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DIRECT OBSERVATION OF A LIGHT-INDUCED ELECTRIC FIELD IN CHLOROPLASTS

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SUMMARY

1. We report the first direct measurement of an electric field generated by the photoevents in photosynthesis.

2. Illumination of structurally intact and photochemically active spinach chloroplasts with a short non-saturating flash generates a transient dipole field.

3. Photosystem I and Photosystem II contribute equally to the steady-state signals in a sequence of flashes.

4. The rise time of the transient is extremely rapid, reflecting early photochemical events. In the time range studied, the decay time of the field is inversely proportional to the conductance of the suspending medium and independent of photosynthetic ion or electron transport.

5. The polarity of the signal is determined by the intensity gradient of the light field; the electrode nearest the light source becomes negative.

6. With increasing flash intensity, the amplitude of the transient goes through a maximum which implies that the signal results from two oppositely directed polarizations.

7. We conclude that the electric field arises from the paired membranes of the chloroplast which contain the photosystems. The phototraps are arranged so that during the photoacts, charges move perpendicular to the plane of each membrane. The donors of both Photosystems I and II are located on the "interior" of the membrane systems and the acceptors are located "outside". During illumination electrons move to the outside, polarizing the two membranes in opposite direction.

INTRODUCTION

Extensive arguments have been presented for the existence of light-induced electric fields across photosynthetic membranes.

These arguments rest mainly on concepts of energy conservation for phosphorylation such as proposed by Mitchell [1] and the Junge and Witt [2] proposal that

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol.

some light-induced absorption changes (e.g. at 515 nm in green cells) are electrochromic in nature. For instance, Junge and Witt [2] reported a strong effect of ionophoric agents upon the life time of these absorption changes while Jackson and Crofts [3] were able to mimick such changes by using salt gradients rather than light.

This paper describes the first direct observation in isolated chloroplasts of an electric potential generated by both Photosystem I and II. A preliminary report of the data was given earlier [4].

MATERIALS AND METHODS

Chloroplasts were isolated by a previous described method from green house spinach [5]. They were maintained in solutions containing 0.4 M sucrose, 50 mM NaCl and 50 mM sodium tricine buffer at pH 7.3. Before use, aliquots of the stock suspension were diluted in the suspending media to the chlorophyll concentration specified in the text.

Two sample configurations were used for the actual measurements. For the study of the effect of electrode geometry on the electrical signal, two identical Ag/AgCl electrodes (Fig. 1a) were used. Each electrode consisted of a silver wire mounted in a glass disposable pipette filled with an agar gel containing 50 mM KCl. Electrical contact with the suspension was made only at the tip of the pipette. Electrodes were mounted in a manner which allowed the distance (d) between the electrodes and the distance (d') of the electrode pair in the sample to be varied. A 1-ml sample was placed in a cylindrical cuvette which would be illuminated from underneath. The remaining experiments were done with an electrode configuration illustrated in Fig. 1b. In this case, the entire bottom (~ 1 cm^2) of a cylindrical cup (~ 1 ml) acted as one electrode,

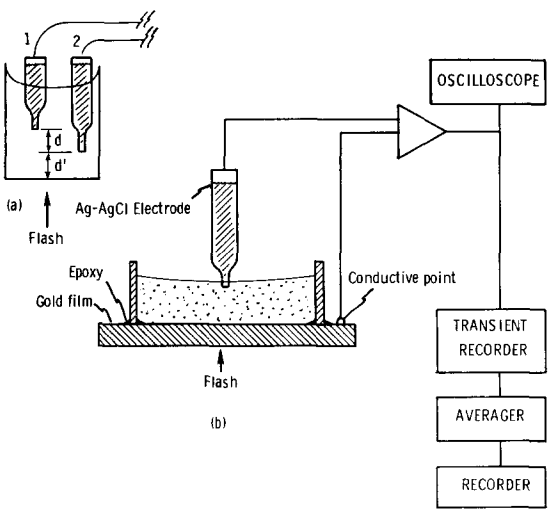

Fig. 1. Diagram of the apparatus for measuring flash-induced electric field changes in spinach chloroplast suspensions. (a) Both electrodes were constrained to move parallel to the light path allowing d' to be varied and d held constant. Electrode 1 can also be moved relative to Electrode 2 allowing the distance d between the electrodes to be varied. (b) Both electrodes were used in a fixed position. See text for the description of the apparatus.

while the other electrode was identical to that described above. The bottom electrode consisted of a gold layer evaporated onto glass, thin enough to allow sufficient light (50 % attenuation) to pass into the sample.

