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EXTREMELY FAST PHOTOELECTRIC SIGNALS FROM SUSPENSIONS OF BROKEN CHLOROPLASTS AND OF ISOLATED CHROMATOPHORES

H.-W. TRISSL, U. KUNZE and W. JUNGE

Schwerpunkt Biophysik, Universität Osnabrück, Albrechtstr. 28, D-4500 Osnabrück (F.R.G.)

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This paper concerns transient photoelectric signals that are observed when a suspension of photosynthetic vesicles is illuminated with flashing light of nonsaturating energy using a pair of electrodes positioned at different depths along the path of the exciting light. With chloroplasts we observed two different types of signals. One rose extremely fast, the rise time (10–90%) was less than 200 ps under excitation with a single pulse from a mode-locked ruby laser, while the other rose more slowly (typically 10 μs). These signals displayed several different properties such as their polarity, kinetics, apparent source impedance, and sensitivity to structural integrity of the chloroplast lamellar system. Experimentally, signal 'Fast' could only be induced by very short light pulses (shorter than approx. 60 ns), whereas signal 'Slow' appeared only under longer excitation. The detection of signal Fast required special instrumentation, particularly fast preamplifiers with low input capacitance. Our results support the more recent idea that signal Slow did not reflect the primary transmembrane charge separation, as postulated in the earlier literature, but rather lateral movement of charge carriers along the intact lamellar system of chloroplasts. On the other hand, signal Fast may reflect the primary outwardly directed electron transfer across the thylakoid membrane. Its polarity, however, was opposite to that previously postulated to appear in response to this event. For comparison we also studied photoelectric effects in a suspension of structurally more homogeneous chromatophores from *Rhodopseudomonas sphaeroides*. These vesicles displayed only a signal of the same polarity and similar kinetics as signal Fast from chloroplasts. When the secondary quinone electron acceptor (Q) was chemically reduced, the decay time was shortened from approx. 30 ns to approx. 10 ns. The acceleration to approx. 10 ns is known for the rapid, supposedly transmembrane back-reaction of the primary charge separation. Therefore, we conclude that the electrodes monitor the primary charge separation. There is still the unresolved problem with the polarity of the electric signal which we attribute to an as yet unidentified property of the pick-up system. Because its signal-to-noise ratio under extremely high time resolution is superior to that obtained in flash spectroscopy, signal Fast represents a very good means to measure the spatial separation between the very primary electron carriers in the photosynthetic reaction center.

Introduction

With a pair of electrodes placed in the propagation direction of an exciting flash of light, Fowler and Kok [1,2], as well as Witt and Zicker [3], observed photovoltage signals from chloroplast suspensions in the milli- and microsecond range.

The authors agreed on the following interpretation of these signals: When excited by a flash of nonsaturating energy, thylakoid vesicles become asymmetrically charged along the direction of the

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; PS, photosystem.

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incident light beam because those membrane segments that face the light source shade the following segments. If two measuring electrodes were positioned at different depths along the propagation axis of the exciting light, a transient photovoltage resulted. If this photovoltage arose from a transmembrane charge separation it was expected that the polarity of the photovoltage coincided with the polarity of the charge separation in that membrane segment that faced the light source.

In the above-cited work, it was observed that the electrode closer to the light source became negative, and both photosystems contributed to almost the same extent to the signal. This seemingly agreed with the fact that electrons are outwardly driven in the primary charge separation in both photosystems of green plants as evident from spectrophotometric and biochemical investigations [4–9].

Recently, however, Becker et al [10]. have questioned this interpretation. These authors measured the amplitude of the photovoltage under excitation with polarized light in magnetically oriented chloroplasts. When the lamellae of the thylakoids were oriented parallel to the light path the photovoltage increased and did not decrease as expected according to the earlier interpretation. These authors therefore proposed that the photovoltage did not reflect the transmembrane charge separation but it resulted from the lateral motion of positive and negative charges of different mobility. If this occurred as a consequence of some photochemical process, which created an inhomogeneous charge distribution, it is analogous to the Dember effect, known from solid-state physics [11,12].

Hence, without additional information measurements of photovoltage with macroscopic electrodes can neither prove a transmembrane charge separation nor assign its direction.

The first report on another, faster photovoltage came from Gräber and Trissl [13]. With laser flashes (10 ns) these authors observed a photovoltage that had a polarity opposite to that previously observed with flash lamps. This photovoltage displayed an instrumentally limited rise time of about 10 ns.

This communication deals with photovoltage signals obtained with suspensions of spinach chloroplasts, of whole cells of the photosynthetic bacterium *Rhodopseudomonas sphaeroides*, and of isolated chromatophores from the same bacterium.

Different types of photoelectric signals can be distinguished: Chloroplasts exhibit (i) a negative voltage (S for slow), characterized by 10 μs rise time and evokable only by excitation with discharge lamps, and (ii) a positive voltage (F for fast), characterized by a rise time of less than 200 ps and evokable only by excitation with Q-switched lasers. (iii) Whole cells of *Rps. sphaeroides* and their chromatophores deliver only a signal with a fast rise time and with a polarity independent of the duration of the exciting flash.

Using chromatophores we were able to observe by electrical measurements the fast back-reaction (approx. 10 ns) that follows the primary transmembrane charge separation in the reaction center when the secondary e^- acceptor, Q-Fe, is chemically reduced [14]. From this we conclude that the photovoltage signals with extremely fast rise times reflect the primary photosynthetic transmembrane charge separation. With sonicated chloroplasts we observed an instrumentally limited rise time of less than 200 ps.

Materials and Methods

The essential parts of the experimental set-up are shown in Fig. 1. The cylindrical measuring

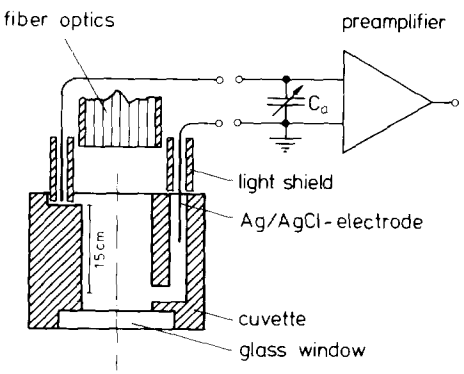

Fig. 1. Schematic view of the experimental setup. Under the standard experimental conditions the flashes were delivered from the top and the bottom electrode was grounded. Thus, polarities of the photovoltages given in this communication refer to the top electrode and illumination from above. To ensure comparable and homogeneous illumination of the reaction volume for the different light sources, all flashes were sent to the measuring cell through scrambler fiber optics.

cuvette was made of black-stained polychlorotrifluoroethylene (Kel F) in order to minimize possible artifacts due to light reflections and stray light. The inner reaction volume had a diameter of 13 mm, a depth of 20 mm, and a sidewall thickness of 8 mm. The bottom was closed with a glass plate to allow for illumination from below as well. A few millimeters above the bottom was a small duct leading through the middle of the wall to the top of the cuvette. From there an Ag/AgCl electrode connected the bottom part of the cuvette via the duct to ground. At the upper rim of the cuvette there was a small groove in the sidewall which contained the measuring Ag/AgCl electrode. In this way a free light path through the cuvette was provided and light prevented from hitting the electrodes. Both electrodes were soldered over the shortest distance possible (approx. 1,5 cm) to a plug that was mounted in the wall of the surrounding Faraday cage.

