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* Present address: Department of Chemistry, State University College of Forestry, Syracuse University, Syracuse, New York 13210.

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Paramagnetic Unit in Spinach Subchloroplast Particles: Estimation of Size

Abstract. *A pulsed ruby laser (wavelength, 694.3 nanometers) was used to measure the dependence on light intensity of light-induced electron paramagnetic resonance (EPR) signal I for short flashes of uniform duration (400 microseconds). Approximately 10^{18} photons per square centimeter per flash from the unattenuated beam were available to the sample of subchloroplast "system I" particles from spinach. The experimental dependence of the EPR signal height plotted as a function of the total number of incident photons per flash was exponential. From measurement of the slope at a very low relative photon flux and the saturated EPR signal amplitude, the value for the cross section or "effective size" of the light-induced paramagnetic unit, σ_{EPR}, was found to be 300×10^{-17} square centimeter. This result is compared with a measured optical absorption cross section, σ_{694nm}, of 2.5×10^{-17} square centimeter, for the identical sample at the laser wavelength. The hundredfold difference in size supports the thesis that the paramagnetic state is a property of an aggregate of chlorophyll molecules of the same general size as the photosynthetic unit.*

As early as 1932 Emerson and Arnold presented evidence that cooperative action of approximately 2400 chlorophyll molecules was required for the reduction of one molecule of CO_2 or the liberation of one molecule of O_2 (*1*). This idea of cooperative action has given rise to the concept of the "photosynthetic unit" whose effective area, or cross section, presented to incident light of a given wavelength, is consistently larger by at least two orders of magnitude than the effective area presented to light absorption by one chlorophyll molecule alone.

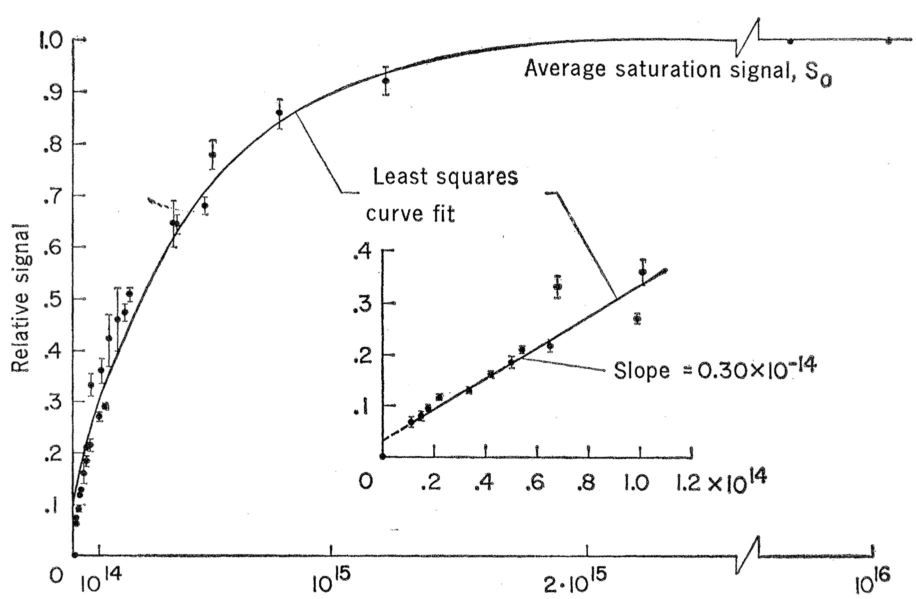

Fig. 1. Height of the induced EPR signal as a function of the number of photons per laser flash. The inset is an expanded portion of the lowest part of the curve. The solid curves are the result of a least-squares fit of all averaged data points including zero, treated with equal weight.