Flashes having a duration of 1–3 μs were obtained from a Xenon flash tube (E.G.G., type FX-101B). Flash energy was controlled by varying the voltage to which the 2 μF discharge capacitor was charged (200–1000 V) or by inserting neutral density screens in the light path. A single lens was used to provide a near parallel light beam which yielded a rather homogeneous light field on the sample.

The flash-induced potential transients were amplified by a d. c. amplifier. The response time in the system was 1–10 μs. Each single transient was recorded by a 200 station Biomation model 610 transient recorder. For small or rapid events, the signal to noise ratio was improved by repetitive observation and averaging. In these instances a number of signals (8–32) were successively transferred from the transien recorder into a signal averager (Biomation model 102). A permanent record was finally obtained using a point plotter (H. P. 7034A). With the fastest instrument time response, flash artifacts contributed considerably to the signal so that corrections were necessary. In these instances, the artifacts had to be measured and subtracted from the signals. The control experiments were made either by placing a shutter in the light beam, which prevented photochemical effects but not the electrical artifacts or by using heat-killed chloroplasts (10 min at 60 °C).

Basic observation and interpretation

When a suspension containing photochemically active spinach chloroplasts is illuminated by a flash under appropriate conditions, a transient electrical potential difference can be measured by electrodes within the suspension. Invariably the electrode whose tip is closest to the light source goes negative. Some examples are shown in Fig. 2.

To facilitate our more detailed description of the phenomena and interpretations in the next sections, we may first present a brief summary:

The occurrence, shape and height of the signal depend upon: (1) the presence of chloroplasts which have some degree of structural integrity and have either or both Photosystems I and II active; (2) a light-intensity gradient across the suspension layer between the electrode tips (d in Fig. 1); (3) the electrical conductance of the suspension medium. The higher the conductance, the more rapid the decay of the field.

The insert of Fig. 2 shows our minimal hypothesis to explain the phenomena. In accord with earlier proposals, [1, 2, 7] we assume (1) that the photosynthetic units of both photosystems are distributed over the surface of some as yet unspecified particle (in Fig. 2 assumed to be spherical); (2) the electron acceptor site(s) of the trapping centers are closer to the surface than the electron donor sites, i.e. in both photoacts the electrons move towards the outside.

Due to the strong pigment absorption in each membrane (a monolayer of chlorophyll absorbs ~2% of a 670 beam [6, 8]) the side of the particle facing the light source receives more excitations than the other side and produces relatively more charge separations, which results in an unbalance of charge. The electrodes observe the field resulting from many such dipoles created by the flash along path d. No net charge differences occur without optical anisotropy, i.e. with both electrodes

