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Electrical events associated with primary photosynthetic reactions in chloroplast membranes

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Abstract

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A study was made of electric phenomena in isolated photosynthetically-active chloroplast membranes upon energization. Energization of chloroplast thylakoid membranes gives rise to a charge separation across the membrane and an associated transmembrane electric field. The kinetics of this field were monitored as potential changes by a micro-electrode inserted in a single chloroplast and they were compared with the absorbance change of an intrinsic pigment (P515) proposed to be a reflection of a transmembrane electric field. The observed discrepancy between the flash-induced P515 response and the electric response was caused by conformational changes in the membranal core that brought about absorbance changes of P515 in addition to its reponsiveness to the transmembrane potential. Most of the conclusions drawn hitherto from P515 kinetics, both after flash excitation and during prolonged illumination, have to be regarded with caution because they commonly are based on assumptions that are invalid under normal physiological conditions. Under certain conditions, if appropriate corrections are made, the P515 kinetics are indeed a reflection of the transmembrane potential. In that case the results appear to be in good agreement with results obtained with the electrophysiological approach.

The component of the P515 absorbance change, that is ascribed to conformational changes in the membranal core, may provide a useful tool for studies of fast structural changes in relation to energy coupling, without affecting the membrane integrity.

Free descriptors: electric potential; P515; 515 nm absorbance change; conformational changes; structural changes; light scattering; thylakoid membrane; photosynthesis; electron transport; surface potential; ATP synthesis.

Contents

Abbreviations

ACMA	9-amino-6-chloro-2-methoxyacridine
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
CCCP	Carbonylcyanide-*m*-chloro-phenylhydrazone
DCCD	Dicyclohexylcarbodiimide
DCMU	3-(3,4,dichlorophenyl)-1,1-dimethylurea
DCPIP	Dichlorophenolindophenol
Diquat	N,N'-ethylene-2,2'-dipyridilium dibromide
EDTA	Ethylenediaminetetraacetate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
MES	2(N-morpholino)ethanesulphonic acid
NADP	Nicotinamide-adenine dinucleotide phosphate
PMS	N-methylphenazonium methosulphate
S_{13}	5-chloro,3-*t*-butyl,2'-chloro,4'-nitro-salicylanilide
SDS	Sodiumdodecylsulphate
Tricine	N-tris(hydroxymethyl)methyl-glycine
Tris	tris(hydroxymethyl)aminomethane

1 Introduction

1.1 HIGHER PLANT PHOTOSYNTHESIS

The overall process of photosynthesis, defined as the light-driven CO_2 reduction with concomitant O_2 evolution, has been known for more than two centuries but it was not before 1943 that the hypothesis of Ruben (1943) arose that photosynthetic CO_2 reduction required the electrochemical generation of two energy-rich products: adenosine triphosphate (ATP) and reduced pyridine nucleotides (NADPH). This hypothesis got strong support in the early fifties (Arnon, 1951; Arnon et al., 1954).

The concept of two light reactions, as proposed by Hill & Bendall (1960), and Duysens et al. (1961), has been an important landmark in the search for the mechanism by which photosynthetic energy conversion leads to $NADP^+$ reduction and ATP production. Duysens & Amesz (1962) proposed that the two light reactions were operating in series: a long-wavelength Photosystem 1 (PS1) and a short-wavelength Photosystem 2 (PS2). This concept can be presented by the so-called Z scheme (Fig. 1). Light energy absorbed by the antenna pigment molecules is transferred to the reaction centers. In the reaction centers of PS1 and PS2, the excited chlorophyll molecules (P700 and P680, respectively) become oxidized with a concomitant reduction of the primary electron acceptors (P430 and C550). The two light reactions are connected by a chain of compounds undergoing reversible oxido-reduc-

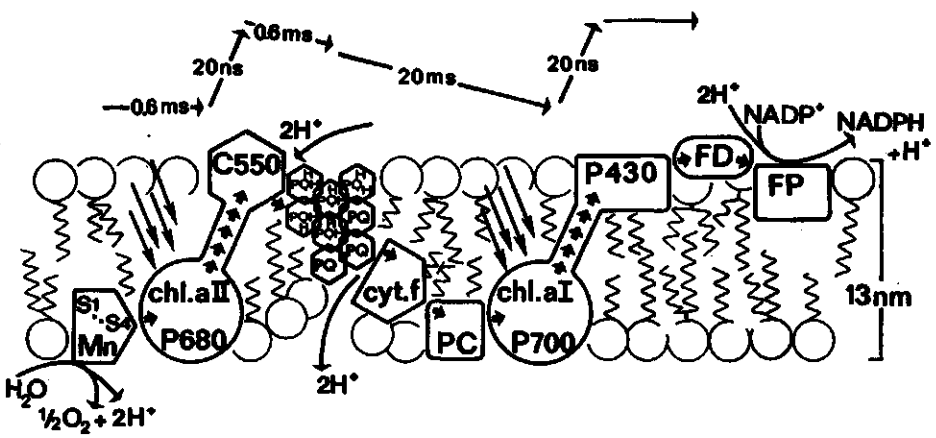

Fig. 1. Simplified visualization of photosynthetic electron flow through the vectorially oriented electron transport chain and coupled proton translocation across the thylakoid membrane. The proton-binding and proton-releasing sites at the outside and the inside of the membrane, respectively are indicated. Reaction sites of cyt b-559 and cyt b-563 are not indicated; P680 and P700: trapping centers of PS2 and PS1, respectively; C550 and P430: primary acceptors of PS2 and PS1, respectively; PQ: plastoquinone; cyt f: C554 (cytochrome f); PC: plastocyanin; FD: ferredoxin; FP: flavoprotein; Mn: mangane-containing water splitting protein; S1-S4: different functional states of the water splitting enzyme.

tion reactions. The strong oxidant that exists after charge transfer in PS2 is capable of oxidizing H_2O. The light-dependent uphill electron and hydrogen transport from H_2O to $NADP^+$ serves the purpose to create reducing power for CO_2 reduction. In addition, following the suggestion of Mitchell (1961, 1966, 1968, 1977), a vectorial orientation of electron and hydrogen carriers in the membrane will lead to the establishment of a proton motive force (pmf). The pmf, which consists of an electrical and a chemical component, is suggested to be the driving force for photophosphorylation. Many experimental data have supported the validity of this chemiosmotic theory for energy coupling in chloroplast membranes (Witt, 1971; Trebst, 1974). Neumann & Jagendorf (1964) recognized that proton uptake in chloroplasts is tightly coupled with electron transport. A few years later, it was suggested that thylakoid membranes were able to use a pH gradient to drive ATP synthesis (Jagendorf & Uribe, 1966; Jagendorf, 1967, 1975). The ability of pH gradients to mimic the effects of light indeed is rather good and extends over a great area of light-induced effects such as Pi-ATP exchange reactions (Bachofen & Specht-Jurgensen, 1967) and conformational changes in CF_1 (Ryrie & Jagendorf, 1972). Recently reversed electron flow caused by a pH gradient has been observed (Schreiber & Avron, 1977).

If the transmembrane electrochemical proton gradient is the energy source for ATP synthesis it would be expected that photophosphorylation starts after a lag period, to allow the establishment of a pmf, sufficiently high to supply the energy required for ATP synthesis. Such a lag period, however, has been observed to be very small or absent and ATP formation was found to proceed during the first few milliseconds of illumination or even in single light flashes, when there is either no or a negligible proton gradient formed (Ort et al., 1976; Ort & Dilley, 1976; Vinkler et al., 1978). Avron (1978) suggested that, in this early time range, the transmembrane electric field was the major driving force. Although the latter suggestion might explain the observed flash-induced ATP synthesis, no convincing evidence for such a mechanism is available. Furthermore, it seems unlikely that the transmembrane electrical potential is high enough to span the phosphate potential (Vredenberg et al., 1973). Within this framework I would like to cast a glance at two alternative models and a variety of intermediate versions, that may provide insight into the mechanism of coupled electron transport (Jagendorf, 1975).

The chemical hypothesis (Slater, 1953; Chance & Williams, 1956) proposes the formation of direct chemical bonds between electron carriers and enzymes or substrates of the phosphorylation reaction. Oxidation of the complex then raises the ligand bond to a high energy level and this energy drives the anhydro-bond formation of ATP. The basic idea behind this mechanism led to the proposal that energy coupling may involve conformational changes (Boyer, 1965). An important feature of this hypothesis, recently revised by Boyer (1974) and Slater (1974), is the release of tightly bound ATP via an energy-linked conformational change at the catalytic site of the coupling ATPase (CF_1) whereas the ATP formation itself requires no energy input. Accordingly, several observations point to the occurrence of conformational changes in the (CF_1) (Ryrie & Jagendorf, 1971; Nelson et al., 1972; Mc Carty et al., 1972; Kraayenhof & Slater, 1975) but no satisfactory explanation as to how the energy is transmitted has been given as yet.

The hypothesis proposed by Williams (1962) emphasizes the importance of electron-transport mediated accumulation of protons in the hydrophylic pockets of the membrane

itself. This may cause a lowering of H_2O activity due to hydronium formation and facilitate the dehydration reaction of polyphosphate formation. Recently, Del Valle-Tascon et al. (1978) gave support for such a mechanism in chromatophores of *Rhodospirillum rubrum*. In addition, local pH changes within the membrane have been proposed to account for an important role of conformational changes of membrane proteins (Williams, 1972). See also Izawa et al. (1975).