The electrical and optical shielding was such that electrical artifacts remained well below 10 μV as repeatedly tested by the addition of the detergent Triton X-100 to the suspension under investigation after an experiment. The same was found when the cuvette was filled with milk providing approximately the same light scattering at the ruby laser wavelength as the chloroplast suspension.

Measurements in the frequency domain up to 100 MHz were carried out with the following components: the signals were fed into voltage preamplifiers of very high impedance that were connected directly to the plug of the measuring cuvette. Up to 15 MHz an electrometer amplifier with an input capacitance of 3 pF was used (M & S Elektronik, model EMV 80). The frequency range between 500 and 10 MHz was covered by an impedance converter with an input capacitance of 3 pF, built by Norbert Spreckelmeyer in our electronic shop. Further amplification occurred with cascaded ultrawide-band amplifiers (B & H Electronics, model DC-3002LN, frequency range: 3 GHz to d.c.). The signals were provisionally stored in a 100 MHz transient recorder (Biomation, model 6500) and then transferred to a signal averaging and processing system (Tracor Northern, model TN 1500).

Measurements in the domain of 1 GHz were carried out by feeding the signals directly into an amplifier of 50 Ω input impedance (DC-3002LN). Two of these amplifiers were cascaded to yield an amplification of 120. The amplified signal was displayed as a single sweep on the screen of a 1 GHz oscilloscope (Tektronix, model 7104). From there it was photographed and digitized by a vidicon camera (Thomson CSF, model TSN 1150) and subsequently transferred to a computer (Digital International Ltd., PDP 11/34).

The measuring cell, filled with electrolyte, together with the electrodes and the plug had a stray capacitance of 11.5 ± 0.5 pF. This capacitance plus the input capacitance of the first amplifier are denoted external capacitance, C_a. In those experiments which were designed to obtain information on the source impedance of the signals, the external capacitance was increased by soldering additional capacitors of known values in parallel with the capacitance of the cuvette and the amplifier.

The biological samples were excited with flash light from one of the following sources: (a) commercial 60 μs photographic flash (Braun, 410 VC), (b) Q-switched and frequency doubled Ne-Yag laser pulse (wavelength: 530 nm) of 10 ns duration, (c) Q-switched ruby laser pulse (wavelength: 694 nm) of 60 ns duration, (d) Q-switched ruby laser pulse reduced to 3 ns by a pulse-clipping system, (e) a single pulse from a mode-locked ruby laser of approx. 30 ps duration. The laser systems were obtained from J.K. Lasers.

Spinach chloroplasts were prepared according to the method of Reeves and Hall [15] with slight modifications described in Ref. 16, and stored in liquid nitrogen until use. The chlorophyll concentration in the storage suspension was 2.6 mM. The storage mediun contained 400 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 4 g/l bovine serum albumin, 50 mM Hepes, pH 7.5, and 5% dimethyl sulfoxide (DMSO). In most cases this chloroplast suspension was diluted 100-fold into the measuring medium. By this procedure a buffer concentration of 0.5 mM and an ionic strength of 0.5 mM was introduced from the stock suspension. Some of the experiments were performed with chloroplasts which had been sonicated with a sonifier (Branson, model B 15; with microtips) for 10 s at a duty cycle of 50%.

Inside-out thylakoid vesicles were prepared from spinach chloroplasts by Yeda press disintegration

following the procedure of Anderson and Åkerlund [17].

Rps. sphaeroides, strain GA, was grown anaerobically under saturating light conditions in the medium described by Ormerod et al. [18]. Chromatophores were prepared from the cells harvested in the late exponential growth phase. The cells were centrifuged and resuspended in a medium containing 50 mM Tris-HCl buffer, pH 7.4, and 5 mM $MgCl_2$, and were then fractionated by means of a Sorvall Ribi Press Fractionator (model RF-1) at 17000 lb/inch^2. Thereafter, the preparation was centrifuged at $190000 \times g$ for 90 min, the supernatant was discarded, and the pellet was resuspended in a medium containing 50 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$ and 60% glycerol. In this medium the chromatophores were stored at −22°C until use.

Results

Experiments with spinach chloroplasts

Two different types of photoelectric signals. With chloroplasts suspended in a medium of low ionic conductivity, transient photovoltages can be measured under excitation with a light flash from either a discharge lamp or a Q-switched laser. The most conspicious observation is the opposite polarity of signals which are evoked by these two kinds of excitation sources (see Fig. 2a). As we used the same wavelength and the same energy for both kinds of flashes the only difference was the duration of the excitation pulse. A positive photovoltage appeared by delivering a given number of quanta within nanoseconds and a negative photovoltage appeared by delivering the same number within microseconds. In the following, the faster, positive photovoltage will be labeled F and the slower, negative one S. The attribution of positive or negative refers to a measuring electrode placed near the entry of the exciting light into the cuvette (Fig. 1).

Signal F and signal S, as well as all other photovoltages described in this communication, had the following common properties: (i) They reversed their polarity but not their shape, when the flashes were delivered from the bottom instead of from the top of the cuvette. This established the necessity of an excitation gradient. (ii) When detergent (1% Triton X-100) was added to the suspension both signals were suppressed (Fig. 2b). This demonstrated the necessity of vesicular struc-