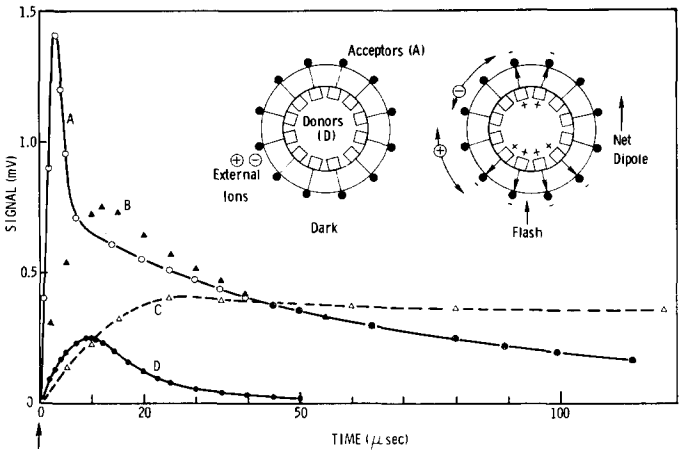

Fig. 2. Typical flash-induced transients obtained for various measuring conditions using Configuration 1b. The chlorophyll concentration was approximately 25 μg/ml and the optical path length between the electrodes was 2 mm. Each curve is the average of 16 flashes given at a rate of 5 per s. Flash artifacts as described in Methods were subtracted from each curve. A, the electrical transient induced by a maximum intensity flash (I_{max}) measured with 1 μs time resolution. Chloroplasts were suspended in a solution containing 0.4 M sucrose and 4 mM NaCl and 100 μM methylviologen. I_{max} is 4–5 times the intensity required to drive photosynthesis to $1-1/e$ of saturation; b, same conditions as Curve A except that a 10 μs time resolution was used; C, The signal obtained with 10 μs time resolution for chloroplasts suspended in 1.4 M sucrose viscosity $\approx$ 25 centipoise, 4 mM NaCl and 100 μM methylviologen; D, same as B except 10 mM NaCl. Insert: Simplified model used to explain the source and various properties of the transient electrical field generated by a flash. The symbols □ and ● represent the primary donor and acceptors respectively for the phototraps. Arrows between the symbols show direction of electron movement in the phototraps generated by the flash.

at the same place in the light field or with a strong flash which excites all trapping centers in light path d in Fig. 1. The charge differences are neutralized by movement of ions in the suspension medium. This neutralization will be more rapid the higher the conductance of the medium, and the smaller the size of the individual dipoles.

Relationship to photosynthesis

The electrical signal is only observed with active chloroplasts under conditions which allow photochemical conversions. This is demonstrated in the experiment shown in Fig. 3, performed with chloroplasts suspended in 0.4 M sucrose and 4 mM salt. The transients shown represent the average of 16 flashes given at a rate of 10 per s and measured with a time constant of 10 μs. Averaging was initiated after the steady state amplitude was attained. This minimized non-random variations in amplitude which occurred in the initial flashes. The curve connecting the open circles is the steady state signal in the presence of 100 μM methyl-viologen. Very similar steady state signals were observed with ferricyanide and other acceptors which sustain electron transport through both photoacts. Addition of 10^{-5} M 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to such systems annihilated the steady state signal (Fig. 3, triangles). Subsequent addition of reduced 2,6-dichlorophenolindophenol (DCIP) and ascorbate to the system restored half the steady state signal as shown in Fig. 3

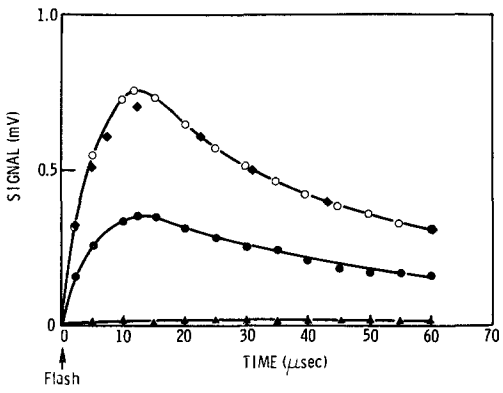

Fig. 3. Electric field transient measured after the addition of artificial acceptors and donors which sustain phototrap activity and DCMU, a Photosystem II inhibitor. All suspensions contained 0.4 M sucrose and 4 mM NaCl in addition to the listed compounds. ○, 100 μM methylviologen; △, 100 μM methylviologen and 10 μM DCMU; ●, 100 μM methylviologen, 1 mM ascorbate, 20 μM DPIP and 10 μM DCMU; ◆, The previous curve multiplied by 2.

(closed circles). The squares show this signal multiplied by 2, indicating that the time course of the two transients is very similar, if not identical.

These data show that both photosystems are contributing equally to the steady state electric field. Additional experiments with various electron donors and acceptors confirmed this conclusion. In a subsequent paper, we will report in more detail on these measurements and, in addition, describe peculiar non-steady state phenomena.

Time course of the transient

Fig. 2a shows a flash-induced electrical transient measured with our shortest response time (~ 1 μs) using chloroplasts suspended in 0.4 M sucrose and 4 mM salt. The recorded potential difference rises rapidly, in a time close to the response of the system. This suggests that the actual rise time of the transient is < 1 μs, reflecting early photochemical events during the photo-trapping process. The decay of the transient shows two phases: (1) a fast-decaying component where observation again seems limited by the time response of the instrument and (2) a slow component; in this experiment its half time is about 50 μs and readily resolvable by the instrument.

