PAPER 17

The indication of a light induced electrical field by pigments incorporated in chloroplast membranes

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SUMMARY

EVIDENCE IS provided that some of the light induced absorption changes in chloroplasts of green plants are due to an electrochromic response of pigments to a light induced electrical field across the thylakoid membrane. These absorption changes can be used as a molecular voltmeter between two aqueous phases of such a small size that they are not accessible to the common electrochemical measuring techniques.

The relevance of the light induced electrical field in photosynthesis is discussed and the extension of the measuring method to other biological systems proposed.

INTRODUCTION

The structure on which the primary processes of photosynthesis of green plants take place is a paucimolecular membrane inside the chloroplasts. According to widely accepted results of several authors (1, 2) this membrane is based on a unimolecular layer of lipids, a second layer of pigments and a third layer of structural protein (Figure 1). The pigment layer is composed of several types of chlorophyll and of carotenoids, which stick to the hydro-

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phobic side of the lipids. These membranes form disk shaped closed vesicles (3, 4, 5), so called thylakoids. The size of one thylakoid can be characterized by the fact that it contains in the order of several 10^5 chlorophyll molecules.

The primary processes of photosynthesis are initiated by the absorption of light energy by the bulk pigments in the second layer and by the final trapping of quanta by two special types of photochemically active chlorophyll molecules. The activation of the two light reactions is followed by three complex events:

(1) Electrons are transferred from water to a terminal electron acceptor $NADP^+$ (6). At least 9 intermediates of the electron transport system have been identified and the kinetic data of their interaction have been

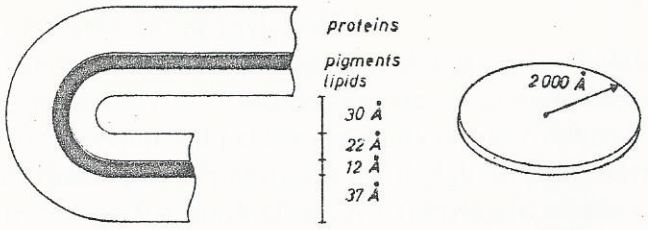

FIGURE 1 Gross structure and average dimensions of one thylakoid.

evaluated (for recent summaries see (7, 8)). The size of one electron transport chain can be characterized by the fact that the two photochemically active pigments are surrounded by a bulk of about 500 chlorophyll molecules (9). Thus one thylakoid includes several hundred electron transport chains.

(2) Protons are taken up by the chloroplasts (10). This proton uptake is accompanied by the release of other cations such as K^+ or Mg^{++} (11) and by swelling and shrinking of the thylakoids (12) ~~that~~ are at least partly due to an osmotic balance of ion fluxes.

(3) ATP is synthesized from ADP and inorganic phosphate (13).

The end products of the primary processes (NADPH and ATP) are consumed in the CO_2-fixation cycle (14).

It is still an open question, in which way part of the absorbed light energy may be transformed into the free energy necessary for the synthesis of ATP. This has to be specified since it has motivated the work reported herein.

In his hypothesis on this subject Mitchell (15) has postulated that on

illumination a concentration gradient of protons and an electrical field may be set on across the photosynthetic membrane. The free energy thus stored in a difference of the electrochemical potential of the protons may be used by a membrane bound anisotropic ATPase, which translocates protons 'down-hill' this potential difference.

Several experimental results on the correlation between the light induced pH difference and the formation of ATP give support to Mitchell's hypothesis (16, 17, 18).

However, the question if there is any electrical field involved in the synthesis of ATP could not be answered by common electrochemical methods. Even microelectrodes are inadequate for measuring the electrical potential in the very small inner phase of one thylakoid.

So a direct response to any electrical field across the thylakoid membrane is reserved to indicators of a molecular size. A straightforward way to get an indicator for an electrical field is the study of electrochromic effects of the bulk pigments, that are incorporated in the thylakoid membrane.