Although all these hypotheses can explain the observed formation of ATP in the absence of either a transmembrane proton gradient or a transmembrane potential (Ort et al., 1976), no convincing evidence for the proposed mechanism is available until now. The chemiosmotic hypothesis so far has provided the most coherent framework to understand and predict many facets of the coupling mechanism. However, some of these predictions have not been verified by experimental findings, especially those concerning the number of ATP molecules formed per pair of electrons moving from H_2O to the reducing site of PS1. A full understanding of this mechanism might request the accomodation of membrane-located events (Harris & Crofts, 1978).

1.2 THYLAKOID STRUCTURE

The ability of the direct conversion of light energy into chemical energy is the unique property of specialized organelles in photosynthesizing organisms. To meet the requirements set by this complex task, the structural framework of these organelles is expected to be highly differentiated and to be provided with appropriate tools which enable a strict functioning of the system. It is now well established that the inner chloroplast membranes, having a folded structure of closely packed membrane stacks (granum lamellae) and interconnecting membranes, called stroma lamellae, separate an inner thylakoid phase from the stroma phase (Menke, 1961).

The thylakoid membrane contains all the chlorophylls and accessory pigments, the components of the electron transport chains and the enzymes for phosphorylation of ADP. The matrix of this structure consists of glycolipids, sulpholipids and few phospholipids. It contains a variety of different proteins (Menke & Ruppel, 1971), either as an intrinsic part, stabilized by hydrophobic interactions and ion pair formation (Gitler, 1972), or losely bound at the surface by salt bridge interaction (Avron, 1963). Chlorophyll a and b, carotenoids (Thornber, 1975) and cytochromes (Bendall et al., 1971; Lemberg & Barrett, 1973) form complexes with proteins in a cooperative and protective interaction. Freeze fracturing, deep etching and serological techniques revealed the morphological and functional asymmetry of the thylakoid membrane (Trebst, 1974; Anderson, 1975).

The compartmentalized three dimensional structure of the chloroplast and the specialized composition of the thylakoid membranes implicitely point to a high accessibility of the primary photosynthetic processes to regulatory activities such as energy-dependent redistribution of ions between the thylakoid inner space and the stroma or structural changes of the membrane. A consideration of this aspect in photosynthesis research emerged in recent years, and many observations confirmed the occurrence of such control mechanisms (Horton & Cramer, 1974; Butler & Kitajima, 1975; Kraayenhof & Slater, 1975). For a more detailed treatise on this subject I would like to refer to recent reviews (Barber, 1976;

Kraayenhof, 1977a, 1977b).

An extensive research on the basic origin of the intrinsic achievements of the thylakoid membrane and the critical importance of the mutual coherence of its components, will lead to a more complete understanding of primary photosynthetic processes.

1.3 GENERATION OF AN ELECTRICAL POTENTIAL ACROSS ENERGIZED THYLAKOID MEMBRANES

Energy captured by the reaction centers is used for electron transfer from a donor to an acceptor molecule. Photo-oxidation of the electron donors Chl a_I dimer P700 and Chl a_{II} P680 (Fig. 1) and vectorial electron ejection towards the externally orientated electron acceptors, P430 and C550, respectively, will give rise to a transmembrane electric field (Junge & Witt, 1968). When both reaction centers are equally activated in a single-turnover light flash, each of them generates half of the electric field (Schliephake et al., 1968; Renger & Wolff, 1975; Schapendonk & Vredenberg, 1979). All further reactions in photosynthesis are subsequent to this process and therefore considerable attention has been paid to understand its origin and function. It may provide further knowledge about the coupling between electron transport and photophosphorylation. A detailed review on this matter has been given recently by Witt (1979).

The magnitude of the electrical potential in a single-turnover light flash measured with different techniques, was reported to be 10-20 mV (Vredenberg & Tonk, 1975), 15-35 mV (Schapendonk & Vredenberg, 1977), 50 mV (Schliephake et al., 1968) and 135 mV (Zickler et al., 1976), although recently one of the authors (Witt, 1979) suggested that the latter value might be an overestimation. Exept for the electrode measurements (Vredenberg & Tonk, 1975), all available information on flash-induced electric field kinetics is obtained from estimates making use of the absorbance response of an intrinsic probe, the P515 pigment complex (Witt, 1971), or of an external potential probe (Bashford et al., 1978; Schuurmans et al., 1978).

Junge & Witt (1968) suggested that the field-dependent electrochromic absorbance shift of P515 was a linear indicator of the electric field across the thylakoid membrane and consequently would follow a single-exponential dark decay with a rate constant, determined by the membrane capacitance and the membrane conductance (Bulychev & Vredenberg, 1976a). Accordingly, a single-exponential decay of the P515 absorbance change in isotonically suspended chloroplasts has been reported to occur under non-phosphorylating conditions (Witt, 1971; Junge & Witt, 1968; Boeck & Witt, 1972). The decay rate, however, seems to be dependent on the membrane integrity rather than on the membrane conductance. This is demonstrated by the scatter in the reported half-life times of the P515 changes, using different membrane isolation procedures (Schmid et al., 1976; Schapendonk et al., 1979).

The occurrence of biphasic decay kinetics under phosphorylating conditions (Rumberg & Siggel, 1968; Junge et al., 1970; Girault & Galmiche, 1978) has been interpreted to be due to an increased proton conductance through the ATP synthesizing enzyme complex above a critical potential (Rumberg & Siggel, 1968; Junge et al., 1970). On the other hand Nelson (1972) and Neumann et al. (1970) observed that the decay kinetics of P515 were independent on the occurrence of ATP synthesis. Moreover, Girault & Galmiche (1976) demon-

strated that the acceleration of the P515 decay could easily be obtained by addition of ATP. These and other observations have given rise to fundamental criticism of the proposed hypothesis (Rumberg & Siggel, 1968) and tend to the conclusion that the observed effects somehow reflect a process which may be associated with nucleotide binding on the membrane or more specifically on the CF_1.

Support was given for the suggestion that at least part of the carotenoid absorbance changes may be caused by conformational changes in the membrane close to the region where the carotenoids are attached (Fleischmann & Clayton, 1968; Baltscheffsky et al., 1971).

Quantitative estimates of the steady-state transmembrane potential across the thylakoid during continuous illumination are also ambiguous as yet. Dependent on the methodological approach, values of 75-105 mV (Barber, 1972a), 30 mV (Strichartz & Chance, 1972), 10 mV (Schröder et al., 1971) have been reported for the transmembrane potential in chloroplasts. Gräber & Witt (1974) concluded from P515 measurements that the steady-state transmembrane potential reached a value of about 100 mV in intact *Chlorella* cells. A direct measurement with micro-electrodes in intact *Peperomia* chloroplasts showed a steady-state potential in the light of less than 10 mV. Moreover, the kinetics of the latter are quite different from those of the 515 nm absorbance changes. Considering the different methods used, the apparently conflicting results in fact are not necessarily contradictory because the intrinsic P515 pigments may react to changes in local electric fields (Fleischmann & Clayton, 1968) and to changes in surface potential (Rumberg & Mühle, 1976). These localized events are not detected with micro-electrodes, that are situated in the bulk phase. Moreover, part of the P515 absorbance changes is caused by slower secondary events, reflected by scattering changes, that are known to occur upon energization (Deamer et al., 1967; Thorne et al., 1975).

1.4 OUTLINE OF STUDY

This study deals with electrical events in thylakoid membranes, associated with light activitation of the photosynthetic apparatus. These events will be discussed in relation to the dynamic function of the membrane in energy transduction and energy coupling. The kinetic pattern of the transmembrane potential will be characterized and discussed in view of the seemingly conflicting results obtained with spectroscopic and electrophysiological methods, respectively. Some additional information will be presented about structural changes in the membrane core induced by electrogenic mechanisms, and their possible function in photophosphorylation will be discussed.

Experiments have been carried out with intact (Class I) and broken (Class II) chloroplasts. Chapter 3 deals with the inhomogeneous distribution of the field-indicating carotenoid-chlorophyll b pigment complex (P515) in the membrane, as concluded from apparent differences in the spectrum, characteristic for the electrochromic response of the complex. A voltage calibration of the P515 absorbance changes caused by a saturating single-turnover light flash is given in Chapter 4. A kinetic analysis of the P515 response in light flashes is given in Chapter 5.

The kinetics of the transmembrane potential changes, reflected by a fast P515 component (resolved in Chapter 5), are studied in relation to ion conductivity and compared

with the transmembrane potential changes as measured with micro-capillary glass electrodes (Chapter 6). The characteristics of a slow P515 component are discussed in relation to conformational changes in the membrane (Chapter 7). Finally, an experimental approach is given, to detect P515 absorbance changes during prolonged illumination after correction for scattering artifacts (Chapter 8).

Part of the work presented in this book has been published (Schapendonk & Vredenberg, 1977; 1979; Vredenberg & Schapendonk, 1978; Tonk et al., 1979; Schapendonk et al., 1979; 1980).