Fig. 4 shows an analysis of these three components as a function of flash intensity. The different intensities were obtained with calibrated neutral density screens. The peak height of the signal which occurs a few μs after the flash (closed circles) attains a maximum level at $\sim 30\,\%$ of the full flash intensity (I_{max}). The initial height of the slow component (measured at the intersection of the fast and slow parts of the transient and plotted as triangles in Fig. 4) also became maximal near ~ 25–$30\,\%$ I_{max}. However, this signal declined with further increase of intensity, decreasing to half at I_{max}. On the other hand, the fast decaying part of the signal (squares) in Fig. 4 increased at higher intensities apparently at the expense of the slow component. The model in Fig. 2 (insert) and the following section dealing with light intensity readily explain the three phases of the transient. In the course of the light flash (~ 5 μs) the total number of excited traps increases. During the early part of the flash, more traps are hit in the front layers than in the back layers, creating a progressively larger charge

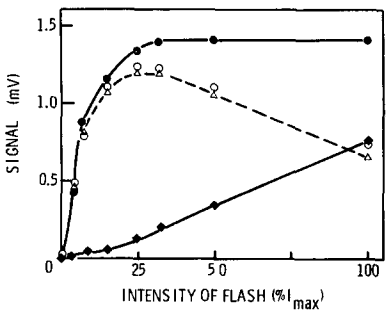

Fig. 4. Dependence of electrical transient upon flash intensity. Chloroplasts were suspended in 0.4 M sucrose and 4 mM NaCl. Chlorophyll concentration was 25 μM. A 2 mm electrode spacing was used. Flash intensity is given as the percent of I_{max}. I_{max} is equal to 4–5 times the intensity needed to drive the flash-induced proton uptake to 1–1/1 of saturation (see text for detail). ●, amplitude of the total electrical transient with 1 μs instrument response; △, amplitude of slow decaying component; ◆, amplitude of spike; ○, amplitude of total electrical signal measured with 10 μs response time.

imbalance (driving up of signal). Subsequently, relatively more traps in the rear layers are excited. Thus, the anisotropy of the charge distribution goes through a maximum leaving at the end of the flash a charge imbalance which is neutralized by ion movement during the slow phase.

The slow phase therefore reflects the net charge asymmetry resulting from the photochemical events during the flash. As shown in Fig. 2b (open circles), this slow phase can be observed with little distortion by using a 10 μs instrument time response. With this time constant the rapid phase and all flash artifacts are greatly attenuated so that corrections are superfluous and measurements are much more convenient. Therefore, whenever the situation allowed, we have used the slower time response for our observations.

Optical parameters which determine the signal

The data plotted in Fig. 5 were obtained with the electrode arrangement of Fig. 1a. One electrode was mounted rigidly, the other could be moved up or down so as to vary distance d. Plotted is the height of the slow phase of the signal induced by two different flash intensities as a function of d. No signal is observed for d = 0, i.e. when the two electrode tips are in the same intensity field. With increasing distance the signal increases to a maximum, the polarity of the signal is such that the electrode in the brighter field goes negative. The time course of the transient (> 10 μs) was unaffected by the geometry.

Fig. 6 shows the effect of flash intensity upon the height of the slow signal component, observed with 3 chloroplast suspension densities and the electrode geometry shown in Fig. 1b. Again the time course of the slow phase of the transient proved invariant. In a manner analogous to Fig. 4, the height of the signal rises and falls with increase of intensity. The three curves have basically the same shape, attaining a maximum amplitude near 0.28 I_{max} and declining to 1/3–1/2 of the maximum amplitude at I_{max}.