Although related to the Stark effect, the splitting in an electrical field of the spectral lines of atoms in the gas phase, the term 'electrochroism' is used in the following in order to designate shifts in the unresolved spectra of larger molecules in the condensed state. The first two terms in a power series expansion of the frequency shift ($\Delta\nu$) of the absorption spectrum of a dye molecule are linear and quadratic in the field strength (E). They depend on the difference of the permanent dipole moments of the ground and the excited state ($\mu^{\dagger} - \mu^{0}$) and on the difference in the polarizability ($\alpha^{\dagger} - \alpha^{0}$) respectively:

$$\Delta\nu = \frac{1}{h}\left[(\mu^{\dagger} - \mu^{0}) \cdot \mathbf{E} + \tfrac{1}{2}\mathbf{E} \cdot (\alpha^{\dagger} - \alpha^{0}) * \mathbf{E}\right]$$

Order of magnitude calculations (19) and experimental results on porphyrins (20) indicate that the shifts of the rotational and vibrational transitions are smaller than those contributed by the electronic transition. Therefore a nearly homogeneous shift of a whole absorption band can be expected. If the frequency shift is small as compared with the bandwidth the difference spectrum of a pigment between the two states with and without electrical field should be simply the derivative of the absorption spectrum.

Of course, one may think of other mechanisms that can give rise to spectral changes of a dye molecule exposed to a transient electrical field: a field induced change of chemical equilibrium as well as local conformational changes in the membrane, that may influence the interaction of pigments.

The question arises as how to detect absorption changes that may be due to the light induced electrical field across a very small membrane.

METHOD

The method of repetitive pulse photometry has been developed as an extremely sensitive tool for the detection of small absorption changes. This technique has been applied especially for those pigments that are involved in the primary process of photosynthesis. Since details of this method are summarized in (21) only the principle may be reported.

A monochromatic beam of weak measuring light (wavelength) passes a cuvette with an aqueous suspension of spinach chloroplasts (Figure 2). The intensity of the transmitted light is detected and recorded. On repetitive excitation ($n \simeq 1000$) of the photochemically active chlorophyll molecules the n transient absorption changes are superposed in a storage device and averaged. In this way the sensitivity and time resolution are increased by a

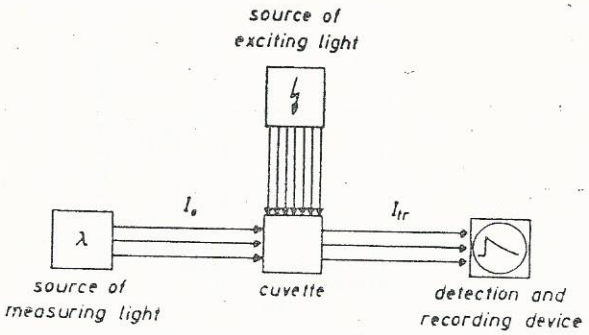

FIGURE 2 Scheme of a flash photometer.

factor $\sqrt{n}$ in comparison with single excitation. Mainly by this method the small absorption changes of 9 intermediates of the electron transport have been detected and the difference spectra clearly separated (7, 8).

Thus if there is any absorption change due to an electrical field across the thylakoid membrane, it has to be discriminated from the aforementioned absorption changes of the electron transport chain and from possible absorption changes due to light induced transients in the concentration of a special type of ions (e.g. H^+).

PROPERTIES OF THE FIELD INDICATING ABSORPTION CHANGES

Tests to discriminate between a field indicating and an electron transport indicating absorption change have been applied to a well known type of absorption change. This type with maxima at 478 nm (neg.), 515 nm (pos.) and 648 nm (neg.) was ascribed to a reaction of chlorophyll-b (22). It revealed

some properties common with phosphorylation but different from electron transport (23, 24). It was proposed that it might be due to a protolytic reaction of chlorophyll-b.

A typical time course of the absorption change at 515 nm is depicted in Figure 3. On excitation with a short flash of light there is a rapid rise of absorption followed by a rather slow decay in the dark. If this absorption change indicates the rapid onset of an electrical field across the thylakoid membrane and the following decay of this field due to an intrinsic conductivity of the membrane (Figure 3), it has to react very sensitively to any change in the membrane's conductivity.