2 Material and methods

2.1 ISOLATION AND CHARACTERIZATION OF CHLOROPLASTS

For all experiments, except those described in Chapter 6, freshly-grown spinach (*Spinacia oleracea*, "Amsterdams Reuzenblad" was cultured on hydroponics in the laboratory green house according to the method of Steiner (1968). For optimum growth in winter the root temperature was maintained at 20°C and during that period the plants were illuminated with high-pressure mercury lamps for 8 hours. Intact chloroplasts routinely were isolated according to a modified method of Nakatani & Barber (1977), except for preparations used in Chapter 4. Washed leaves (25 g) were cut into small pieces (≈ 0.5 cm^2) and ground for 2 s, using an Ultra Turrax 18/10 cavitation disperser provided with 18N shaft, in 50 ml of a medium containing 0.33 mol/l sorbitol, 0.2 mmol/l $MgCl_2$, 20 mmol/l MES and 5 mg egg albumen, adjusted to pH 6.5 with tris. After filtration through 8 layers of perlon net (pore diameter 40 µm), the filtrate was centrifuged in two tubes for 40 s at 815 *g* in a MSE Chill Spin centrifuge. The pellets were resuspended by gentle hand-shaking in 10 ml of 0.33 mol/l sorbitol, adjusted to pH 7.5 with tris. However, in the course of the investigation, it was discovered that replacement of tris by tricine - NaOH gave better CO_2-dependent O_2 evolution, see also Cheniae & Martin (1978). A subsequent centrifugation for 30-40 s at 815 *g* yielded a preparation of 90-100% intact chloroplasts as determined by ferricyanide reduction (Heber & Santarius, 1970). The assay medium consisted of 0.33 mol/l sorbitol, 0.5 mmol/l K_2HPO_4, 10 mmol/l $NaHCO_3$, 2 mmol/l HEPES, pH 7.5, unless otherwise indicated in the text. The CO_2-dependent O_2 evolution rate usually was in the range between 60 and 80 µmol O_2 per mg chlorophyll per hour.

The isolation procedure did not take more than 4-5 min. Broken chloroplasts were obtained by a 30 s osmotic shock in either H_2O, 10 mmol/l $MgCl_2$ or 10 mmol/l KCl and subsequent addition of double strength assay medium, containing 0.66 mmol/l sorbitol, 4 mmol/l HEPES, pH 7.5 and either 10 mmol/l $MgCl_2$ or 10 mmol/l KCl, unless otherwise indicated in the text.

2.2 FLASH-INDUCED ABSORBANCE DIFFERENCE MEASUREMENTS OF P515

Flash-induced absorbance changes were measured in a modified Aminco-Chance absorption difference spectrophotometer (Fig. 2a). The apparatus operated in the dc single-beam mode. A 0.3 x 0.3 cm sample cell was inserted into a special holder that could be thermostated. Flash illumination (half-time 8 µs) with a General Electric FT-230 flashtube or a Xenon 457 flashtube of wavelengths above 665 nm reached the sample via a flexible light guide. The photomultiplier was shielded from stray light by a Wratten 58, BG 38 and BG 18 filter combination. Single flashes or a group of 2-6 flashes with time intervals of 100 ms were

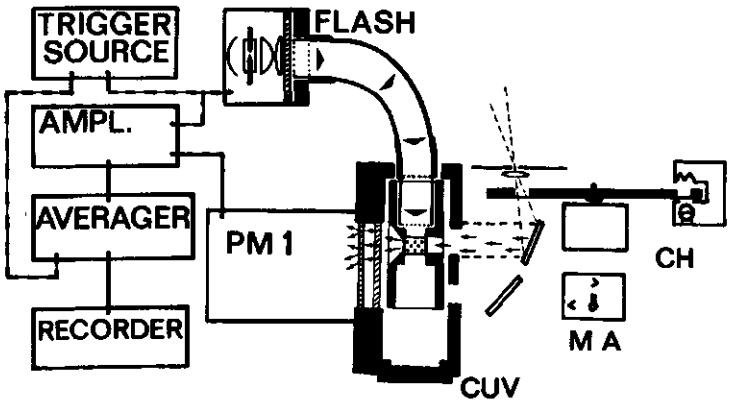

Fig. 2a. Schematic diagram of the equipment to measure flash-induced absorbance changes in chloroplast suspensions. The chopperblade (CH) is at a fixed position allowing one measuring beam of a certain wavelength to pass the cuvette via an adjustable mirror assembly (MA). Transmitted light is detected by a photomultiplier (PM1). After compensation with a dc voltage and subsequent amplification, the signal caused by a flash-induced change in transmission, is fed into a Data lab. 102A signal-averager. Several flash responses are averaged and the signal is monitored on a recorder or an osciloscope. A pulse generating system, Tektronix Rate/Ramp generator 26G1 triggering a Ramp generator 26G2 and two pulse generators 26G3, controls the time of flash firing and provides trigger pulses for the averager and the osciloscope (Tektronix 7313).

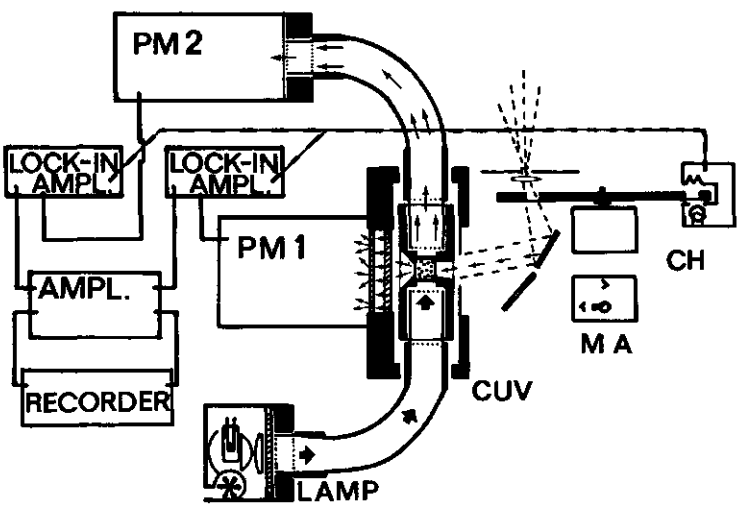

Fig. 2b. Operation mode of equipment to measure absorbance changes and 90°-scattering changes simultaneously. Chopper rotation causes a 300 Hz modulated passage of the measuring beam of a fixed wavelength through the cuvette, which allows an absorbance (and scattering) measurement at this wavelength against a dark reference. Light, scattered by the chloroplast suspension is detected by a second photomultiplier (PM2). Further details are in the text.

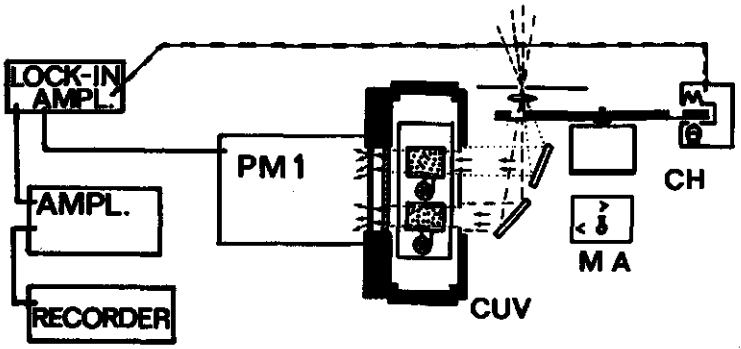

Fig. 2c. Equipment to measure absorbance changes caused by addition of substances to one of the cuvette compartments. Chopper rotation directs measuring light of a selected wavelength alternatively through the measuring and reference compartment of the cuvette. The ac signal is fed into a Lock-in amplifier and the resulting dc signal is recorded.

fired. The repetition rate usually was 0.05 Hz. A number of either 32 or 64 absorbance responses were sampled and averaged using a Data Lab 102 A signal-averager. In some experiments 38 or 70 flashes were fired at a repetition rate of 3-4 Hz. In this case 6 flashes were used for pre-conditioning the sample.

2.3 ABSORBANCE CHANGES OF P515 AND 90°-SCATTERING CHANGES DURING PROLONGED ILLUMINATION

Absorbance changes and 90°-scattering changes were measured simultaneously in the same apparatus (Fig. 2b) now operating in the chopped single-beam mode (monochromatic measuring beam alternating against dark). Scattering changes were monitored by a second photomultiplier via a flexible light guide, placed against the side wall of the cuvette. The photomultipliers were shielded from stray light by a Wratten 58, BG 18 and BG 38 filter combination. The sample was illuminated by light from a 250 W tungsten lamp placed in a modified lamphouse assembly of a lightprojector. The excitation light was of wavelengths above 665 nm. The temperature of the sample was kept at 2°C. Beam modulation (300 Hz) was achieved by a rotating chopper blade. The signal of a light-emitting detection system (Data Technology AD 2010 E) was used as the reference input for a Brookdeal Lock-in Amplifier Type 401 A and a Brookdeal Phase sensitive Detector 9412, that served to convert the modulated response of the photomultiplier into a proportional dc signal. This signal was amplified and recorded.