In a photosynthetic system, whether intact algae or chloroplast preparations, a light-induced paramagnetic state can be observed by the technique of electron paramagnetic resonance (EPR) spectroscopy. The precise identity and function of the photoprocess of the observed paramagnetic states have been under investigation (*2*). Evidence is accumulating for the assignment of the two distinctive EPR signals to photosystems I and II in the plant photosynthetic process. One piece of evidence for this assertion is the finding of Vernon *et al.* that particles could be isolated from spinach which represent an efficient photosystem I as it exists in the intact chloroplast with little contaminating inactive chlorophyll (*3*). They present a number of criteria for the activity of photosystem I, among them the facts that the spin intensity of signal I was considerably greater than that for intact chloroplasts, and that P_{700} and signal I exhibited similar behavior under a variety of conditions. These subchloroplast particles proved to be suitable material for a cross-section determination for the following reasons: an aqueous suspension of them in the EPR cuvette was optically thin and nonscattering; induction effects were absent; the rise time of the signal was less than a millisecond, while the paramagnetic intermediates were relatively stable, taking several seconds to decay in the dark. The suspension yielded reproducible results over several hours at room temperature.

A pulsed light experiment analogous to that of Emerson and Arnold can be performed on the paramagnetism in such particles, and from the resulting data a measure of the cross section for the process can be made.

The relation between the EPR signal and the incident number of photons is assumed to be

$$S(n) = S_0[1 - \exp(-\sigma \cdot n)]$$

with

$$\sigma = \frac{\left(\frac{\Delta S}{\Delta n}\right)_{n \to 0}}{S_0}$$

where

$$\left(\frac{\Delta S}{\Delta n}\right)_{n \to 0} = \text{initial slope}$$

n is the number of photons per square centimeter, σ is the cross section in square centimeters, $S(n)$ is the relative

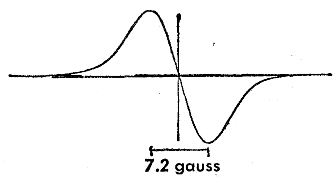

Fig. 2. Steady-state EPR signal resulting from illumination (680 nm, 8×10^4 erg cm^{-2} sec^{-1}) of an aqueous suspension of **the** spinach subchloroplast particles at room temperature. The EPR settings are as follows: time response, 1 second; modulation amplitude, 4.45 gauss; microwave power, 35 mw; scanning rate, 20 gauss min^{-1}.

EPR signal, and S_0 is the saturation value of the EPR signal.

Figure 1 illustrates the observed behavior of the EPR signal as a function of incident photons. The low scatter of the experimental points allowed a reasonable fit to the above expression.

An X-band EPR spectrometer (Varian model V4500) and associated 23-cm magnet were used. The sample was contained in a flat quartz sample cell, 0.036 cm deep. All measurements were made at ambient temperature. In order to establish a consistent value for the saturated EPR signals over one or more decades of incident photon flux, it was necessary to use short and intense flashes of light. For this purpose a pulsed ruby laser (Optics Tech model 130) was used. Figure 1 shows that the saturation of the EPR signal could be determined with good accuracy. Equally pertinent in the determination of σ_{EPR} is the determination of the initial slope of the experimental curve at very low photon flux. This condition placed a constraint on the choice of sample material required to yield a high EPR signal-to-noise ratio (Fig. 2) in dilute solutions with low optical density at 694.3 nm. Particles consisting mainly of photosystem I [freshly extracted from commercially available spinach by the method of Vernon *et al.* (*3*)] met these requirements and those mentioned in the introduction rather well. The particles exhibited complete reversibility even at full laser beam intensity. The decay course of the EPR signal in all cases exhibited first-order kinetics with a measured decay half-time of the order of 10 seconds. A typical response to a moderate photon flux is illustrated in Fig. 3. The signal rise time was instrument-limited but appeared to coincide with the laser beam in onset and duration, when recorded on a dual channel recorder (Clevite-Brush Mark 280) with a chart speed of 200 mm per second.

