plants were grown in the same way with the addition of $5 \cdot 10^{-5}$ M of the herbicide Norflurazon. Secondary leaves were used in all experiments with maize. Seedlings of squash (*Cucurbita pepo* L.) were grown for 5 d in the dark on moist vermiculite and variegated fig plants (*Ficus benjamina*, cv. Hawaii) were obtained from a local store. For infiltration of the intercellular air spaces with water, green leaves were immersed in tap water for several hours at room temperature. This procedure provided reproducible data.

Optoacoustic measurements

The optoacoustic spectroscopy arrangement was similar to the one described before [9,10] except for the following changes. Rhodamine B (590–620 nm), DCM (620–680 nm), and Pyridin 1 (655–730 nm), dissolved in methanol, were used as laser dyes (Lambda Physics, Göttingen). The energy output of the 15 ns dye laser pulse was adjusted with a variable neutral density glass and ranged between 0.15 and 200 μJ. The laser beam normally had a diameter of 0.8 mm. In order to reduce the fluence the beam was widened up to 3 mm diameter, which still resulted in a narrow first peak of the voltage deflection H (see Fig. 2). The original C-clamp geometry [12] was modified as depicted in Fig. 1. The leaf was mounted on a Plexiglass

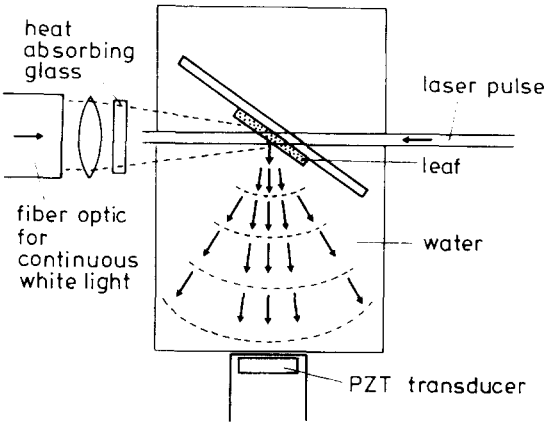

Fig. 1. Cuvette arrangement for the measurement of optoacoustic signals from highly scattering plant tissue. The leaf is mounted on a plexiglass holder and immersed in water. The laser pulse traverses the leaf in such a way that only remitted, and no reflected, light can reach the transducer.

holder and inserted in a Plexiglass cuvette (10 × 15 mm) with a variable (5–10 mm) distance between the leaf and the PZT-5a (Vernitron, OH) transducer. Tap water served as coupling medium for the acoustic signals, and the leaves were carefully coated with silicon grease to enhance acoustic coupling.

An Oriel 7750 Broad Band 100 W Quartz Halogen Source with DC regulated power supply designed for fiber optic applications was used as a continuous white light source; a glass fiber optic cable (Oriel 77525; 0.9 m length, 3.2 mm diameter), mounted approx. 4 cm from the leaf, provided a maximum energy output of 1000 $W \cdot m^{-2}$. A heat-absorbing glass (KG 3, Schott, Mainz) was inserted between the cuvette and the fiber optic in order to prevent inadvertent heating of the sample by the white light. As shown in Fig. 6 (cf. O), the removal of the KG 3 glass normally did not lead to any noticeable effect. Neutral density glasses were used to reduce the white light fluence when required.

Care was taken to keep the temperature inside the cuvette constant [13] at 28 ± 0.1°C during the measurements. The first 10 μs of the optoacoustic signal trace were monitored and stored in a Biomation 8100 transient recorder. Ten signals (1 Hz repetition rate) or 200 signals (10 Hz repetition rate) were averaged by the computer system (PDP 11/04-VAX 11/780).

Heating of the sample by the incident laser pulse should be negligible: when light absorption is assumed to be complete and the heat capacity of the leaf to equal that of water, the temperature in the irradiated leaf part can be estimated to increase less than 0.1°C at 0.1 mJ pulse energy.

In vivo absorbance spectra were recorded on a Perkin-Elmer 356 spectrometer with the leaf mounted on a paper mask. For measurements of the fig leaves, the variegated white part of the leaf served as a reference, while for maize a Norflurazon-treated photobleached leaf was used.