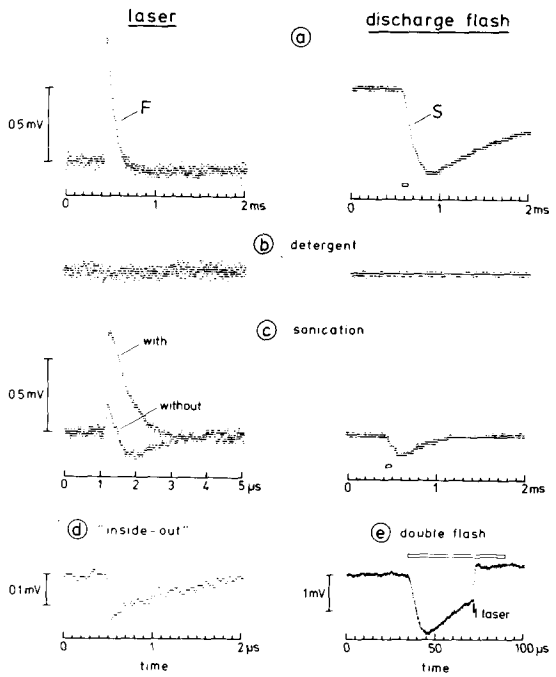

Fig. 2. Original recordings of the transient photovoltage from spinach chloroplasts under different experimental conditions. Chloroplasts were suspended in 2 M sucrose to give a final chlorophyll concentration of 26 µM. The absorbance at a wavelength of 694 nm was about 0.25. All traces represent the average of four flashes given every 10 s. The amplitudes are scaled in mV per flash. (a) Excitation by a ruby laser flash ($\lambda = 694$ nm) at an energy of 30 µJ/cm^2 and a duration of 60 ns full width at half maximum (left trace) and by a discharge flash through an interference filter ($\lambda = 689$ nm, $\Delta\lambda = 7$ nm) at an energy of 85 µJ/cm^2 and a duration of 60 µs (right trace). The positive signal evoked by the laser flash is labeled F and the negative signal S. (b) As in a but after addition of detergent Triton X-100 (final concentration 1%) to the suspension. Energy of the ruby laser flash 0.3 mJ/cm^2. (c) The effect of sonication. The traces on the left side were made with chloroplasts diluted 100-fold with 10 mM KCl. In this medium sonication was carried out. The trace on the right side was made by diluting the stock solution of chloroplasts 50-fold with distilled water, sonicating the suspension, and then diluting it twice with 2 M sucrose. Excitation conditions as given in a. (d) Inside-out vesicles prepared from chloroplasts suspended in 1 M sucrose. Excitation conditions: ruby laser, 0.3 mJ/cm^2; 3 ns. (e) Double flashes to chloroplasts suspended in 300 mM sucrose, 1 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM benzyl viologen. Excitation by discharge flash (from top) at $\lambda = 430$ nm, $\Delta\lambda = 40$ nm, energy 1.5 mJ/cm^2 and ruby laser flash (from bottom) at an energy of 1 mJ/cm^2.

tures. (iii) The decay rate was proportional to the overall electric conductivity of the suspending medium. This showed that the decay resulted from a relaxation of an inhomogeneous charge distribution via ionic currents through the medium (as contrasted by 'through the membrane').

(iv) Another common property of signals F and S was their dependence of the peak amplitudes on the flash energy. In Fig. 3 the amplitudes of the two signals are plotted double-logarithmically versus the absolute flash energy. Within the measured range both signals were in parallel. At low energies the curves were approximately linear with a slope of 0.8. This result may indicate that there is a considerable distribution width of the apparent photosynthetic unit size in chloroplasts. Maximal photovoltages were reached between 10^{14} and 10^{15} quanta/cm^2. Laser flashes of higher energies than those included in Fig. 3 evoked smaller amplitudes (for instance: $3 \cdot 10^{16}$ quanta/cm^2 evoked 0.1 mV/flash). Higher energies of the discharge flash caused kinetic problems, which shall be considered later (Fig. 5). The saturation at relative low energies proved that both types of signals involved the absorption of light by the antennae system.

On the other hand, signals F and S differed in

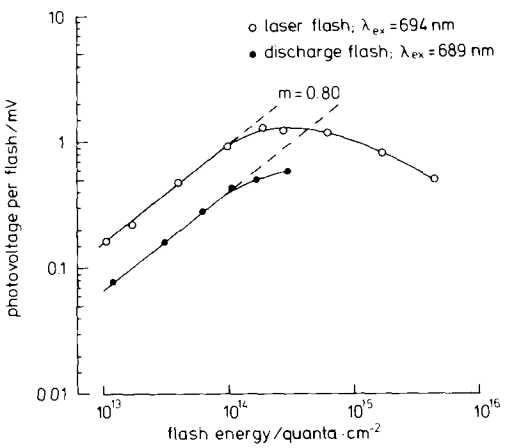

Fig. 3. Dependence of signals F and S on the excitation energy plotted in double-logarithmic scales. The stock solution of chloroplasts was diluted 100-fold in 2 M sucrose. Signal F was evoked by a pulse from a ruby laser ($\lambda_{ex} = 694$ nm; duration 60 ns) and signal S by a flash from a discharge lamp ($\lambda_{ex} = 689$ nm; duration 60 μs.). The abscissa is scaled in number of quanta per 1 cm^2 as measured in the middle of the measuring cell.

their dependence on the structural integrity of chloroplasts. To examine this, chloroplasts were comminuted by sonication or, alternatively, they were osmotically swollen by suspending them into distilled water. Sonication resulted in an enhancement of signal F (note the different suspension medium in Fig. 2a and c), and in a diminution of signal S (Fig. 2c). The enhancement of signal F upon sonication can be understood in terms of a better electrical access of the measuring electrodes to the thylakoid vesicles. The diminution of signal S upon sonication could be partially explained by an acceleration of the decay: the inhomogeneous charge distribution relaxed more rapidly around a smaller vesicle. When the suspension medium of the chloroplasts did not contain a high concentration of sucrose, sonication caused the complete disappearance of signal S (data not shown). In suspensions of osmotically swollen chloroplasts, blebs, signal F as well as signal S were principally observable. However, amplitudes and kinetics were not stable in time and were therefore not investigated further.

Some further experimental results are worth mentioning: (i) The amplitude and polarity of signal F did not change under variation of the pulse duration of the ruby laser (wavelength, 694 nm) from 60 ns to 3 ns or 30 ps, as well as under excitation with the frequency doubled Nd-Yag laser (wavelength, 530 nm) at a pulse duration of 10 ns. (ii) The negative photovoltage S appeared independently of the excitation wavelength of the discharge flash (chlorophyll concentration, 13 μM). (iii) Up to 10-fold dilution of chloroplasts yielded smaller amplitudes of signal F and S, but neither effected their kinetics nor their polarity. Higher concentrations of chloroplasts (chlorophyll concentration > 50 μM) did not significantly effect signal F, but caused signal S to become bipolar with a reversed polarity at short times. This effect depended on the wavelength of the exciting discharge flash (cf. Ref. 13). (iv) Signal F could be observed under repetitive excitation with constant amplitude up to a repitition rate of 1 Hz. (v) Signal F was almost insentitive to DCMU.

Control experiments, in which the two Ag/AgCl electrodes were connected with the chloroplast suspension via salt bridges, showed the same photovoltages as before.