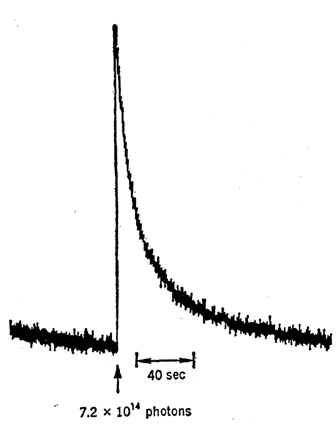

Fig. 3. The EPR signal monitored at maximum low-field deflection, resulting from a single laser pulse of 7.2×10^{14} photons. The EPR settings are: time response, 0.3 msec; modulation amplitude, 11 gauss; power, 35 mw; recorder chart speed, 20 sec cm^{-1}.

An illumination system described by Margozzi *et al.* (*4*) was used to produce the saturated steady-state signal. A measure of the number of spins in this signal (Fig. 2) was obtained by comparison of a first moment determination of the resonance with that of a nitroxide radical (a "spin label") which is a permanent free radical.

The optical cross-section measurements were performed on a spectrophotometer (Cary model 14) within a few hours on the same material in the same cuvette as that used for the EPR experiment. The depth of the cuvette was determined with a known concentration of $CuSO_4$, and chlorophyll was determined by the method of Arnon (*5*). Ratios of chlorophyll a to chlorophyll b ranged from 5.7 to 7.4.

The laser output was calibrated with a TRG (Control Data model 107) calorimeter used in conjunction with a Hewlett-Packard microvoltmeter and a Moseley *X-Y* recorder. The effect of attenuating elements was directly measured. An interference filter with peak transmission at 694 nm (Baird-Atomic, B-1, half-bandwidth 10 nm) was taped directly on the EPR cavity in order to exclude pumping light to a level which induced no measurable effect. The filter plus the slotted cavity grid allowed an incident photon flux on the sample of 8.0×10^{17} photon/cm^2 for each 1.5-joule flash from the laser. In order to further attenuate the laser beam, calibrated neutral density filters (Special Optics) were placed in its path, and in some experiments filters were combined with calibrated $CuSO_4$ solutions in flat bottles.

A total of five separate sets of data were collected from aliquots of particles prepared from two different batches of market spinach. Each experimental point presented in Fig. 1 is an average of several determinations. The solid curves (exponential and linear) (Fig. 1) are the result of a least-squares fit of all the average data points, including the origin. Utilization of the linear approximation to the exponential curve at low values of incident photons yields the following value for σ_{EPR}

$$\sigma_{EPR} = 300 \times 10^{-17}\ cm^2$$

Measurements of an optical density of 0.24 at $\lambda = 694$ nm (for a light path of 0.036 cm) for the same sample in the same cuvette yielded a value for the molecular absorption coefficient of

$$\sigma_{694nm} = 2.5 \times 10^{-17}\ cm^2$$

or a ratio of

$$R = \frac{\sigma_{EPR}}{\sigma_{694nm}} = \frac{300}{2.5} = 120$$

for material made up largely of system I particles in which chlorophyll was the chromophore. This result supports the assumption that an aggregation of chlorophyll molecules is required to produce the observed paramagnetic intermediate.

Determination of spin concentration as a function of chlorophyll indicated that there were between 112 and 116 chlorophylls per spin; thus the quantum efficiency for spin production is unity. Vernon *et al.* report a chlorophyll:P_{700} ratio of 100 (*3*).

These results, when considered in addition to previously reported work (*2*, *3*) make it seem certain that the light-induced paramagnetism observed as signal I is an intrinsic feature of photosynthesis and direct evidence of the earliest chemical act following light absorption.

E. C. WEAVER
Exobiology Division, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, California 94035
H. E. WEAVER
Analytical Instrument Division, Varian Associates, Palo Alto, California 94303

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6. We are indebted to Dr. W. Arnold for suggesting this incisive experiment and for valuable discussion, and we acknowledge the technical assistance of Mrs. N. Willetts.

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