Results and Discussion

The scattered light problem

In an optoacoustic arrangement, scattered light is absorbed by various parts of the system, including the detector (a PZT transducer), each part contributing a signal after a time d/c (where d is the distance to the detector and c the sound propagation velocity). Proper light absorption in the sample, on the other hand, sets a signal after a transit time corresponding to the propagation of the sound wave over the distance between the sample area of absorption and the detector. In order to reduce the scattered light problem in optoacoustic signals, Tam and Patel [14] suggested a multi-bend arrangement of the quartz plate that serves as the acoustic coupler between the sample and the detector. The authors noted that the scattered-light-induced signal is strongly reduced in such an arrangement, while the amplitude of the acoustic pulse is not affected by the longer propagation distance. Since in our hands such an arrangement required laser pulse energies of several millijoules to induce a noticeable signal, we devised a different arrangement, in which the laser beam passed through the leaf and the acoustic pulse emerging from the rear side of the leaf was detected (Fig. 1). The resulting oscillations of the transducer appear to be a superposition of the scattered-light-induced signal and of the acoustic pulse (expected delay approx. 6 μs) due to prompt heat emission caused by primary photochemistry and radiationless deactivation (Fig. 2a). White photobleached maize leaves, the white part of the variegated fig leaves or white paper instead of a leaf produced signals (Fig. 2b) which served as a reference to minimize the scattered-light-induced signal. Scattered light induces signal waves, both with the green leaves and with the reference samples, starting immediately after the laser pulse. With the green leaves, the immediate signal waves are wavelength-dependent, whereas they are not with the colorless reference materials. The reference-to-sample ratio of these deflections were found suitable for the correction of the original signals. In order to achieve the correction the scattered light signal was subtracted after weighting it with the wavelength-dependent ratio of the immediate voltage deflections. The resulting signal (Fig. 2c) was similar to that from transparent solutions [10]. It can be considered as being pro-

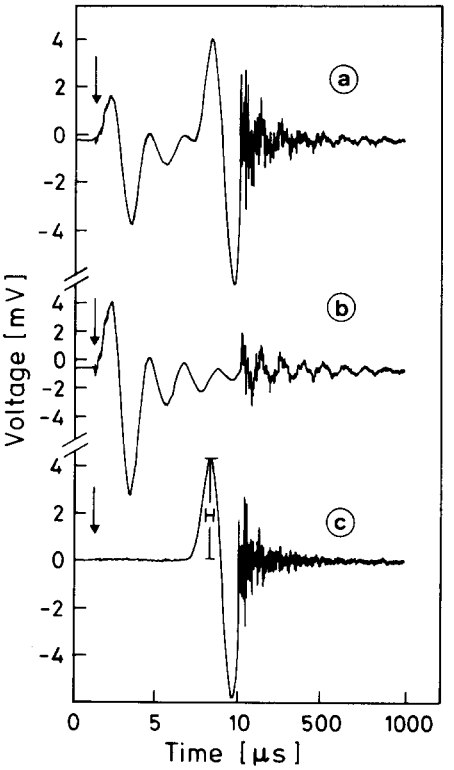

Fig. 2. Optoacoustic signals from a variegated fig leaf after irradiations with 15 ns pulses (λ^{exc} = 700 nm; pulse energy 10 μJ, beam diameter 0.8 mm). (a) Signal from the green part of the leaf. (b) Signal from the white part of the same leaf. The signal from a white paper showed a similar trace. (c) Difference from signal (a) minus 0.6-times signal (b). The vertical arrows indicate the laser pulse incidence. The traces shown are averages of ten single traces; note that the signals are recorded and corrected on a dual-time base.

duced solely by the heat-generating photophysical processes in the leaf. The spectral dependence of the correction factor closely resembles the reflectance spectrum of green vegetation (Fig. 3), which additionally justifies the claim that the original signals are corrected for the scattered light effects only.

The amplitude of the first maximum (H) was taken as the sample response [9]. Since the beam diameter was 3 mm, the acoustic transit time, τ_t, was about 2 μs, with water being the rate-limiting contributor to the velocity of sound in living tissue.

After correction for light scattering, variation of the incident pulse energy between 5 and 15 μJ in the wavelength range 590–730 nm resulted in linearly increasing heat emission (data not shown), as is normal in this energy range of optoacoustic spectroscopy [16]. The action spectrum for heat emission (per incident energy) by a green fig leaf was obtained by plotting the slopes of the energy-response curves vs. wavelength. It resembles the overall absorption spectrum from the same leaf (Fig. 3). The only notable difference is that the maximum of the action spectrum is somewhat depressed. This can be attributed to a stronger light attenuation in the wavelength region of the absorption maximum. Furthermore, since the absorption can differ very much between the inside and outside of the leaf [17], and since the origin(s) and the propagation of the acoustic pulse(s) in the irradiated tissue are not known, no attempt has been made to relate the action spectrum quantitatively to the absorbed energy at the site of effective absorption.