Dependence of the photovoltage on the external capacitance . It was found that the amplitude of signal F depended on the type of preamplifier used. Amplifiers with a small input capacitance yielded larger signals than amplifiers with a large input capacitance. However, signal F was only slightly dependent on the input resistance of the preamplifier. In the experiments designed for extremely high time resolution, where the conductivity of the suspension medium was very high, signal F could be measured even with an amplifier of 50 Ω input impedance (Fig. 5b). Signal S was found to be rather insensitive to the input characteristics of the preamplifiers used.

A systematic examination of the influence of the input capacitance of the amplifier on the photovoltage was carried out with a preamplifier of the highest input impedance available: an electrometer amplifier with an input capacitance of 3 pF and an input resistance of greater than 10^{14} Ω (see Materials and Methods). Using this amplifier the external capacitance was increased by adding capacitors of known value parallel to the input. Without additions the total external capacitance was 14.5 pF (smallest value in Fig. 4). This comparatively high value resulted mainly from the stray capacitance between the measuring cell and the surrounding Faraday cage.

It was found that in all cases examined, the experimental data fell on straight lines when the reciprocal peak amplitudes were plotted against the external capacitance, C_a (Fig. 4). As seen in Fig. 4, signal S from chloroplasts showed almost no dependence on C_a whereas signal F decreased about 4-fold when C_a was increased from 14.5 to 70 pF. The extrapolation of the line connecting the data for signal F to ideal measuring conditions, $C_a \rightarrow 0$, points to the origin of the ordinate. This suggests that much larger photovoltages would be measured with a smaller stray capacitance of the setup. This also indicated a small apparent source impedance of signal F.

Rise times. The only factor determining which of the two signals, F or S, was elicited proved to be the time interval in which a given number of quanta (same wavelength) was offered to the chloroplasts. This raises the question as to the critical excitation interval where signal S disappears and signal F appears.

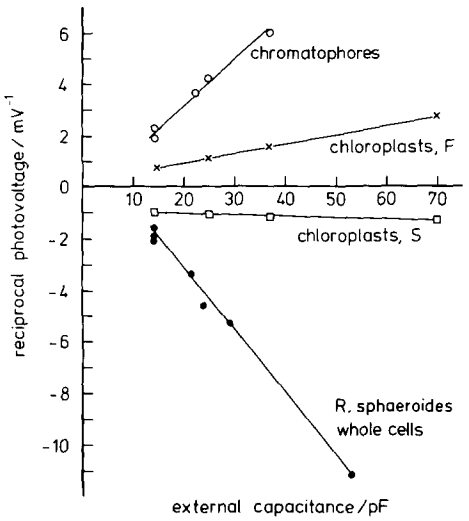

Fig. 4. Plots of the reciprocal photovoltage versus the external capacitance. Spinach chloroplasts were diluted 200-fold in 300 mM sucrose, 1 mM KCl, 10^{-4} M $MgCl_2$, 10^{-4} M benzyl viologen. Excitation conditions: signal F: $\lambda = 694$ nm, $\Delta t = 3$ ns; signal S: $\lambda = 430 \pm 30$ nm, $\Delta t = 60$ μs. Cells of *Rps. sphaeroides* were suspended in 4-fold diluted growth medium, as given in Ref. 17. The cell concentration was $1.5 \cdot 10^6$ cells/ml. Excitation conditions: $\lambda = 694$ nm, $\Delta t = 3$ ns. Chromatophores of *Rps. sphaeroides* were suspended in 6 mM Tris-HCl buffer, pH 7.4, 0.6 mM $MgCl_2$ to give a final BChl concentration of 160 μM. Excitation conditions: $\lambda = 694$ nm, $\Delta t = 3$ ns.

To follow up this question, the rising phase of signal S was examined during excitation by a discharge lamp at different flash energies, thus varying the initial quantum fluxes. At nonsaturating flash energy (curve 1 in Fig. 5a) the photovoltage developed slowly (slew rate, 14 V/s) during the time course of the flash (documented in the bottom part of Fig. 5a). When the total flash energy was saturating (curve 2) the photovoltage developed faster (slew rate, 88 V/s) within the earlier parts of the flash. A maximum was reached and then the photovoltage decreased when further input of quanta saturated the sample (cf. also Fig. 2e). A further increase in the flash energy (curve 3) did not further accelerate the development of the photovoltage (same slew rate, 88 V/s) but saturation was reached earlier and the maximal amplitude decreased.

These experiments show that a limiting slope of the rising phase of signal S exists which cannot be passed over by increasing quantum fluxes. It was

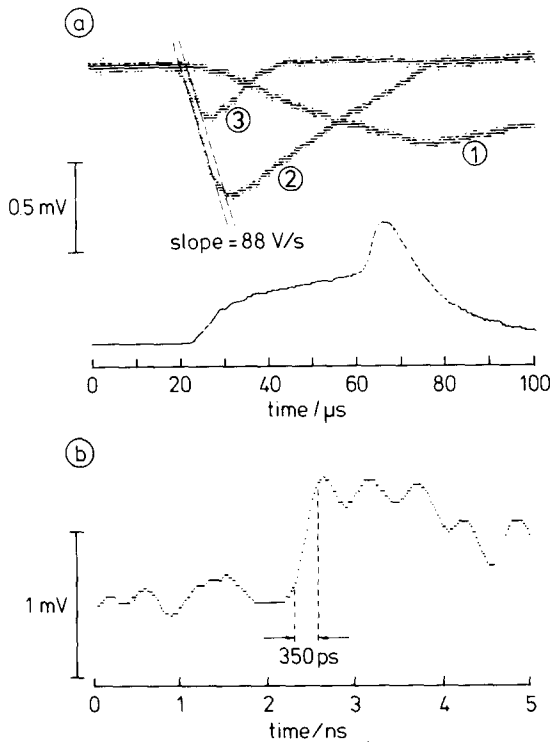

Fig. 5. Rising phases of the two photovoltage signals F and S. (a) The superimposed rising phases of signal S are traced during the discharge time of a photographic flash at three different energies (upper traces). The time course of the light intensity is shown in the trace below. Curve 1 results from a flash energy of 50 $\mu J/cm^2$ using a narrow-band interference filter centered at 682 nm. Curve 2 results from a flash energy of 1.5 mJ/cm^2 using a wide-band interference filter centered at 425 nm, and curve 3 results from a flash energy of about 12 mJ/cm^2 white light. The stock solution of chloroplasts was diluted 100-fold into 1 M sucrose. (b) The rising phase of signal F. The chloroplasts were diluted 100-fold into 3 M KCl and sonicated. The photovoltage was preamplified with a 3 GHz–50 Ω amplifier and displayed on a 1 GHz oscilloscope. A single sweep on the oscilloscope screen triggered by a single pulse (approx. 200 $\mu J/cm^2$) of a mode-locked ruby laser ($\lambda_{ex} = 694$ nm; $\Delta t \approx 30$ ps) was digitized as described in Materials and Methods.

further observed that the limiting slope depended on ionic strength, osmolarity, and mild sonication. A variation of the temperature between 10 and 30°C (all other experimental parameters remaining constant) had no measurable effect on the limiting slope. This suggests that the slope is limited by electrical factors rather than by electrochemical ones which are inherent to the biological charge separation.