2.4 SALT-INDUCED ABSORBANCE CHANGES OF P515

Absorbance changes caused by the addition of salts at characteristic wavelengths in the region between 460 and 700 nm were measured in the split-beam mode of operation (Fig. 2c). The monochromatic beam of measuring wavelengths alternatively passes sample and reference compartment of a special cuvette (Fig. 3), see also Tonk et al. (1979). The motor-driven pinions (a and b) coupled by the string (e), rotate synchronously in two small cylindrical channels (5 mm diam.) which have open connections with the upper and lower parts of sepa-

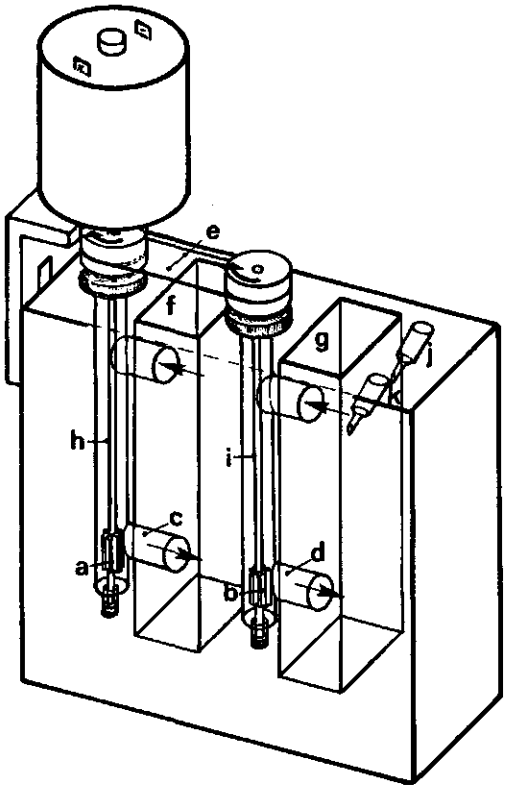

Fig. 3. Cuvette, designed to measure absorbance changes in chloroplast suspensions caused by addition of substances through inlets k and j in one of the compartments (g), with reference to compartment f.

rated reference (f) and measuring (g) compartment (34 x 8 x 10 mm). The centers of the lower holes (c and d) are off-line with the shafts (h and i) of the rotating pinions. Clockwise-directed rotation therefore causes a suction into the upper holes and an upward directed pressure in the compartments, which leads to a counter-clockwise circulation of the suspensions in both compartments. Because of the parallel and synchronized flow of particles, split-beam absorbance difference measurements are much less limited in sensitivity and accuracy by whirling effects. The mixing time after the pulsed injection (through inlets j or k) of a small volume of a concentrated chemical effector in the measuring compartment was found to be less than 1 s. The double-beam mode (monochromatic beams of measuring and reference wavelength alternatively passing through a single compartment of cuvette) was used to measure accurately absorbance difference spectra of KCl-induced changes in the presence of 3 µmol/l valinomycin and 2 mmol/l $MgCl_2$. Salts were added as concentrated solutions (1 mol/l via micro-syringes).

2.5 POTENTIAL AND RESISTANCE MEASUREMENTS BY MEANS OF MICRO-CAPILLARY GLASS ELECTRODES

Measurements were performed on chloroplasts of *Peperomia metallica* in a thin leaf section (Fig. 4). Growth conditions of plants and experimental procedures were essentially the same as described by Vredenberg et al. (1973) and Bulychev et al. (1973). Fig. 5 shows the diagram of the experimental arrangement. Potentials were measured by means of a micro-capillary glass electrode (Fig. 6) filled with 3 mol/l KCl in contact with a Ag- AgCl wire, via an agar KCl bridge (Fig. 7). The potential (change) of the electrode, inserted into a single chloroplast, was measured with reference to an external electrode. The electrode signal was fed into a high-impedance unity gain amplifier (IL PICO- metric Amplifier - 181), and recorded on an oscilloscope or a galvanometric recorder with a time resolution of 0.5 ms. Photoreactions were induced either by prolonged illumination from a modified lamphouse assembly of a lightprojector (250 W), or by flashes with half-life times of 8 μs and 80 μs using a Xenon or a Rollei flash device, respectively. The light reached the sample via a flexible light guide (1 mm diameter). The position of the light guide was at a distance of 1 to 4 mm from the leaf section. The position of the electrode was stabilized, quenching environmental vibrations by a specially constructed table (W. Tonk, unpublished). Photoresponses could be observed during a period of 5-15 min after insertion of the electrode.

The membrane resistance was recorded with switch S in position 1 (Fig. 5). With the 10^9 Ω resistor in the current circuit, the current pulses, either hyperpolarizing or depolarizing the membrane, were of low amplitude (less than 1 nA). The current pulses

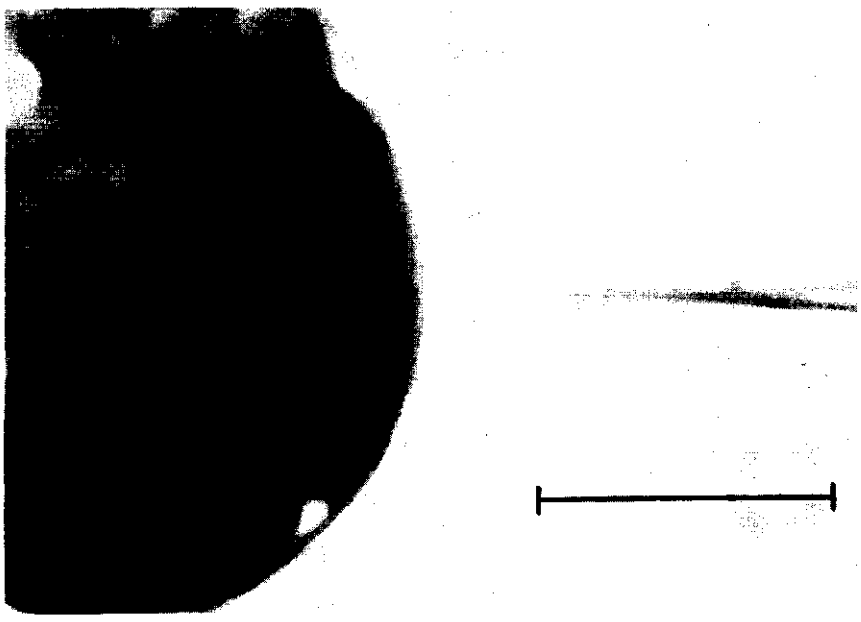

Fig. 4. Light-microscope picture of a leaf section of *Peperomia metallica* with one cell, containing four chloroplasts. The glass electrode is positioned near to the cell wall. The bar corresponds to a length of 20 μm.

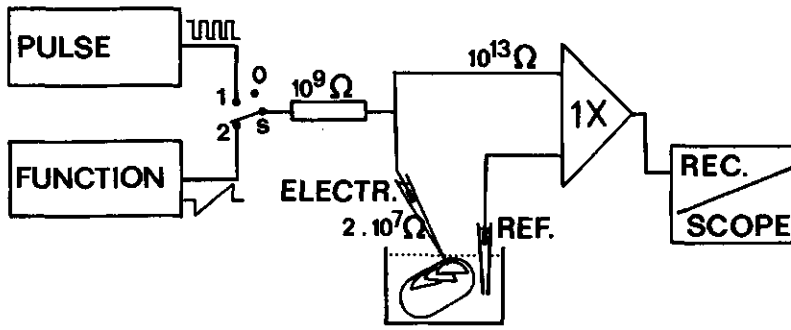

Fig. 5. Scheme of electric potential measurements in a leaf section of *Peperomia metallica*. The potential is measured across chloroplast membranes by a micro-capillary glass electrode inserted into a single chloroplast with reference to an electrode in the surrounding solution. The signal is fed into a high-impedance unity gain amplifier and subsequently monitored on an osciloscope or recorder.
A pulse generator (switch S in position 1) or a function generator (switch S in position 2) allowed current injection, limited by a 10^9 Ω resistor in the circuit, across the chloroplast membranes. Light-induced potential changes were measured with the switch in position 0.

passed across the membrane through the electrode towards the external reference electrode. The capacitance of the electrode was compensated by a negative-capacitance coupling circuit of the PICO-metric amplifier.

Current-voltage characteristics of the thylakoid membrane were measured by means of a current-scanning technique, basically similar to the one described by others (Coster, 1965; Vredenberg & Tonk, 1974b). Switch S is put in position 2, connecting the current circuit with the output of a function generator, operating in a single-sweep mode, which generates a voltage that changes at a uniforme rate. The current limited by the 10^9 Ω load resistor changed from -1 (inward current) to +1 nA (outward current) at a rate of 0.66 nA/s. Special attention was paid to the linearity of the I-V characteristic of the measuring electrode.