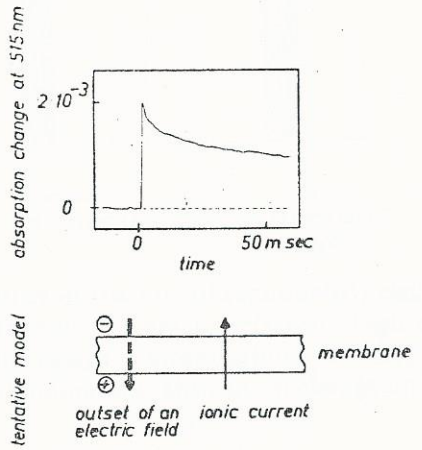

FIGURE 3 Typical time course of the absorption change at 515 nm (above) and tentative model for the interpretation of this absorption change (below).

There are two conclusive experiments that demonstrate that the absorption change at 515 nm does not reflect direct properties of the electron transport chains but fulfill the expectation for a field indicating absorption change perfectly. While the electron transport should work in principle even on membrane fractions that contain only a minimum set of about 500 chlorophyll molecules, the electrical field should disappear (respectively become not resolvable quickly) if the inner and the outer phase of thylakoids are short circuited by grave ruptures of the membrane.

Indeed, this behavior is reflected by the 515-absorption change (Figure 4) on treatment of chloroplast membranes with the permeability increasing antibiotic gramicidin D. In contrast to this there is no influence on the rate of (uncoupled) electron flow.

On the other hand only one pore per each thylakoid ($\simeq 10^5$ chlorophyll molecules) that is permeable for ions should yield an acceleration of the field

decay. This has been tested (25) with the antibiotic gramicidin D which in rather low concentrations increases the permeability of several biological membranes for alkaline ions (26, 27). Again the expected effect, an acceleration of the field decay on gramicidin treated chloroplasts, is reflected by the 515-absorption change (Figure 5). Moreover, a minimum concentration of only one molecule of gramicidin on $2 \cdot 10^5$ chlorophyll molecules induced an acceleration of only one half of the amplitude of the absorption change while

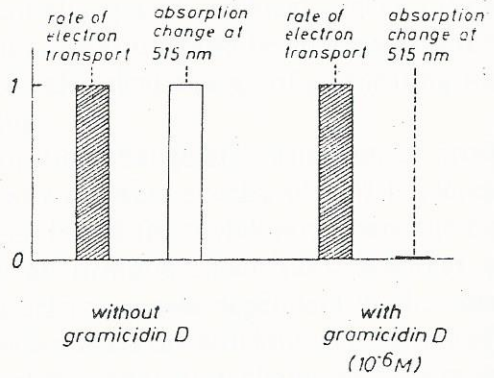

FIGURE 4 Response of the rate of (uncoupled) electron flow and of the absorption change at 515 nm to relatively high concentration of gramicidin D. (Chlorophyll concentration: 10^{-5} M; rate of electron flow in continuous illumination; absorption change on flash excitation).

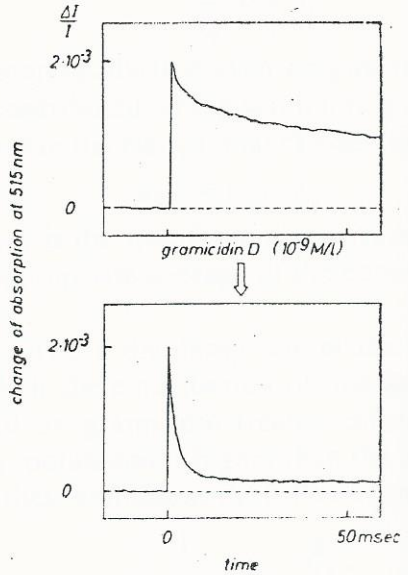

FIGURE 5 Acceleration of the absorption change at 515 nm by small concentrations of gramicidin D. (Chlorophyll concentration: 10^{-5} M).

the other half decayed slowly as before (25). This demonstrates that the absorption change at 515 nm reflects a property that is defined on one whole thylakoid ($\simeq 10^5$ chlorophyll molecules). This is conclusive to discriminate between the 515-absorption change and absorption changes of the electron transport chain, and it agreed with the assumption, that the 515-absorption change indicates an electrical field across the thylakoid membrane.