The mere existence of signal F under experimental conditions where the limiting slope of signal S is observed excludes an electrical bandwidth limitation of the measuring system (Figs. 2a and 6). This is further verified in the experiment shown in Fig. 2e, where a discharge flash was sent from the top and a saturating laser flash from the bottom during the existence of signal S. As seen in this figure signal S is eliminated more rapidly than it has arisen. (When only the laser flash was applied signal F was negative, as expected from the opposite direction of light incidence.)

The rise time of signal F was also dependent on the structural integrity of chloroplasts. Broken chloroplasts displayed rise times of 10–100 ns, varying slightly with the electrolyte concentration. The rise times became faster after sonication. In sonicated chloroplasts the rise times decreased considerably with increasing salt concentration and stronger sonication. It appeared that under certain experimental conditions the rise time would be limited by an $R \cdot C$ time which results from the resistance of the suspension medium and the external capacitance.

The fastest rise time obtainable with our present instrumentation is shown in Fig. 5b. For this experiment chloroplasts were sonicated in 3 M KCl. The preamplifier had 50 Ω input impedance and an upper limiting frequency of 3 GHz. The slowest electronic component was the 1 GHz oscilloscope. In response to a 70 ps step voltage from a rise time tester (Tektronix, model 284), amplifiers and oscilloscopes showed a smooth rise with a 10–90% rise time of 350 ps. The excitation source was a mode-locked ruby laser from which we selected one single pulse of about 30 ps duration with an energy of approx. 200 µJ.

As seen in Fig. 5b the photovoltage develops within 350 ps (10–90%) and is therefore instrumentally limited. The error of this number is estimated to be $\epsilon = \pm 20$ ps. Taking into account the instrumental limitation, the true 10–90% rise time of signal F, τ_F, must be shorter than about 200 ps according to standard Laplace filter analysis [19,20].

$$\tau_F = \sqrt{(\tau_m + \epsilon)^2 - (\tau_m - \epsilon)^2}$$

$$= \sqrt{370^2 - 330^2}\ \text{ps} \lesssim 200\ \text{ps} \qquad (1)$$

When retreating to true half rise times (instead of 10–90% rise times) the calculated half rise time of the source voltage is 100 ps.

Hall effect? If lateral movement of charge carriers were responsible for the photovoltage signals by laser flashes, then one would expect a kind of Hall effect to be observed [38], i.e., a photovoltage measured with a pair of electrodes positioned perpendicular to the light direction and perpendicular to a magnetic field. We looked for this effect by exposing chloroplasts to a magnetic field induction of 7.5 kG. It was found that there was no Hall photovoltage exceeding the experimental resolution of 50 μV. This result also points to a transmembrane charge separation as the origin of the fast photovoltage. Mobile charge carriers are seemingly not involved!

'Inside-out' thylakoid vesicles. Using the discharge flash we were unsuccessful in finding a suspension medium that would allow the observation of a photovoltage from inside-out thylakoid vesicles. Excitation with the ruby laser, however, evoked a negative photovoltage in the submicrosecond range (Fig. 2d). In agreement with the expectation from the inverted sidedness, the polarity of this photovoltage was opposite to that from chloroplasts (laser excitation). In view of the classical understanding of the light gradient effect, however, the polarity is opposite to that expected (see Discussion).

Experiments with Rhodopseudomonas spaeroides

Only one type of photovoltage. Photosynthetic bacteria represent a less complicated photosynthetic system as compared to chloroplasts, since they contain only one photosystem instead of two and the photosynthetic membranes, at least in isolated chromatophores, have a simple vesicular structure instead of the complicated stacked and interconnected grana and stroma lamellae [21]. The following experiments were done with whole cells of *Rps. sphaeroides*, strain GA, and with isolated chromatophores from the same species.

With a suspending medium of low ionic conductivity for whole cells of *Rps. sphaeroides*, a negative photovoltage could be measured either by laser flash excitation or by discharge flash excitation (Fig. 6a). However, the amplitude of the photovoltage evoked by the laser flash was much larger than that evoked by the discharge flash. An inspection of the signal evoked by the discharge flash revealed that its time course closely followed that of the flash lamp (cf. Fig. 5a). This indicated that the photovoltage did not fully increase, because the decay kinetics of the photovoltage are significantly faster than the flash duration, as seen in the left part of Fig. 6a.

Compared to whole cells of photosynthetic bacteria, chromatophores are inside-out vesicles [21] (see Fig. 8). Consequently, one would expect opposite polarities of the photovoltages from both structures, if the photovoltage originated from a transmembrane charge separation.

In agreement with this expectation, chromatophores from *Rps. sphaeroides* displayed a positive photovoltage as seen in Fig. 6b. The polarity did not depend on the excitation source. Again, the photovoltage evoked by the laser flash was much larger than that evoked by the discharge lamp. This can be explained by the argument given before. Due to the smaller size of chromatophores, the delocalization of the charge asymmetry by ionic fluxes occurs faster (approx. 1 μs) than in whole cells (approx. 10 μs). Accordingly, the amplitude of the photovoltage is reduced by a factor of 10 (cf. right traces in Fig. 6).

As with chloroplasts, the polarities of the photovoltages from both *Rps. sphaeroides* and their chromatophores were opposite to those expected from the traditional arguments cited in the Introduction (see also Fig. 8).

Control experiments showed that also these signals were intimately related to a light gradient and to a vesicular structure. Delivering the flashes from the bottom instead of from the top reversed the polarities of all photovoltage signals shown in Fig. 6. Furthermore, the presence of 1% Triton X-100 in the suspending medium abolished the signals.

Dependence of the photovoltage on the external capacitance. The influence of the external capacitance on the amplitude of the photovoltages from whole cells of *Rps. sphaeroides* or chromatophores was studied analogously to chloroplasts. Similarly to signal F from chloroplasts, these photovoltages decreased sharply when the external capacitance was increased.