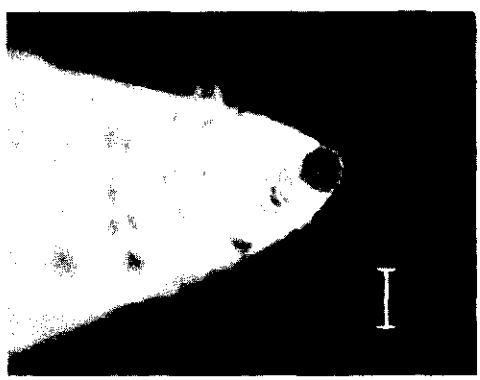

Fig. 6. Scanning electron micrograph of the open electrode tip. The bar corresponds to a length of 1 µm. The micrograph was made by the Technical and Physical Engineering Research Service, Electron Microscopy Section at Wageningen.

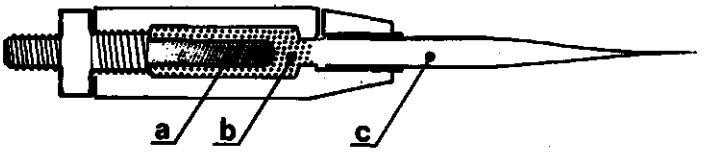

Fig. 7. Perspex holder for micro-capillary glass electrode. The Ag-AgCl wire (a) is in contact with the KCl-filled glass electrode (c) via a KCl-agar bridge (b).

In some experiments chloroplasts were isolated in a medium containing 0.25 mol/l sucrose, 25 g/l Ficoll, 100 mg/l bovine serum albumen, 12 mmol/l NaCl, 20 mmol/l HEPES--NaOH, pH 7.6. One single chloroplast was immobilized by suction on a fire-polished tip of a glass pipette (tip diameter 10-12 μm), and the electrode could be inserted. Further procedures were as described in this section and appeared to be applicable to this system without further adaptations.

2.6 PARTICLE ELECTROPHORESIS OF CHLOROPLASTS

The electrophoretic mobility (u) of individual chloroplasts was determined in a thermostated (25°C) laterally orientated rectangular quartz chamber (depth 0.7 mm, height 14 mm) connected to reversible Cu-$CuSO_4$ electrode compartments. The instrument, basically similar to the one described by Fuhrmann et al. (1964), was equipped with phase contrast optics (Leitz, large working distance) and a fibre-optics actinic illuminator providing red light (650 nm cut-off filter) with an intensity of 220 W/m^2 at the electrophoresis chamber. Measurements were performed at constant currents (1-5 mA) resulting in electric field strengths between 5 and 35 V/cm. The observation light was filtered by a green (540 nm) interference filter and kept at the lowest possible intensity in order to minimize actinic effects. The objective was focussed on one of the two pre-determined stationary phases. The electrophoretic migration was timed for both forward and backward (reversed field) runs over a known distance (37.5 or 75 μm). Usually 10 consecutive runs of different chloroplasts of the same type were registered and the mean values of u are given in tables and figures; within the same chloroplast preparation the standard deviations were mostly 1 to 3%, never exceeding 8%. The electrical conductance and viscosity of the different media, including chloroplasts were measured with conductivity bridges (Philips PR 9501 and Radiometer CDM 3) and an Ostwald-type viscosimeter, respectively.

Glutaraldehyde-treated chloroplasts were obtained by incubating broken chloroplasts for 2 min in 2.5% glutaraldehyde, followed by two washing steps.

Electrophoretic measurements were done in close collaboration with Dr. A. Theuvenet, Dept. of Chemical Cytology of the University at Nijmegen and Dr. R. Kraayenhof and workers, Dept. of Biology of the Free University at Amsterdam.

2.7 CHEMICAL ANALYSES

- Chlorophyll concentrations were determined by the method of Bruinsma (1961).
- Protein was determined by the Biuret reaction of Gornall & Bardawill (1949).
- Light-dependent Mg^{++} flux across thylakoid membranes was detected by a Mg^{++}-selective electrode placed in a cylindrical perspex cuvette (W. Tonk, unpublished). Stirring of the suspension was achieved by rotation of the cuvette caused by a constant flow of air against a paddle wheel. The advantage of this stirring method above the more commonly used magnetic stirrer is a suppressed deterioration of the chloroplasts and an improvement of the signal to noise ratio. The suspension was illuminated by light from a 250 W tungsten lamp placed in a modified lamphouse assembly of a lightprojector (Leitz).
- Light dependent oxygen evolution was monitored polarographically with either a separate oxygen electrode (Rank Brothers) or a Gilson Oxygraph. The temperature was kept constant at 20°C.
- Phosphorylation activity of chloroplast preparations was measured by ATP consuming luciferine/luciferase mediated light emission. The chloroplast suspension was illuminated under phosphorylating conditions with light of wavelengths above 665 nm. Immediately after cessation of the illumination, the quantum yield of the ATP hydrolyzing luciferine/luciferase reaction was detected by a photomultiplier. Calibration was carried out by titrating known amounts of ATP.
- A CF_1 preparation from spinach chloroplasts was isolated by a chloroform-water extraction procedure of fragmented thylakoid membranes and subsequent ammoniumsulphate precipitation of the protein fraction in the water phase. Further purification by Sepharose 6B chromatography yielded a highly purified CF_1 fraction. Antibodies against CF_1, elicited in rabbit, were a donation of Dr. J. Verheyen of the Biochemistry Department, University of Amsterdam. In the experiments dealing with these antibody preparations, the serum from the same animal before immunization was used as a control.

3 Evidence for a light-induced blue bandshift of part of the P515 pigment pool in intact chloroplast membranes

3.1 INTRODUCTION

The light-induced difference spectrum in the 460-540 nm wavelength region, measured in algae and chloroplasts, is characterized by a maximum around 518 nm, a minimum at about 480 nm and an inflection in the 490-500 nm wavelength region (Duysens, 1954; Roux & Faludi-Daniel, 1977; Bouges-Bocquet, 1977; Conjeaud et al., 1976). Evidence has been given that this spectrum is the result of an electrochromic response of chlorophyll *b* and carotenoid pigments to the electric field, generated by the primary light-induced charge separation in the membrane (Junge & Witt, 1968; Reich et al., 1976).

Reich et al. (1976) showed that the frequency shift of the absorption of a molecule was related to the strength of the electric field. The relation is given by:

$$h\Delta\nu = -|\vec{\mu}e - \vec{\mu}g|\vec{F} - \tfrac{1}{2}(\alpha e - \alpha g)\vec{F}^2,$$

in which $\Delta\nu$ is the frequency shift of the absorption, due to the electrical field $\vec{F}$, $\vec{\mu}g$ and $\vec{\mu}e$ present the permanent dipole moment of the molecule in its ground state and excited state, respectively; αg and αe present the polarizability of the ground state and the excited state respectively; h is the constant of Planck. Because carotenoids lack a permanent dipole moment $\vec{\mu}$, only the quadratic part of the relation would be expected to contribute to the shift. Experiments, however, showed a linear relationship between the electric field and the absorbance shift at a fixed wavelength (Jackson & Crofts, 1969a; Amesz & De Grooth, 1976; Chapter 4). Thus, it has been suggested that the carotenoids are exposed to a high permanent field ($\vec{F}$int), due to asymmetrical complex formation with polar molecules (Sewe & Reich, 1977a; 1977b; Reich et al., 1976; Reich & Scheerer, 1976). In the case of carotenoids such a ligand formation has been shown to induce a dipole moment related to the strength of the permanent field (Reich et al., 1976) with:

$$\Delta\vec{\mu} = \Delta\alpha// \cdot \vec{F}\text{int},$$

in which $\Delta\alpha//$ is the polarizability of the carotenoid molecule parallel with the long axis of the molecule. Consequently:

$$h\Delta\nu = -|\Delta\alpha// \cdot \vec{F}\text{int}|\vec{F} - \tfrac{1}{2}(\alpha e - \alpha g)\vec{F}^2$$

Sewe & Reich (1977b) estimated that the magnitude of the permanent field, caused by asymmetrical complex formation is of the order of 1.7×10^6 V·cm^{-1}. For chromatophores an even higher field strength has been reported (Amesz & De Grooth, 1976), that may exceed

7×10^6 V·cm^{-1}. In comparison, the photo-electric field across a thylakoid membrane has been approximated to be about 1.2×10^5 V·cm^{-1} (Reich & Scheerer, 1976). Therefore, the quadratic term in the equation can be neglected. Witt (1979) inferred that the absorbance change $\Delta A(\lambda)$ is related to a generated electric field of moderate strength according to:

$$\Delta A(\lambda) = c\left(\frac{\delta A}{\delta \lambda}\right) |\vec{F}|$$

The constant c is proportional to the scalar product of the magnitude of the induced dipole moment and the cosine of the angle between the molecular axis and the field direction. This means that:
- $\Delta A(\lambda)$ is linearly dependent on the field strength
- the shape and wavelength location of the difference spectrum $\Delta A(\lambda)$ is invariable and proportional to the first derivative $\left(\frac{\delta A}{\delta \lambda}\right)$ of the absorbance spectrum.

De Grooth & Amesz (1977) suggested that, at least in bacterial chromatophores, the pool of carotenoids showing electrochromism may not be completely homogeneous and may consist of molecules with slightly different peak wavelengths and bandshifts depending on the strength and orientation of local electric fields.