It has still to be demonstrated that the absorption change at 515 nm does not reflect transient changes of the local concentrations of only one special type of ion. In this context it would be conclusive if the absorption change is reactive only to the electrical charge of permeating ionic species but not to their chemical nature.

In fact the absorption change at 515 nm can be accelerated if the concentration of chemically different species of ions is increased (e.g. K^+, Mg^{++}, Cl^-). This has been found for thylakoids where the membrane permeability was increased by an osmotic shock (28). Whereas on gramicidin treated chloroplasts the acceleration was dependent on the concentration of alkaline ions only (25), which reflects the well known property of gramicidin (26, 27).

If the decay rate of the absorption change at 515 nm reflects the discharge of the thylakoids capacity by ion fluxes across the thylakoid membrane, the observed first order decay rate ($1/\tau_{\frac{1}{2}}$) should be proportional to the sum of several conductivity terms taken over all permeating ionic species.

$$\frac{1}{\tau_{\frac{1}{2}}} \sim \sum_i \sigma_i$$

Ciani has shown theoretically that even on paucimolecular membranes the conductivity (σ_{K^+}) contributed by a given ion (e.g. K^+) can be approximated by the value contained in the Nernst–Planck equation (29).

$$\sigma_{K^+} = u_{K^+} \cdot \bar{c}_{K^+}$$

In this equation u_{K^+} is the specific conductivity coefficient for the given ion (K^+) and $\bar{c}_{K^+}$ an appropriate average of the concentration of this ion in the membrane phase.

The relationship between the decay rate of the field and the conductivity becomes very simple if the contribution of one ionic species is dominating. This can be realized on gramicidin treated chloroplasts provided that the concentration of e.g. potassium is higher than the concentration of any other alkaline ion. Under these conditions the following relation has to be expected:

$$\frac{1}{\tau_{\frac{1}{2}}} \sim u_{K^+} \cdot \bar{c}_{K^+}$$

Thus the specific conductivity u_{K^+} and therefore the decay rate $1/\tau_{\frac{1}{2}}$ should increase with increasing gramicidin concentration (the number of pores for

K^+) if the potassium concentration is held constant. Indeed this behavior is reflected by the decay rate of the 515-absorption change (Figure 6, right side). On the other hand, the gramicidin concentration being constant, it has to be expected that the average concentration ($\bar{C}_{K^+}$) of potassium in the membrane and therefore the decay rate increases with the potassium concentration in the suspension. That this holds in a certain concentration range is shown in Figure 6, left side (28).

Thus the properties of the absorption change at 515 nm are in agreement with the assumption, that this absorption change indicates a light induced electrical field across the thylakoid membrane. The question arises as to the mechanism of this indication.