Similar action spectra as depicted in Fig. 3 have been obtained also for other plants including maize, oleander, and tradescantia (data not shown). Even etiolated white mustard and squash seedlings gave reasonable optoacoustic signals (e.g., Fig. 4); the correction factors for scattering light were larger (between 0.50 and 0.95) than those shown in Fig. 3 for green leaves. The maximum of the

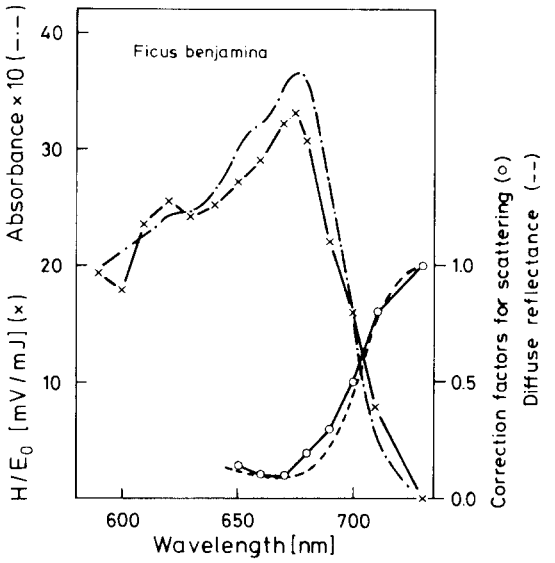

Fig. 3. (× ——— ×) Action spectrum for prompt heat emission after flash irradiation of the green part of a variegated fig leaf (closed photosynthetic reaction centers). It was constructed from the slopes of the energy-response curves after correction for scattering (cf. Fig. 2c; averages of ten single traces). The laser energies were between 5 and 15 μJ; beam diameter 0.8 mm. The correction factors (scattering coefficients; ○ ——— ○) were obtained through optimizing the correction to yield an optoacoustic signal as in Fig. 2c. Below 650 nm they were around 0.1. For comparison, the absorption spectrum (·—·—·; the two spectra are normalized at 700 nm) and an example of diffuse reflectance variation with wavelength (- - - - - -) are given. The reflectance data, which are characteristic for green vegetation, are for green bean leaves and have been taken from Ref. 15.

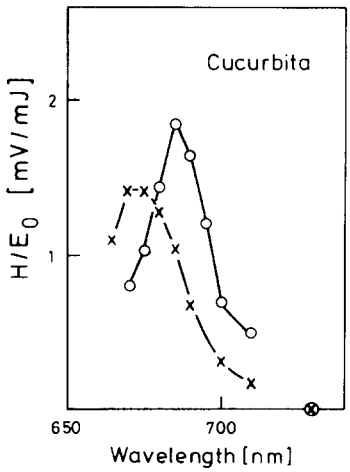

Fig. 4. Action spectra for heat emission from an etiolated squash leaf. The excitation conditions, measuring procedure and scattering coefficients were the same as described for Fig. 3. The optoacoustic experiments were performed immediately after illuminating the leaf for 1 min with continuous background white light (○ ——— ○) and after waiting for 1 h in the dark (× ——— ×).

action spectrum of heat emission taken immediately after the phototransformation of protochlorophyll(ide) to chlorophyll(ide) in etiolated squash seedlings shifted to shorter wavelengths during the following hour in the dark. This phenomenon has also been observed by in vivo absorption spectroscopy and is well known as the Shibata shift [18]. The weak prompt heat emission compared to that from green leaves probably reflects the small number of chlorophyll molecules present at this stage of plant development, while the difference in heat emission (H/E_0) between the measurements taken at different time intervals after the protochlorophyll(ide) phototransformation may reflect a somewhat greater loss of excitation energy through nonphotochemical radiationless deexcitation processes. This may be due to the proposed relocation of the chlorophyll molecules to a more stable site [19].