In this chapter we discuss an effect of cations on the light-induced difference spectrum of P515 in chloroplasts. It is shown that in intact chloroplasts, as well as in broken chloroplasts in the presence of cations, part of the pigments in the pool respond in a way that, upon flash illumination, their absorption band is shifted towards a shorter wavelength. This blue shift, which gives rise to the inflection around 500 nm in the overall difference spectrum, is interpreted in terms of a cation-dependent change in the orientation of the pigment complex with respect to an induced photo-electric field, or of a cation-induced migration of the primary acceptor of Photosystem 2 into the hydrophobic region of the membrane. Thus, in the latter case, part of the pigments might become exposed in the light to an oppositely orientated field between the primary acceptor and the outer surface of the membrane.

3.2 MATERIAL

Intact chloroplasts from fresh spinach leaves were isolated as described in Chapter 2 except for the following modifications. The incubation medium of intact chloroplasts contained 330 mmol/l sorbitol and 30 mmol/l tricine pH 8.0. Broken chloroplasts were obtained by brief osmotic shock either in water, or salt solution containing 10 mmol/l $MgCl_2$ and 15 mmol/l KCl and subsequent washing in incubation medium containing, in addition to 330 mmol/l sorbitol and 30 mmol/l tricine, no salt or 10 mmol/l $MgCl_2$ and 15 mmol/l KCl, respectively. Stock suspensions of chloroplasts were diluted 6-10 fold in assay medium which was similar to the incubation medium. In suspensions of broken chloroplasts, 20 µmol/l diquat was added as electron acceptor. Final chloroplast concentrations were equivalent to approx 100 µg/ml chlorophyll.

3.3 FLASH-INDUCED ABSORBANCE DIFFERENCE SPECTRA

Fig. 8a shows the spectra of flash-induced absorbance changes in intact and broken chloroplasts in the presence and absence of ions, respectively. The two spectra are distinctly different. The ratio of the absorbance changes at the maximum 515 and the minimum 480 nm is higher in intact chloroplasts than in broken chloroplasts. In addition, the spectrum of the intact chloroplasts shows an inflection in the 490-500 nm region. The spectrum of broken chloroplasts, isolated and incubated in a salt-containing medium was found to be similar to the spectrum of intact chloroplasts (not shown). Thus, the spectral differences

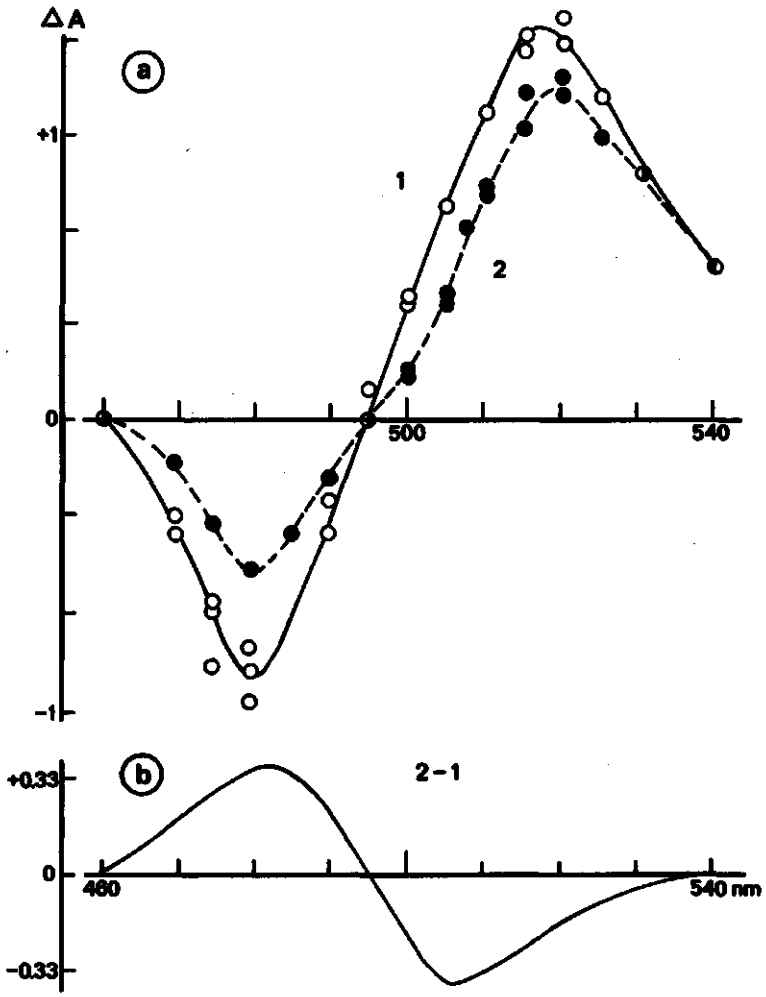

Fig. 8. a) Spectra of flash-induced absorbance changes in intact and broken chloroplasts, (curves 2 and 1, respectively).
Intact chloroplasts suspensions contained in addition to sorbitol and tricine, 10 mmol/l $MgCl_2$ and 30 mmol/l KCl. Broken chloroplasts were prepared and incubated in the absence of cations. The absorbance changes are given in relative units. b) Spectrum obtained by subtraction (curve 2-1) of the two spectra in Fig. 8a.

appeared to be due to ionic effects.

Induction and recovery of the flash-induced absorbance changes for each preparation had the same kinetics at all wavelengths studied, but no attempt was made to distinguish the kinetics of ΔA515 in different chloroplast suspensions. A detailed study of these kinetics is given in Chapter 5. The magnitudes of ΔA515 were found to vary linearly with chlorophyll concentration.

As shown in Fig. 8b, subtraction of the two spectra of Fig. 8a gives a spectrum with a minimum around 505 nm, a maximum at 483 nm and zero points at 460, 495 and 535 nm. Thus, it appears that the difference spectrum of intact chloroplasts is caused by two different bandshifts, one similar to the red bandshift observed in broken chloroplasts (Fig. 8a, see also Chapter 4) and one (Fig. 8b), indicative for a blue bandshift.

3.4 SIMULATION OF BANDSHIFTS

Fig. 9 (curve "a + b") shows a computed difference spectrum composed of a 1 nm red bandshift of a single Gaussian band A (maximum at 497 nm, half width 44 nm) and a 1 nm blue bandshift of a Gaussian band B (B = 0.18 A, maximum at 495.5 nm, half width 25 nm). For comparison the spectra of bandshifts A (curve a) and B (curve b) are given. The computed difference spectra a and a + b appear to be a reasonably good simulation of the spectra 1 and 2 measured in the chloroplasts (Fig. 8). Obviously, the width of the negative band of the experimental difference spectra is smaller than that of the computed spectra. Moreover, the latter would need a small baseline shift (Fig. 9) to match the asymmetry observed in the experimental spectra.

3.5 DISCUSSION

Incubation of chloroplasts in an ion-free environment will lead to unstacking (Gross & Hess, 1974; Telfer et al., 1976), changes in the membrane surface charge (potential) (Barber & Mills, 1976), and to structural changes in the membrane (Gross & Hess, 1974; Murakami et al., 1975). The structural changes will affect the mutual orientation of pigments.

Thus, in the presence of high concentrations of Mg^{++} and K^{+}, which for instance exist in the intact chloroplasts (Nobel, 1969; Vredenberg, 1977), the carotenoids in the membrane might become exposed to electric fields with a different orientation and magnitude than in an ion-free environment. If so, our results can be interpreted in terms of two alternative models: (i) In stacked chloroplasts, in the presence of mono- and divalent cations, part of the electrochromically responding carotenoids have a dipole orientation which, with respect to the light-generated electric field, is different from the one of the bulk of the carotenoids. But in the absence of ions all field-indicating pigments are orientated in the same direction. (ii) All carotenoids have the same dipole orientation, both in the absence and presence of ions, but a small part of them, notably those located in the vicinity of the primary acceptors of the reaction centers at the outer core of the membrane, become exposed to an oppositely orientated light-generated electric field between the charged primary acceptors and the outer membrane surface. This could perhaps occur if the acceptor has migrated more into the hydrophobic region of the membrane under high-salt