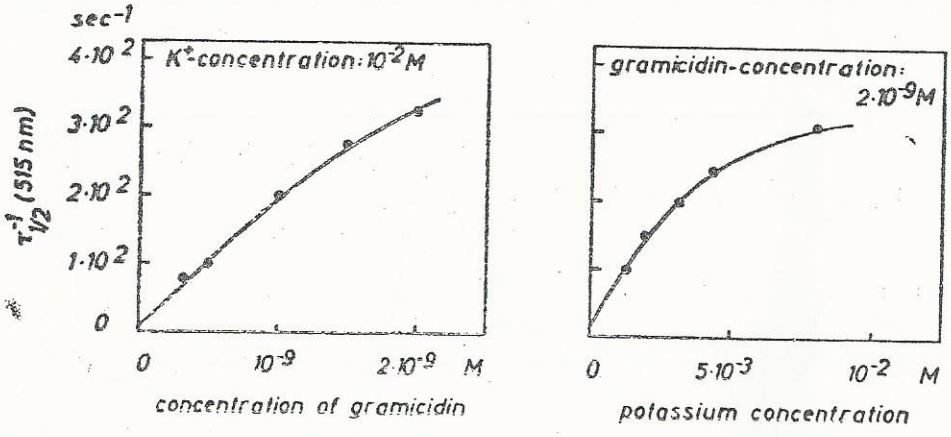

FIGURE 6 Dependence of the decay rate of the absorption change at 515 nm ($\tau_{1/2}^{-1}$) on the concentration of gramicidin (left side) and on the concentration of potassium (right side).

If the response to an electrical field at the wavelength 515 nm is due to an electrochromic effect, similar absorption changes can be expected in all those spectral regions where the bulk pigments absorb. Since these pigments stick directly to the non-aqueous lipid layer in the thylakoid membrane across which the field only can exist (see Figure 1), they are exposed to the stray-field at the membrane. Indeed the overall spectrum of the field indicating absorption changes (Figure 7) reveals maxima in the blue where the carotenoids and chlorophylls absorb and in the region of the red bands of chlorophylls *in vivo* (chl-b_{653}, chl-a_{673}, chl-$_{683}$, chl-a_{695}).

Only those components of the complex transient absorption changes at each wavelength have been included in this difference spectrum, which reveal the same characteristic properties as the absorption change at 515 nm. This spectrum can be obtained by a kinetic analysis of two difference spectra with and without gramicidin for instance.

The superposition of spectral shifts due to several pigments makes the analysis of the spectrum very difficult. ~~[A detailed analysis will be published elsewhere.]~~ However, at least the maxima at 648 nm (neg.) and 660 nm (pos.) that are nearly antisymmetrical to the center frequency of chlorophyll-b *in vivo* (653 nm) correspond to the expectation for a difference spectrum due to a small homogeneous electrochromic shift of the chlorophyll-b band to the red. (The minor height of the 660-change may be due to the superposition with the negative lobe of a similar differential spectrum centered around chlorophyll-a_{673}.)

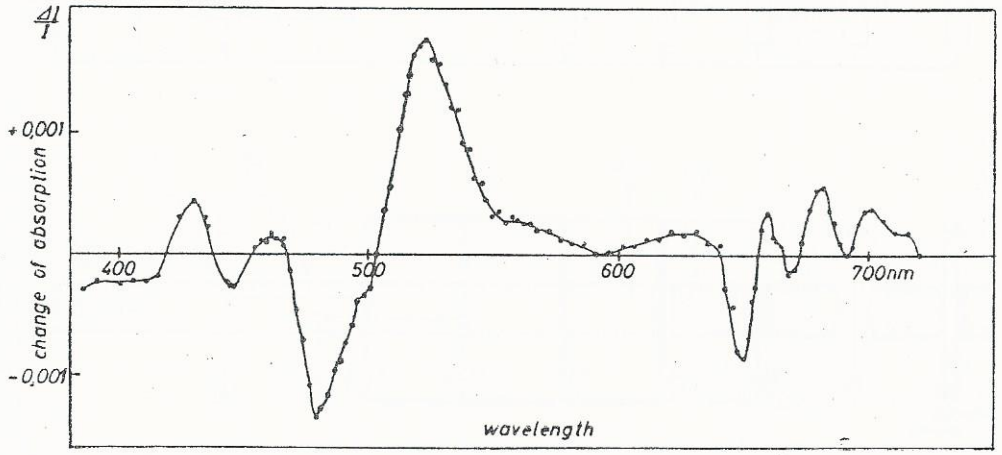

FIGURE 7 Separated difference spectrum of the field indicating absorption changes on spinach chloroplasts.

Under the assumptions of a homogeneous shift of the whole chlorophyll-b *in vivo* band and using the spectral profile of the *in vitro* band (30) the wavelength of the shift can be calculated from the height of the maximum at 648 nm of the difference spectrum. It corresponds to a shift of about 0, 1 Å.