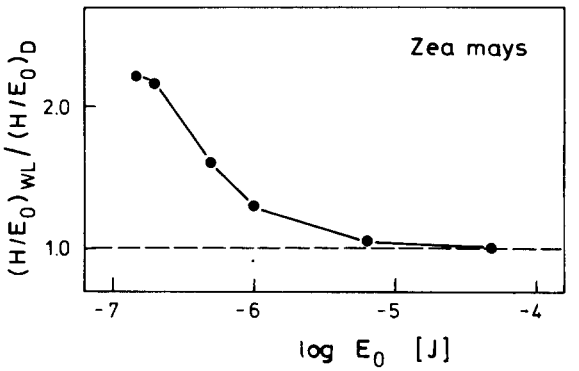

Fig. 5. Optoacoustic signal (●———●) obtained when a water-infiltrated maize leaf was irradiated with $\lambda^{exc} = 675$ nm in the presence of continuous background white light. The measured signal, $(H/E_0)_{WL}$, is normalized to the signal without background light, $(H/E_0)_D$, and plotted as a function of the incident laser pulse energy. Note: The beam diameter was 3 mm and the correction factor 0.1. The points are averages of 200 traces and were taken with a 10 Hz pulse repetition rate.

Reference system

At incident laser pulse energies of around 1 μJ and less, the signal was too small for our detection system, unless it was enhanced by infiltration of the intercellular air spaces with water (which changes the optical properties of the sample [20]). This procedure increased the acoustic signal markedly (data not shown), presumably by improving the acoustic coupling between the chlorophyll-containing cells and the aqueous medium in the cuvette. In this way, at pulse energies of less than 1 μJ spread over about 5 mm^2 of the target surface, the prompt heat emission from maize leaves became sufficiently strong to be recorded.

For a quantitative interpretation of the optoacoustic signals it was necessary to have a suitable reference system totally lacking any photochemistry. In photosynthesis this is achieved either by poisoning the system or by applying saturating continuous high-intensity white light, which converts – in a reversible way – the photochemical reaction centers of the Photosystems II and I to photochemically inactive (so-called closed) forms [4].

Accordingly, the prompt heat emission from water-infiltrated maize leaves increased significantly when at irradiation with low laser pulse energy strong background white light was applied (Fig. 5). At a pulse energy of 0.15 μJ the white light enhanced the signal by a factor of about 2.2. However, at higher pulse energies (at least 6 μJ) the white-light effect was cancelled. It appears that the pulse energy became so high that photosynthetic units were hit several times within the duration of the laser pulse. As a consequence, the reaction centers were already closed for most of the laser pulse time, at sufficiently high pulse energies.

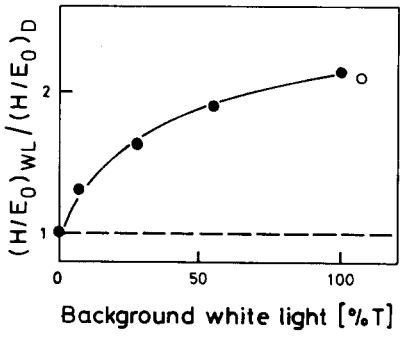

Fig. 6. Optoacoustic signal (●———●) from a water-infiltrated maize leaf plotted as a function of continuous background white light fluence. The measured signal, $(H/E_0)_{WL}$, is normalized to the signal without background light, $(H/E_0)_D$. The laser energy was 0.2 μJ; $\lambda^{exc} = 675$ nm. ○, Measurement without heat-absorbing filter (cf. Materials and Methods). See also Note for Fig. 5.

As can be seen from Figs. 5 and 6, the ratios $(H/E_0)_{WL}/(H/E_0)_D$ show a tendency to level off at the minimum pulse energy and the maximum fluence of white light, respectively. The procedure thus promises to provide a suitable reference system. However, since the lowest pulse energy which our present detection and averaging system was able to resolve reasonably was around 0.15 μJ, the accurate saturation level remains to be more firmly established. The measured value of 2.2 for the enhancement factor may in fact be a lower limit only. The following quantitative considerations (see Calorimetric evaluation) are subject to this caution.

At very low pulse energies (under 1 μJ) the prompt heat emission and the incident light energy show a relationship which can be fitted as a biphasic curve (Fig. 7), with an initial small slope changing into a steeper one with increasing energy. The change occurs in the energy range, in which the white light illumination had an enhancing effect on the total heat emission (Fig. 5). The biphasic behavior can be rationalized by assuming that additional contributions to heat emission, which arise specifically upon photochemical saturation, become more important at higher laser energies. Such contributions are internal conversions and intersystem crossings in photosynthetic units which have received multiple hits. This phenomenon then should allow discrimination between different primary photoprocesses by varying the laser energy. Since the slopes used for the spectrum in Fig. 3 had been obtained at laser energies of greater than 1 μJ, this means that the spectral amplitudes of that experiment reflect photosynthetic systems being partly closed by the flash. It should be noted that differentiation between the photosynthetic systems I and II is not possible. The wavelength resolution is insufficient, and the relative contributions of the two systems to the total heat production are unknown.