A plot of the reciprocal photovoltage peak am-

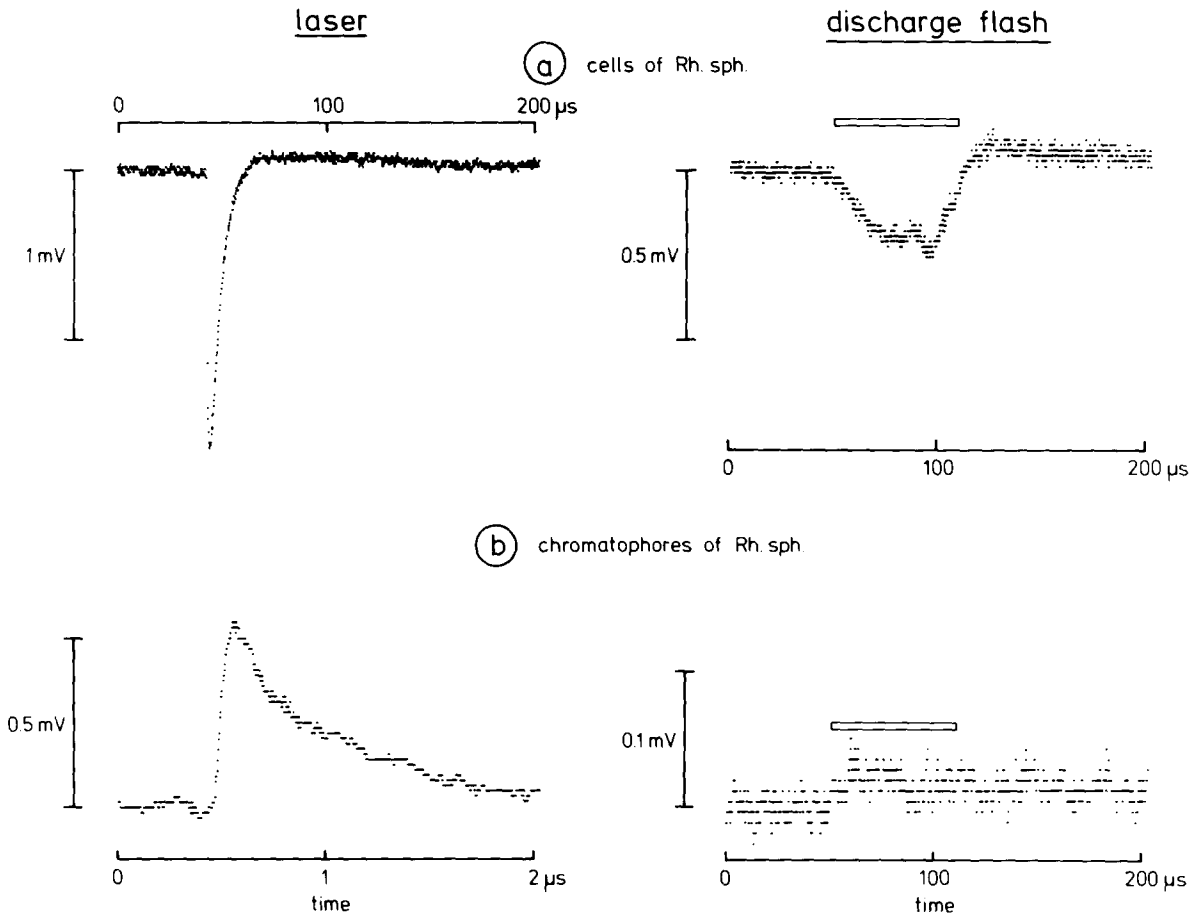

Fig. 6. Photovoltage signals from whole cells of *Rps. sphaeroides* and chromatophores of the same bacterium. (a) The cells were diluted 2-fold from the growing medium with 1 M sucrose to give a final cell concentration of $3 \cdot 10^6$ ml^{-1}. Excitation occurred either with a ruby laser (694 nm; 0.13 mJ/cm^2; 3 ns) or with a discharge lamp (495–570 nm; 0.3 mJ/cm^2; 60 µs). (b) Chromatophores were diluted 8-fold into distilled water to a final concentration of 0.2 mM BChl. Excitation occurred either with a ruby laser (694 nm; 0.6 mJ/cm^2; 3 ns) or with a discharge lamp (white; 10 mJ/cm^2; 60 µs).

plitudes versus the external capacitance, C_a, yielded straight lines for the signals from whole cells and chromatophores (Fig. 4). In both cases, the extrapolation to ideal measuring conditions ($C_a \rightarrow 0$) points to the intersection of the coordinates. The significance of this finding will be discussed later.

Detection of a fast charge-recombination reaction. Excitation of reaction centers of photosynthetic bacteria results in the oxidation of a bacteriochlorophyll dimer, P, and the reduction of a primary electron acceptor, also a bacteriochlorophyll, B, within 1 ps [22]. The electron is then transferred in about 4 ps to a bacteriopheophytin, H, and in about 200 ps to a first ubiquinone, designated Q [22–24]. Following Shuvalov and Parson [14] the components of the reaction center are written as PBHQ. The reaction center is thought to lie perpendicular to the plane of the photosynthetic membrane in such a manner that P is located close to the center of the hydrophobic core of the membrane and Q is located near to the outer membrane/water interface [25–32]. Thus, the primary photosynthetic charge separation ($P^*BHQ \rightarrow P^+BHQ^-$) occurs across the outer half of the membrane. If Q is chemically reduced before the reaction center traps an exciton, the charge separation proceeds up to bacteriopheophytin ($P^*BHQ \rightarrow P^+BH^-Q^-$). From this state, denoted P^F, a

back-reaction takes place with a time constant of about 10 ns [14].

The time domain of this back-reaction is within the time resolution of our electrical measuring system. We used this reaction to probe whether the fast photovoltages monitor the described primary charge displacement.

Experiments were performed with chromatophores suspended in 50 mM KCl with and without $Na_2S_2O_4$, to reduce the quinone acceptor, Q [14]. The excitation source was a Q-switched and pulse-clipped ruby laser at 694 nm with 3–4 ns pulse duration. The electronical upper limiting frequency was limited by the 100 MHz transient recorder, so that a total time resolution of 10–90% rise or fall times of 5–6 ns was achieved.

Fig. 7 shows that photovoltages developed with the instrumentally limited rise time of the apparatus both in the presence and in the absence of $Na_2S_2O_4$. The decay time of the photovoltage due to ion fluxes around the chromatophore was about 30 ns ($-Na_2S_2O_4$). It decreased considerably upon addition of $Na_2S_2O_4$. An analysis of the results from several different experiments for single exponential decay time constants on a semilogarithmic plot yielded a time constant of about 30 ns when Q was oxidized and about 9 ns when Q was reduced. After taking into account the limited time resolution of the apparatus, a decay time constant of 7 ± 2 ns results which equals the time constant spectroscopically determined for the back-reaction in reaction centers with reduced Q [14].