Note that the amplitude of the signal is very nearly proportional to the chloroplast concentration. In these experiments the chloroplast concentrations were so low

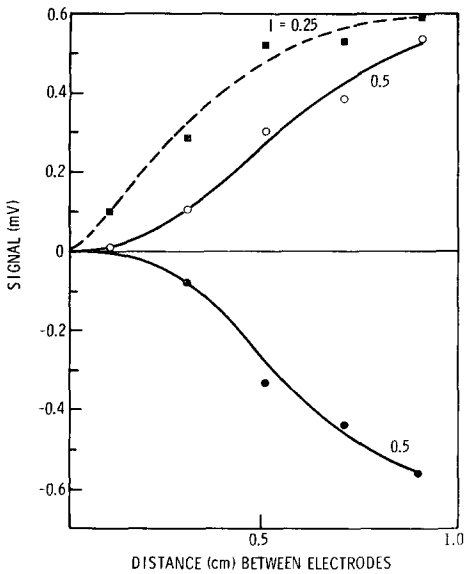

Fig. 5. Dependence of the amplitude of the electrical transient upon eletrode spacing d for two flash intensities. Electrode Configuration 1a was used. Chlorophyll concentration was 25 μM. Chloroplasts were suspended in 0.4 M sucrose and 4 mM NaCl. Polarity was measured relative to the movable electrode. □, intensity 25% I_{max}; ○, intensity 50% I_{max}, movable electrode farther from light source than the fixed electrode; ●, intensity 50% I_{max}, movable electrode placed closer to light source than fixed electrode.

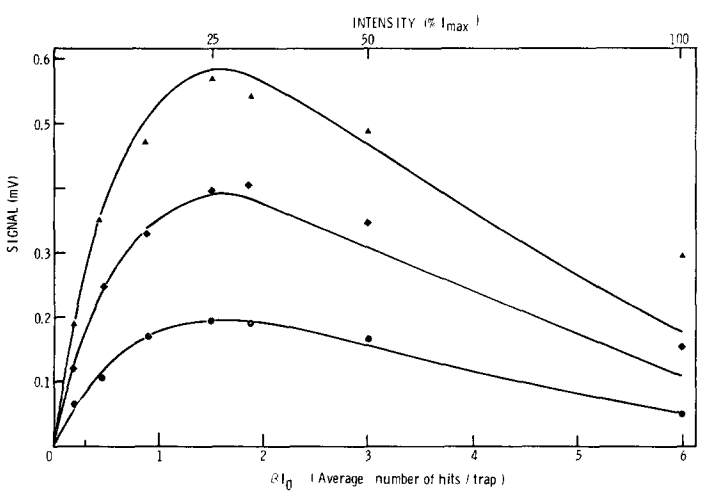

Fig. 6. Dependence of peak amplitude (10 μs resolution) on the intensity of the flash for three different concentrations of chloroplasts. Symbols are experimental data and the drawn lines are calculated curves. See the text for details. ●, 5 μg chlorophyll/ml; ■, 10 μg chlorophyll/ml; ▲, 15 μg chlorophyll/ml.

that on the average, few if any light rays traversed more than one chloroplast. Thus the signal versus intensity curves should be determined by the absorption characteristics of a single chloroplast and the signal amplitude should be directly proportional to the number of chloroplasts in the suspension.

Mechanism of decay: conductance of the medium

One can consider several mechanisms to explain the slow part of decay of the signal after the flash: (1) Spatial reorientation of the particles due to Brownian motion. However, the rotational relaxation time for 1 μm particles (the approximate size of chloroplasts), is in the order of seconds and therefore much too slow. (2) Photosynthetic electron transport reactions in which the electrons and holes are consumed. Also the rates of these reactions appear too slow. (3) The most likely explanation assumes that a redistribution of ions of the suspension medium neutralizes the dipoles. This hypothesis predicts that the relaxation of the dipole field will be influenced by the conductance of the medium, i.e. the concentration of ions and the viscosity. The experiments shown in Fig. 7a and b illustrate that this expectation is actually fulfilled at least over the time range which could be studied with our instrument. The viscosity was controlled by varying the sucrose concentration. A complication encountered in these experiments was that the signal height proved to be inversely proportional to the salt concentration. We have no ready explanation for this, but suspect that salt penetrating inside the chloroplast causes an internal short circuit.