Similar dose-response curves for the initial slope at other wavelengths are not available at the moment because of an insufficient signal-to-noise ratio. However, with further improvements in the detection and averaging systems it should become feasible to study also states in which all photosynthetic systems are fully active (all reaction centers open).

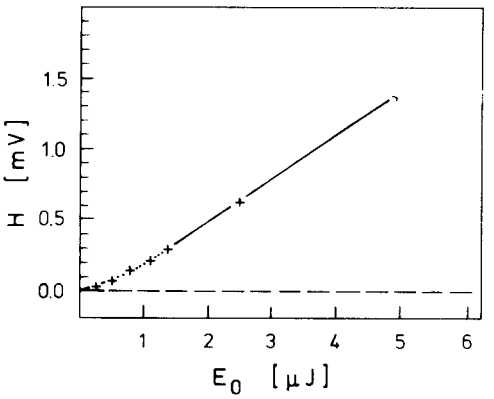

Fig. 7. Dependence of the optoacoustic signal (H) on the incident laser energy (E_0). A water-infiltrated maize leaf was irradiated with a 15 ns pulse of 675 nm. See also Note for Fig. 5.

Calorimetric evaluation

The measurements of this work provide a basis for the quantitative evaluation of the radiationless physical and chemical processes which deactivate the photoexcited chlorophylls. It is pertinent to stress that the values given below represent data for the total of the chlorophyll in the intact leaves (i.e., light harvesting and reaction center molecules). Butler [21] calculated that the radiationless decay should be the major pathway for energy dissipation when the Photosystem II reaction centers are closed. The white-light-illuminated sample was taken for the calibration of the optoacoustic signal: the absorbed light energy is either dissipated as fluorescence (about 10% from closed reaction centers [22] * vs. about 3% from open centers [23]) or as prompt heat emission (α). The latter will include quenching of triplet chlorophyll (e.g., by carotenoids and by oxygen), which is estimated [24] to occur in vivo within the optoacoustic transit time of 2 μs.

For open reaction centers then the 'photophysical and photochemical losses' can be evaluated

* In an investigation by conventional photoacoustic spectroscopy of intact leaves illuminated with continuous white light, Cahen, Malkin and co-workers [4,25,26] neglected the fact that strong background illumination enhances fluorescence. The results of their quantitative evaluation may therefore require revision.

using the energy balance expressed in the following equation [7]:

$$N_A h\nu = \alpha N_A h\nu + \phi_f N_A h\nu_f + \phi_r \Delta E,$$

where N_A is Avogadro's number, h Planck's constant, α the prompt heat dissipation calibrated against the signal obtained under the conditions of background white light illumination, ΔE the internal energy content difference per mole of photoproduct formed, ν and ν_f are the frequencies of incident irradiation and weighted average of fluorescence, respectively, and ϕ_f and ϕ_r the quantum yields of fluorescence and primary photoreactions, respectively. The term $\phi_r \Delta E/N_A h\nu$ (for open reaction centers) is calculated to be about 0.6, using values of $\phi_f = 0.03$, $\nu_f/\nu = 1$, and $\alpha = 0.4$. The value for α was obtained from the expression $(1 - \phi_f)(H/E_0)_D/(H/E_0)_{WL}$, with ϕ_f and $(H/E_0)_{WL}$ taken under closed reaction center conditions (see Fig. 6). In view of the above-mentioned caution considering the enhancement of heat emission by white light, the value $\alpha = 0.4$ should be taken as an upper limit for the radiationless processes, and $\phi_r \Delta E/N_A h\nu \geqslant 0.6$. The values for ϕ_r and $\Delta E/N_A h\nu$ must then both be larger than 0.6 for processes occurring within 2 μs. Values for $\Delta E/N_A h\nu$ have to match those expected on the basis of independent considerations: from the difference between midpoint redox potentials of the components involved in electron transport processes within 2 μs alone, a value of 0.7–0.8 can be estimated. Furthermore, the theoretical maximum for photosynthetic energy conversion efficiency – not limited to the 2 μs time window – is calculated to be about 0.7 [27,28].

Acknowledgement

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