However, this is the order of magnitude that has been measured for the shift of the Soret band of porphyrins (*in vitro*) due to an electric field of about 10^6 V/cm (20).

THE ROLE OF THE LIGHT INDUCED ELECTRICAL FIELD IN PHOTOSYNTHESIS

The field indicator properties of the absorption changes at 515 nm have been used for detailed studies of the role of the light induced electrical field in photosynthesis.

The results have been published elsewhere (25, 31, 32, 33, 36, 37) therefore only some of them shall be briefly summarized in the following scheme (Figure 8). In a single short flash of light (time of duration 10^{-4} sec) the two light reactions promote the transport of one electron from water to $NADP^+$. Additionally each of the two light reactions sets on one half of the electrical field strength across the thylakoid membrane and translocates one proton into the inner phase of the thylakoid (32). The time for the onset of the electrical field measured as the rise time at 515 nm on excitation with an extreme short saturating giant laser pulse is extremely fast ($\leq 2 \cdot 10^{-9}$ sec (7, 33)).

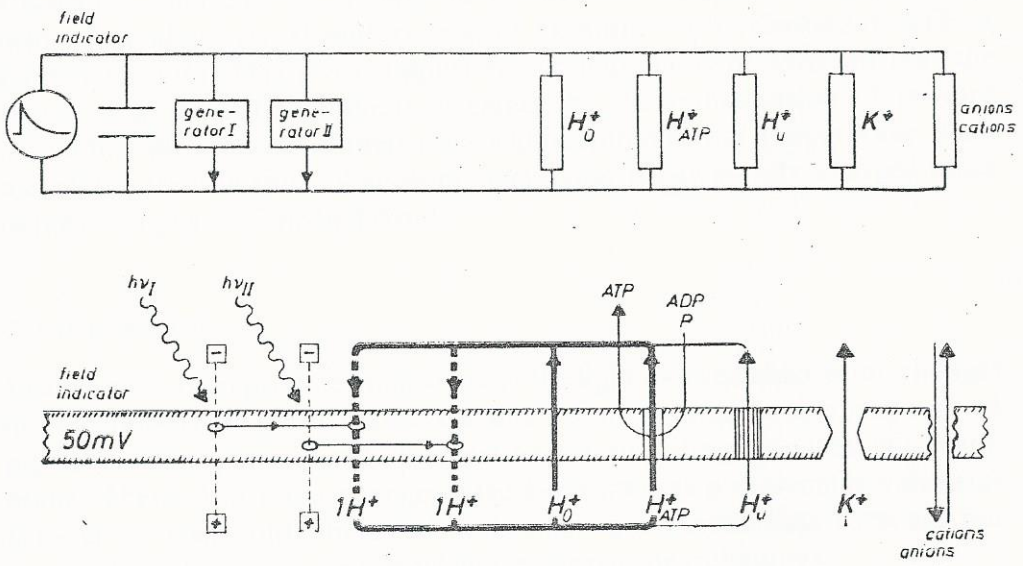

FIGURE 8 Model for the role of the light induced electrical field in photosynthesis.

Although the stoichiometric coupling between field onset and the proton translocation does not imply a kinetic coupling it can be concluded that the field strength corresponds to a charge separation of two elementary charges per electron transport chain. Using X-ray diffraction data (34) on the area of one electron transport chain ($\simeq 10^5$ Å) and on the thickness of the insulating lipid layer (see Figure 1), the order of magnitude of the voltage across the membrane can be calculated. The voltage amounts to 50 mV on excitation with the single short flash (32). (This corresponds to a field strength of about $2 \cdot 10^7$ V/cm.) In continuous saturating illumination this value can increase about fourfold to about 200 mV and in the steady state it decreases to 100 mV (32). (These conclusions are based on the assumption that the absorption change at

515 nm is a linear indicator of the field strength. This can be concluded from (32) and it has been demonstrated in a wider range in (35).