A most important conclusion derived from these and similar experiments is that the decay time of the slow phase of the signal is determined exclusively by the conductance of the medium. When all physical parameters including intensity, conductance, electrode geometry are held constant, the signal height is determined solely by the type and efficiency of the photochemical reactions carried out by the chloroplasts (cf. previous section).

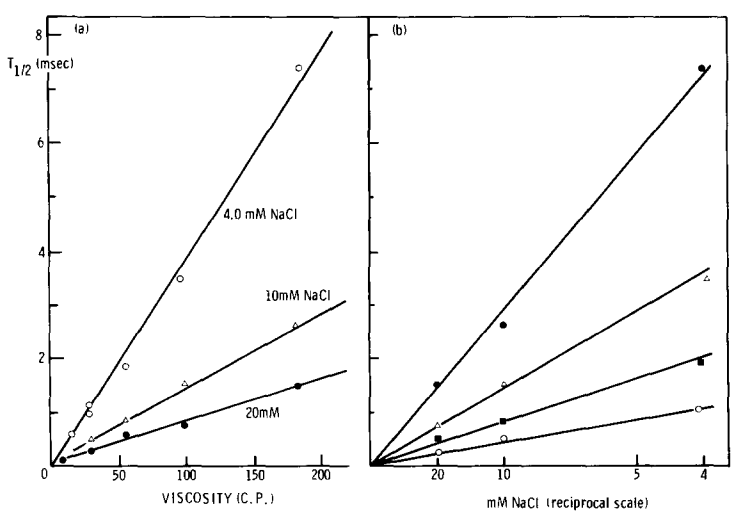

Fig. 7. (a) Dependence of the decay time of the flash-induced electric field transient on viscosity for 3 different salt concentrations: ○, 4 mM NaCl; △, 10 mM NaCl; ●, 20 mM NaCl. (b) Dependence of decay time of the flash-induced electric field transient on concentration of NaCl for 4 different viscosities given in centipoise. ○, 30 C.P.; ■, 60 C.P.; △, 100 C.P.; ●, 200 C.P.

Dependence on chloroplast structure

We have only been able to observe the signal with isolated spinach chloroplasts having a certain degree of structural integrity. Attempts to measure such a field in suspensions of *Chlorella vulgaris* or *Anacystis nidulans* have been unsuccessful. Briefly sonicated spinach chloroplasts gave no signal, regardless of the acceptor used. Microscopic observation of these chloroplasts showed that the original structure was destroyed and only barely visible subchloroplast particles were left. To explain this we suggested that, with small particles, the collapse of the field is extremely fast in comparison to the response time of the instrumentation, or even the duration of a Xenon flash. Another possible explanation may be that membrane pairs are separated so that dipole orientation becomes random (see next section).

Attempting to observe the effect of very low salt and osmotic pressure, we made some observations with chloroplasts which were suspended in distilled water a few minutes before and during the experiments. Microscopic observation showed that most of these chloroplasts were still "intact" but had formed large balloon shapes, consisting probably of a continuous outer membrane and no internal lamellar structure (Arnold, W., personal communication). We observed that rapid shaking of suspensions of these low osmotically treated chloroplasts resulted in a collapsing of the balloons into shapes which were very nearly the same size of normal chloroplasts. These treatments had very little effect upon the amplitude of the flash-induced transients. Compared to control samples suspended in 0.4 M sucrose and 4 mM NaCl, the variation was no more than a factor of two.

Physical significance

It should be stressed that our observations yield unique information about the structural arrangement of the photosynthetic apparatus. We should consider:

1. The chloroplasts are randomly dispersed in the suspension between the electrodes so that the light field is the only directional vector which can underly the polarization.

2. With increasing flash intensity the field first increases and then declines. This implies two oppositely directed polarizations which become equal in strong light.

3. The electrode nearest the light source goes negative.

While the simple dipole model in Fig. 2 adequately explains the data, the paired membrane lamellae of the chloroplast actually accommodate the above criteria quite readily. In accordance with commonly accepted models for the lamellar system [2, 7] we assume that:

a. The photoacts of Systems I and II are arranged perpendicular to the plane of the unit membrane.

b. In a double lamella the traps of the one membrane are directed oppositely to those in the other.

c. The primary electron donors of the photosystems are inside the membrane pair and the electron acceptors are on the outside [1, 2, 7]. This model is illustrated in Fig. 8.