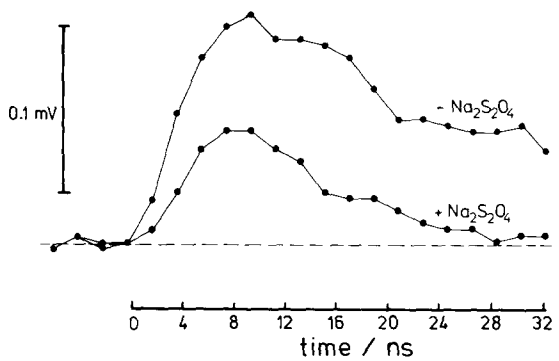

Fig. 7. Detection of a fast back-reaction following the primary charge separation in reaction centers of chromatophores with reduced Q. Chromatophores from the storage medium (see Materials and Methods) were diluted 6-fold into 50 mM KCl to yield a final bacteriochlorophyll concentration of 0.27 mM. The absorbance at the excitation wavelength of 694 nm was approx. 1. Chemical reduction of the quinone acceptor was achieved by the addition of 0.5 mg/ml $Na_2S_2O_4$ to the suspension. The photovoltage picked up by the electrodes was first converted into a 50 Ω signal by a fast (500 MHz) impedance converter, then amplified by two 3 GHz amplifiers, and finally recorded on a 100 MHz transient recorder. To increase the signal-to-noise ratio 16 signals were averaged for each trace. Excitation conditions: ruby laser, 0.4 mJ/cm^2; 3 ns, repetition rate 0.2 Hz.

Simulation showed that the decrease of the amplitude of the photovoltage was not primarily due to interference with the accelerated decay, but indicates that the charge separation occurred over a shorter distance when Q was in the reduced state. In principle, therefore, the results in Fig. 7 could be used to estimate the relative distances between PH and PQ. A more thorough approach aimed at determining these distances using different electrical methods will be the subject of a subsequent paper (Trissl, unpublished data).

Discussion

As cited in the Introduction, a photoelectric signal from chloroplast suspensions was previously observed by other authors. In this communication we report on two different types of photovoltages from a chloroplast suspension. One photovoltage, signal S, had properties compatible with the photovoltage previously described [1–3] and another, signal F, had a polarity opposite to signal S [13] and a very fast rise time (less than 200 ps). We further report for the first time fast photovoltages in the submicrosecond range from a photosynthetic bacterium and from isolated chromatophores.

An obvious first question is why was the positively directed signal F from chloroplasts not observed in the earlier work by other authors? The most probable explanation is that both the stray capacitance and the input capacitance of their preamplifiers were not small enough to obtain reasonable amplitudes. As shown in Fig. 4 the amplitudes of the photovoltages evoked by laser flashes decreased sharply with increasing external capacitance. If no special care is taken, usual external capacitances lie around 100–200 pF (for example: 1 m coaxial cable corresponds to about 100 pF and a typical value for the input capacitance of an oscilloscope amplifier is 50 pF). This

means that the photovoltage under such a condition would be less than about 0.1 mV, a figure equivalent to the characteristic peak-to-peak noise of good 1–10 MHz amplifiers. Hence, the photovoltage signals would be buried in noise. In addition, external capacitances of 100 pF or more cause slower rise times due to a low pass filter formed by the resistance of the suspending medium and electrodes, and by the external capacitance. Since the decay time is in the same range, the rise and fall times approach each other and clip the amplitude of the photovoltage even more.

The most notable observation in the experiments reported here is the polarity of the photovoltages elicited by short laser flashes. All these polarities are opposite to those expected, if one assumes that the signals arise from a transmembrane charge separation which pushes an electron from the inside to the outside in thylakoid vesicles or in chromatophores. It is remarkable that the polarity of the fast photovoltage reversed when the sidedness of the vesicles was inverted. This was observed for chloroplasts and inside-out vesicles (cf. Fig. 2a and d) as well as for whole cells of *Rps. sphaeroides* and their chromatophores (cf. Fig. 6a and b).

The various types of signals observed of different biological systems, as well as their respective polarities are summarized in Fig. 8. In the upper line of this figure the vesicular structures of the photosynthetic systems are schematically drawn and the known direction of the charge separation is identicated by the vectorial movement of an electron across the membrane that faces the light source. The arrows also indicate the polarity which is expected for the photovoltage from the arguments of Fowler and Kok [1,2] and Witt and Zickler [3].

From Fig. 8 it is apparent that, with one exception, all polarities were opposite to the expected ones. The exception is the photovoltage from chloroplasts which is evoked by discharge flashes. This is the signal already noted in the literature [1–3,10]. Before discussing this exception, we will first discuss the photovoltages from the simplest vesicular system, bacterial chromatophors.

As seen in Fig. 4, the photovoltage from chromatophores was strongly dependent on the exter-