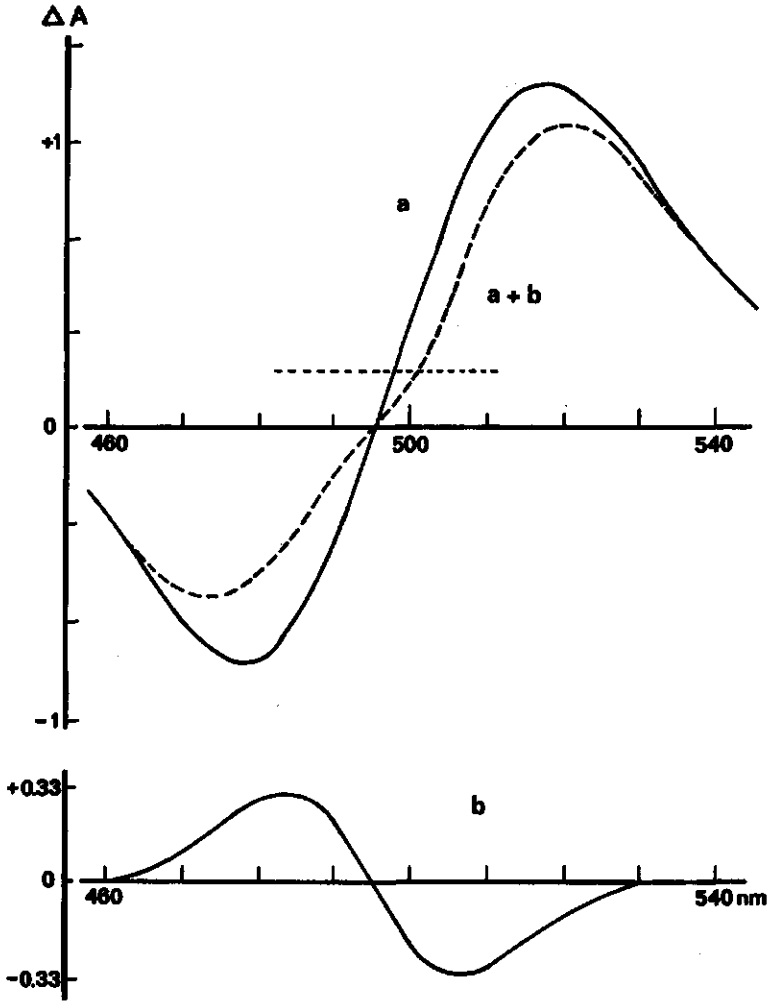

Fig. 9. Computed difference spectrum (curve a + b) composed of a 1 nm red bandshift (curve a) of a single Gaussian band A and a 1 nm blue shift (curve b) of a Gaussian band B (B = 0.18 A). The broken horizontal line is the original zero line of the spectra. The spectra are plotted in relative absorbance units.

conditions. The postulated migration would become reversed in the absence of ions. According to an interpretative model proposed by Renger (1975) the migration of the primary acceptor Q of Photosystem 2 including its proteinaceous shielding complex, could take place in the presence of surfactants. Furthermore, a field-driven movement of Q into the membranal plane has been suggested (Joliot & Delosme, 1974).

Although I am not in the position to discriminate between the two alternatives, I consider the first one as the most plausible one. The fraction of pigments that shows a blue bandshift cannot be estimated with precision as yet. Analyses of the data of Figs 8 and 9 indicate that when the blue bandshift is assumed to be equal to the one of the red bandshift of the bulk pigments, the former one is caused by 18% of the bulk pigments. The

absorption band of the blue-shifting fraction presumably has a somewhat smaller half-width (25 nm) than that of the bulk (44 nm) and is located at a slightly lower wavelength. These differences suggest a different chemical environment which would be in harmony with the models discussed.

4 Salt-induced absorbance changes of P515 in broken chloroplasts

4.1 INTRODUCTION

Voltage calibration of the 515 nm absorbance change usually is made with reference to the absorbance change in response to a single-turnover saturating light flash. According to approximations about the number of charges loading the membrane capacitance, a single flash would generate a transmembrane potential of approx. 50 mV (Junge & Witt, 1968; Emrich et al., 1969; Witt & Zickler, 1973; Witt, 1971). Potential measurements with micro-capillary glass electrodes inserted in a single granum stack of chloroplasts of *Peperomia metallica* have indicated that a single-turnover light flash generates a potential across the thylakoid membrane in the range between 10 and 50 mV (Bulychev et al., 1972; Vredenberg et al., 1973; Vredenberg & Tonk, 1975; Vredenberg, 1976).

According to the results of studies on the effect of the voltage-dependent ionophore alamethicin on the kinetics of the 515 nm absorbance change, it has been concluded that the potential generated by a single flash is of the order of 100-200 mV (Zickler et al., 1976; Boheim & Benz, 1978). This value is at variance with the potential concluded from the micro-electrode approach. The discrepancy emphasizes the need for another independent voltage calibration method of the electrogenic charging of the thylakoid membrane.

Attempts have been made to measure electrochromic bandshifts, brought about by a transmembrane (diffusion) potential, generated by salt additions (Strichartz & Chance, 1972; Gross & Libbey, 1972). These experiments were successful in bacterial chromatophores (Crofts et al., 1972; Jackson & Crofts, 1969a) and have been used as a calibration method for determining the magnitude of the transmembrane potential, associated with photosynthetic energy conservation, in bacteria. However, this method could not be applied to chloroplast (thylakoid) membranes because substantial changes in light scattering occur upon salt additions, which prevented accurate measurements of the 515 nm bandshift (Strichartz & Chance, 1972; Gross & Libbey, 1972).

This chapter reports on experiments in which KCl-induced absorbance changes are measured in the 460-540 nm wavelength region under proper conditions at which scattering changes are minimized. The results indicate that a 515 nm absorbance change of 1.1×10^{-4} corresponds with a potential across the thylakoid membrane of 1 mV and that the electrogenic potential generated by a single-turnover saturating light flash is in the range between 15 and 35 mV.

4.2 MATERIALS AND METHOD

Chloroplasts were isolated from fresh leaves of spinach, grown in the laboratory glass house. Washed leaves were homogenized in an isolation medium containing 0.33 mol/l sor-

bitol and 2 mmol/l HEPES, adjusted to pH 7.1 with NaOH. The homogenate was filtered through four layers of perlon net (pore diameter 40 μmol/l), and centrifuged for 2 min at 2000 *g*. The sedimented chloroplasts were subjected to an osmotic shock resuspending them in 10 ml distilled water. After 1 min, 10 ml of double strength isolation medium was added to the suspension. The stock suspension of broken chloroplasts was diluted 10 to 15 fold in isolation medium giving a final concentration, equivalent to 15-25 μg/ml chlorophyll. Chloroplasts were used within 1 h after preparation.

Absorbance changes induced by salt addition or by single-turnover light flashes were measured as described in Chapter 2. The actinic flash was of wavelengths above 665 nm. The absorbance changes were measured at various wavelengths, selected by interference filters, in the region 460-540 nm. A number of flashes were fired at dark intervals of 0.3 s. Usually the signals of 64 flashes were sampled and averaged.

4.3 SALT-INDUCED ABSORBANCE CHANGES

The characteristics of the absorbance changes at 515, 540, 460 and 475 nm upon successive additions of 10 mmol/l KCl and 2 mmol/l $MgCl_2$, or *vice versa*, to a suspension of chloroplasts are shown in Fig. 10. Addition of 10 mmol/l KCl causes an absorbance decrease at these wavelengths. At 515 and 540 nm the decrease is preceded by a transient increase. It appears that the decrease in absorbance is composed of a fast and a slow component. The relative magnitude of these components is different at the wavelengths used. Subsequent addition of 2 mmol/l $MgCl_2$ causes an increase in absorbance at 515 and 540 nm, which approximately equals the preceding decrease caused by the addition of KCl (Figs. 10a and 10b). As can be seen, the kinetics of the $MgCl_2$-induced absorbance increase are multiphasic. The increase at 475 and 460 (Figs. 10c and 10d) caused by $MgCl_2$ in the presence of KCl is relatively small as compared to the increase at 515 and 540 nm. Addition of 2 mM $MgCl_2$ to a suspension of chloroplasts in the absence of KCl causes an increase in absorbance at 515 and 540 nm and a decrease at 475 and 460 nm. The absorbance changes caused by $MgCl_2$ at these wavelengths appear to be composed of a slow and a fast component. Subsequent addition of 10 mmol/l KCl causes a small and reversible absorbance increase at 515 and 540 nm. Absorbance changes of this kind at 475 and 460 nm could not be resolved with sufficient accuracy from the response caused by the dilution effect. The absorbance changes brought about by $MgCl_2$ addition in the absence and presence of KCl, respectively, were found to be saturated at a $MgCl_2$ concentration of 1-2 mmol/l (not shown).

The characteristics of the absorbance changes upon KCl addition in the presence of $MgCl_2$, as observed at 515 (and 540 nm) (Figs. 10a and 10b), were studied in more detail. The accuracy of the measurements was improved by performing them in the dual-wavelength operation mode of the spectrophotometer. In order to suppress disturbances caused by the dilution effect, measurements were done with reference wavelengths in the 570-680 nm wavelength region, at which the absorbance of the chloroplast suspension was equal to the one at the measuring wavelengths in the 460-540 nm region. For example, 570 and 642 nm were used as reference wavelengths for measurements at 540 and 515 nm, respectively. It was verified that the absorbance changes at the reference wavelengths upon addition of KCl in the presence of $MgCl_2$ and valinomycin, were negligibly small as compared to the ones at the