For a more detailed analysis, we assume that the observed electric field is a resultant of the potential differences across R-paired membranes in one chloroplast. We assume that the layers are traversed perpendicularly by the light (random orientation modifies the answers by a constant factor). If the light is attenuated from I_0 to I

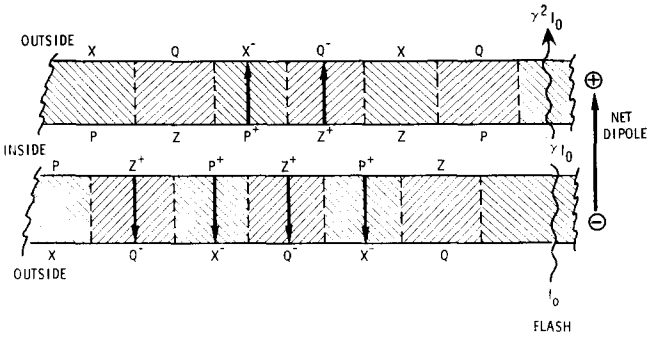

Fig. 8. Proposed model showing how the electric field measured in a chloroplast suspension could be generated in the double membrane of chloroplasts. P and Z are the primary donors and X and Q are the primary acceptors of Photosystem I and Photosystem II, respectively. Arrows between P and X and Z and Q denote direction of electron movement during phototrapping.

by traversing R-double membranes, the unit transmission of each single membrane is $\gamma = \sqrt[2R]{I/I_0}$. Suppose there are N trapping centers per unit area of the membrane layer, then the dipole field in the first layer will be proportional to the number of traps excited by the flash: $E_1 = k\mathrm{N}(1-\mathrm{e}^{-\gamma\beta I_0})$. β is equal to the cross section for absorption times the quantum yield.
Thus the resulting field across the first bilayer is

$$E_{R_1} = E_1 + E_2 = k\mathrm{N}(\mathrm{e}^{-\gamma\beta I_0} - \mathrm{e}^{-\beta I_0})$$

The net electric field established over R-double layers will be

$$E_{R_T} = k\mathrm{N} \sum_{n=0}^{n=R-1} (\mathrm{e}^{-I_0\gamma^{2n+1}} - \mathrm{e}^{\beta I_0\gamma^{2n}}) \qquad (1)$$

A single membrane typically has an absorbance near 0.01 and there are 30–60 single membranes in a chloroplast [6, 8]. Using Eqn 1 we calculated potential versus intensity curves (βI_0), for a system with 15–30 paired membranes, (one layer of chloroplasts), in the light path. We chose one of these curves (R = 25) and normalized it in terms of peak position and amplitude with the experimental data of Fig. 6a (full line). Curves b and c were obtained by multiplying Curve a by two and three respectively. In independent experiments we used the same geometrical arrangement to measure flash-induced changes of pH which reflect electron transport (unpublished observations). Here we determined that (1 − 1/e) of saturation was reached with a flash intensity between 20–25 % of I_{max}. Therefore, in the experiment shown in Fig. 6 the electrical signal attains a maximum close to the predicted intensity. Additionally, there is reasonable agreement between the overall shape of the experimental and the predicted curves.

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REFERENCES

1 Mitchell, P. (1966) Biol. Rev. 41, 445–502
2 Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 244–254
3 Jackson, J. B. and Crofts, A. R. (1969) FEBS Lett. 4, 185–189
4 Fowler, C. F. and Kok, B. (1972) in Abstr. 6th Int. Congr. Photobiol., Bochum, No. 417
5 Schwartz, M. (1966) Biochim. Biophys. Acta 112, 204–212
6 Izawa, S. and Good, N. E. (1966) Plant Physiol. 41,544
7 Menke, W. (1966) in Biochemistry of Chloroplasts (Goodwin, T. W., ed.), Vol. I, pp. 1–18, Academic Press, New York
8 Olson, R. A. and Engel, E. K. (1958) in The Photochemical Apparatus, Its Structure and Function, Brookhaven Symp., No. 11, 303–309