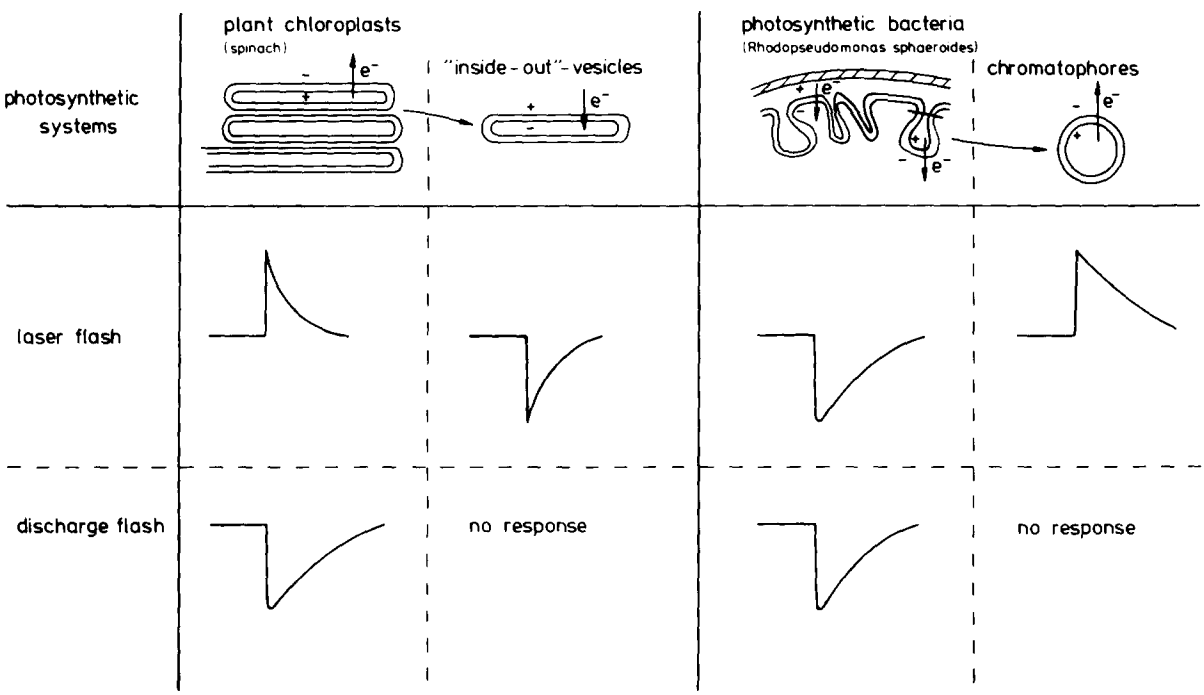

Fig. 8. Schematic illustration of the photosynthetic systems examined (upper line) and the polarities of the photovoltage evoked by short ruby laser pulses (middle line) or by discharge flashes (lower line).

nal capacitance. It was empirically found that the amplitude of the photovoltage increased in an approximately hyperbolic manner as the capacitance decreased. When extrapolated to ideal measuring conditions ($C_a \rightarrow 0$) very high photovoltages can be expected and, eventually, even reversal of the polarity. We interpret this as an indication of the small power of the suspended chromatophores, i.e., a very high access impedance. Consequently, it is conceivable that the impedance of the individual vesicular dipoles in the solution is not properly matched to the impedance of the measuring cell and the preamplifier. In the present experiments all efforts undertaken to overcome a possible impedance mismatching failed, mainly because the residual stray capacitance of about 15 pF could not be further minimized. The problem might be overcome by redesigning the measuring cell as a coaxial line tuned to the amplifier [33].

The only electrogenic event known for bacterial photosynthesis in the time domain of the photovoltages observed, is the primary charge separation in the reaction centers. Therefore, it is suggestive that the photovoltage indicates this charge displacement in the hydrophobic core of the chromatophore membrane. This interpretation could be corroborated by the observation of an approx. 10 ns reversal of the photovoltage under conditions when the photochemical charge separation is known to back-react with this time constant [14] (Fig. 7). In view of the detailed literature which demonstrates that the primary photochemical electron transfer is outwardly directed in chromatophores [25–32], we have to admit that the observed fast photovoltage is inverted in polarity by some as yet unknown property of our measuring device. The alternative, that it did not reflect the primary transmembrane charge separation, seems unlikely.

One might be inclined to attribute signal F from sonicated chloroplasts also to the transmembrane charge separation. One argument in favor of such an interpretation is the similar polarities of signal F and the signal from chromatophores. But there are three further lines of evidence for relating signal F to such an event: (i) The extremely fast rise of less than approx. 200 ps (Fig. 5b), (ii) the independence of the amplitude from the ionic strength, and (iii) the absence of any detectable Hall effect.

The observation that signal F rises in less than 200 ps argues against the possibility of attributing this signal to secondary reactions following the primary charge separation. In agreement with an attribution of signal F to a charge separation in the hydrophobic part of the membrane is the observation that the amplitude of signal F was independent of the ionic strength (cf. Figs. 2a and 5b). Furthermore, signal F can be measured with almost equal peak amplitudes over a time range covering 5 orders of magnitude, although under different experimental conditions (0.35 ns in Fig. 5b and 35 μs in Fig. 2a). Such a characteristic would be met only by the primary photosynthetic charge separation. The argument is furthermore strengthened by the absence of any Hall effect.

Our attempts to correlate signal F from chloroplasts with Photosystem (PS) I and/or PS II activity have not provided any distinct conclusions at this point. However, most likely the photovoltage in the experiment showing the fast rise time (Fig. 5b) is due to the charge separation in reaction centers of PS I for the following two reasons. This experiment was done at an excitation wavelength of 694 nm (ruby laser), a wavelength that, under nonsaturating conditions, gives preference to PS I over PS II, according to their action spectra. On the other hand, the extremely fast rise time of less than approx. 200 ps is only in agreement with the trapping process in PS I and not in PS II, since the energy-transfer time through the light-harvesting pigments to PS I reaction centers is faster than the measured rise time of the photovoltage [39], whereas the energy-transfer time to the PS II trap is slower (approx. 400 ps) [39–42].

The attribution of signal F to a primary transmembrane charge separation is in conflict with the previous attribution of signal S to such an event [1–3]. We found at least six properties in which signals F and S differ from each other. These properties are listed in Table I. In view of this we regard it as probable that signal S reflects some other photosynthetic event, perhaps a lateral charge displacement consecutive to the primary charge separation, as suggested by Becker et al. [10]. This proposal is supported by the extreme sensitivity of signal S to structural disintegration (Fig. 2c). When the chloroplasts were suspended in hypoosmolar medium and then sonicated the signal disappeared

TABLE I

DIFFERENCES BETWEEN THE PHOTOVOLTAGE SIGNALS F AND S FROM SPINACH CHLOROPLASTS

Item	Signal F	Signal S
Polarity	+	−
Amplitude (extrapolated to ideal measuring conditions)	$\lesssim$10 mV	$\approx$1 mV
Minimal rise time	$\lesssim$200 ps	$\approx$10 μs
Source impedance	small	large
Sonication	enhancement	vanishes
Sensitivity to DCMU	little	vanishes

completely. Thus, when the grana stacks and their membranous interconnection by stroma lamellae were broken the structural basis for sufficiently slow lateral movement of charge carriers (ions) also may be lost.

The observed negative polarity and the observed pH dependence of signal S (data not shown) would be compatible with H^+ as the more mobile ion. If this could be proved, signal S might be a useful tool to study the concept of lateral pH heterogeneity in the thylakoid membranes as suggested by Kouchkovsky and Haraux [34,35]. This concept is relevant to the proton-coupling mechanism, to the ATP synthetase that is thought to occur either via localized circuits [36] or a delocalized electrochemical gradient [37] (chemiosmotic theory).

Although the electrical measuring system described in this communication contains polarity and impedance matching problems, it is nevertheless well suited to the investigation of early electrogenic events in photosynthetic membranes at an unprecedented signal-to-noise ratio. At present, we are investigating the relative positions of the primary redox components (P, B, H and Q [14]) in bacterial reaction centers. Similar experiments are feasible with vesicles from chloroplasts enriched either in PS I of PS II.

In addition, the high time resolution of the method opens the perspective of studying the kinetics of exciton transfer times from light-harvesting pigment complexes to photosynthetic reaction centers (traps) in various photosynthetic organisms and organelles.

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