In the scheme (Figure 8) three artifically induced ways to accelerate the field decay have been indicated by thin lines (osmotic shock increases the membranes conductivity for different types of ions (e.g. Cl^-, Mg^{++}, gramicidin treatment for alkaline ions only, so called uncouplers for protons).

Relevant for *in vivo* photosynthesis are only the events on intact membranes (indicated by thick lines). Studies on the correlation between the absorption change at 515 nm and the synthesis of ATP have shown, that the field decay after several milliseconds of excitation is accelerated if ATP is synthezised (36). This gives support to the hypothesis of Mitchell that the energy used for ATP synthesis is gained by the translocation of protons 'down hill' the gradient of their electrical potential across a membrane. That means the translocation of protons back from the positively charged inner to the outer phase of the thylakoid.

CONCLUSIONS

Evidence has been provided that some of the light induced absorption changes in chloroplasts of green plants are due to an electrochromic response of pigments to a light induced electrical field across the photosynthetic membrane. These absorption changes have been used as a molecular voltmeter between two aqueous phases of such small dimensions that they are not accessible to the common electrochemical measuring techniques.

The photometric detection of an electrical field across a microscopical membrane (in combination with the repetitive technique) should be extendable to other biological systems, since it has two valuable properties: its extremely fast response to transients in field strength and its ability to detect fields that are localized in very small regions of space.

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DISCUSSION

GREEN I would like to ask whether one could interpret your experiments very simply in terms of a conformational change in the system which is intrinsic to an energy cycle of the membrane. When gramicidin is present, the energized system becomes discharged and ions are moved. When ADP is present, the energized system becomes discharged and ATP is synthesized. This is a rather simple interpretation. Is there any reason why the particular interpretation I am suggesting should not be considered? It accounts for all the facts very simply.

JUNGE As yet it is very hard to discriminate whether the ion fluxes are caused by conformational changes or vice versa. This is partly due to the fact that ion fluxes are coupled to osmotic phenomena, which induce conformational changes in a very trivial sense.

However, the Nernst–Planck-like behavior of the ion fluxes that we studied by means of absorption changes gives evidence that the ions are driven by an electrical field across the thylakoid membrane. Whether this field is set on by a conformational change or not has to be studied in the future.

ADAM You have a very fast rise of the absorption change with 2×10^{-8} seconds. Now, do you think that this is an indication of an electric field built up by reaction of the reactive center to the light? I guess you do. Then, this time is very fast indeed, since, for capacitive changes of the axon membrane potential you have microseconds as the duration in which the field is established. So, I see a discrepancy of two orders of magnitude.

JUNGE This discrepancy between the axon and the chloroplast may be due to a difference between both systems. The field in the chloroplast is set on in the course of photochemical reactions, which are very rapid.

ADAM How do you imagine that this field comes about? Is it a movement of ions or is it something else?

JUNGE As yet there is no evidence favoring a special mechanism of the field onset. One may imagine that the translocation of either a proton or an electron changes the membrane's capacitance.

As Professor Onsager has shown, the translocation of a proton across the membrane can be sufficiently rapid.

On the other hand one may assume that a slower hydrogen translocation follows the field onset. The field may be set on by a rapid electron transport from the inner to the outer side of the membrane. This may be followed by the uptake and neutralization of a proton from the outer phase, a transport of one hydrogen to the inner side and the final release of one proton to the inner

phase. By this antitransport: e^-, H the field can be set on very rapidly, but the net translocation of a proton may be much slower. This has been discussed in Refs. 8 and 25.

ADAM If I may add one comment, I would find it rather improbable that such a fast rising electric field can come about by ionic movements. I would think that these are rather electronic shifts, maybe in charge transfer complexes.

JUNGE Well, that is one of the possibilities I tried to point out.

GREEN I can't resist making a comment here. This is a general comment for so many systems of this kind. On the one hand one has physical methods of great precision where very precise calculations are made, and on the other hand complete ignorance about the system one works with, so that you have this contrast of incisive physical methods in total darkness with the most elementary features of the system itself.