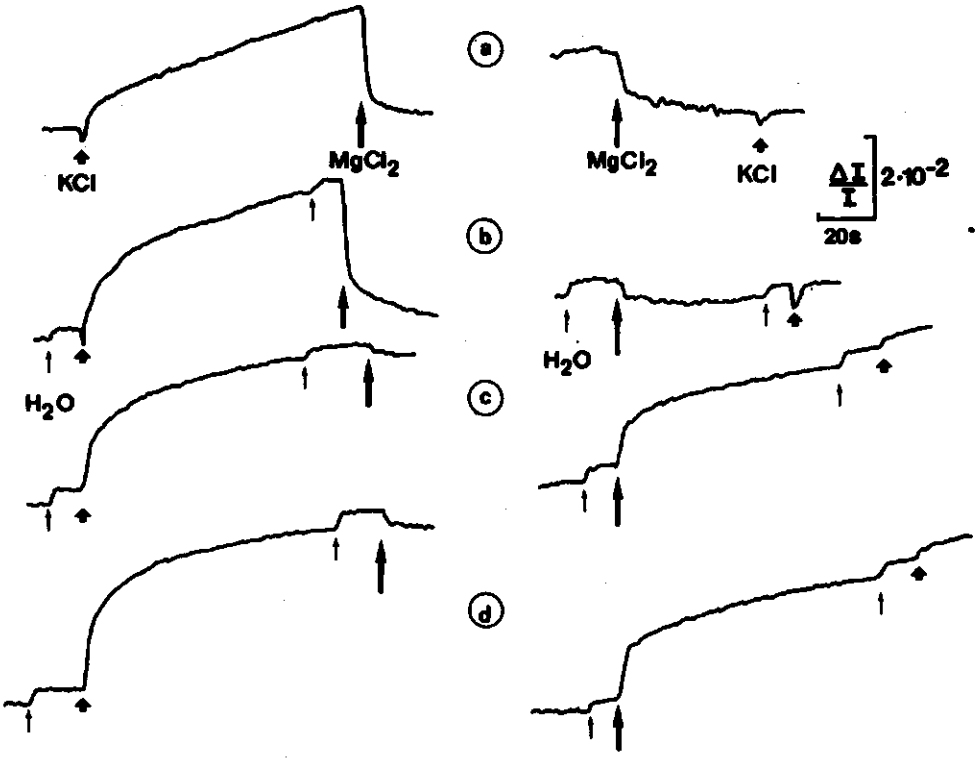

Fig. 10. Kinetics of salt-induced absorbance changes at 540 (a), 515 (b), 475 (c) and 460 nm (d) in a cation-free suspension of broken chloroplasts (15 µg/ml chlorophyll). Final concentrations of KCl and $MgCl_2$ were 10 and 2 mmol/l respectively. Addition of $MgCl_2$, KCl and H_2O are indicated by long, short and thin upward pointing arrows, respectively. The response upon additions of H_2O at the same amount as the salt solution indicates the dilution effect. The spectrophotometer was operating in the split-beam mode. An upward inflection means a decrease in absorbance.

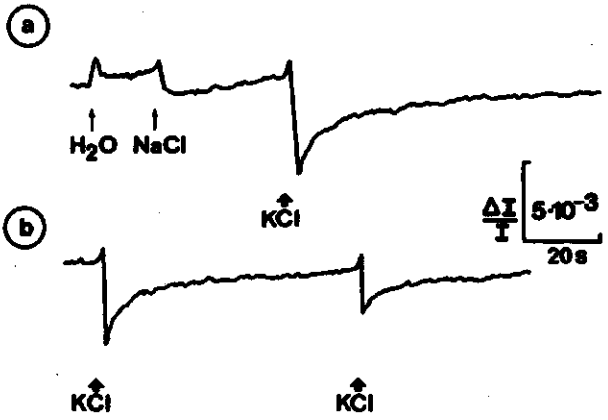

Fig. 11. Absorbance changes at 515 nm upon two subsequent additions of 10 mmol/l KCl (b) or of 10 mmol/l NaCl and 10 mmol/l KCl (a) to a chloroplast suspension (25 µg/ml chlorophyll) in the presence of 2 mmol/l $MgCl_2$ and 2 µmol/l valinomycin. A downward inflection means an increase in absorbance. The spectrophotometer was operating in the dual-wavelength mode. The reference wavelength was 642 nm. The small initial transient signal was caused by the addition of the salt solution.

measuring wavelengths.

Fig. 11b shows the KCl-induced absorbance change at 515 nm in the presence of 2 mmol/l $MgCl_2$ and 3 µmol/l valinomycin. A fast increase is followed by a slow decrease to the initial level. A second addition of 10 mmol/l KCl causes a similar but smaller absorbance change. A small effect is also observed upon a prior addition of 10 mmol/l NaCl (Fig. 11a). A subsequent addition of 10 mmol/l KCl, in this case, gives rise to an absorbance change that is about equal to the one observed in the absence of NaCl and KCl in the suspending medium.

4.4 DATA FOR A VOLTAGE CALIBRATION OF THE P515 ABSORBANCE CHANGE

In Fig. 12 the absorbance change at 515 nm is plotted as a function of the KCl added to the chloroplast suspension. It appears that the increase in absorbance is proportional to the logarithm of the KCl concentration gradient, initially induced across the thylakoid membrane, at least for a concentration below 50 mmol/l. This suggests that the absorbance change is proportional to the membrane diffusion potential, set by the salt addition. KCl concentrations up to 50 mmol/l did not affect the magnitude of the light-induced absorbance changes at 515 nm. At a KCl concentration above 50 mmol/l the absorbance increase was higher than would be predicted from the relationship at the lower concentrations (not shown).

The time course of the light-induced absorbance change at 515 nm in the presence and absence of $MgCl_2$, respectively, is shown in Fig. 13. The magnitude of the fast initial

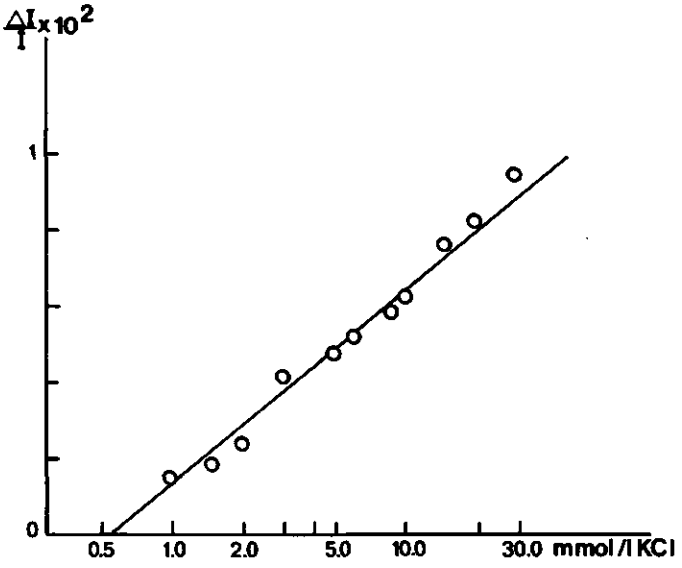

Fig. 12. The magnitude of the KCl-induced absorbance changes at 515 nm, measured in the presence of 2 mmol/l $MgCl_2$ and 3 µmol/l valinomycin, plotted as a function of KCl concentration. Chlorophyll concentration was 25 µg/ml. Other conditions were as described in the text.

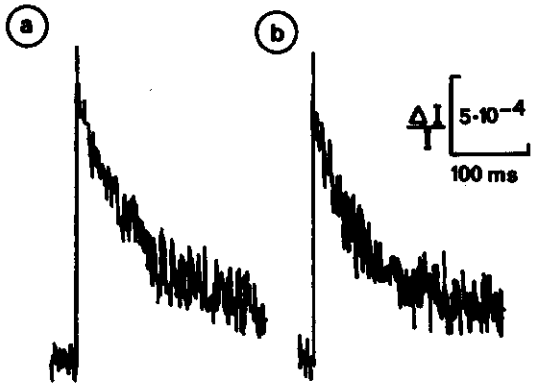

Fig. 13. Absorbance change at 515 nm upon single-turnover saturating light flashes in the presence (a) and the absence (b) of 2 mmol/l $MgCl_2$, respectively. Chlorophyll concentration was 23 µg/ml. Other conditions were as described in the text.

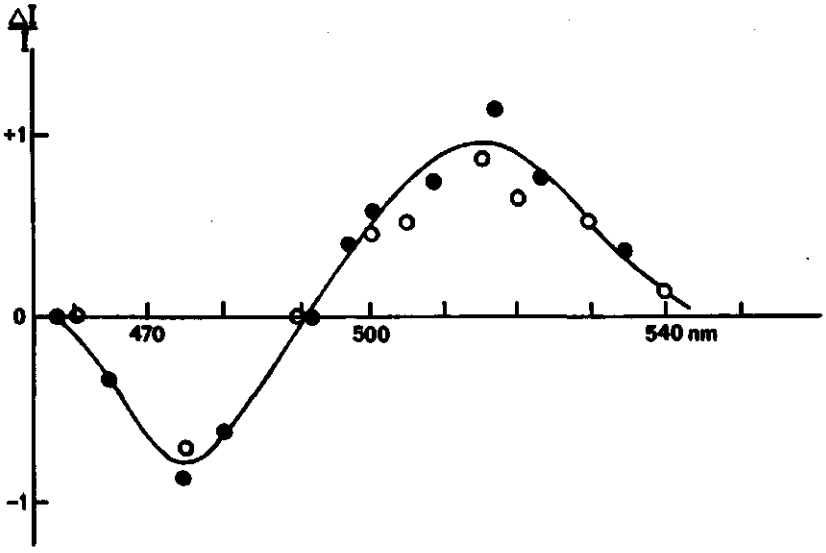

Fig. 14. Spectrum of absorbance changes induced by addition of 10 mmol/l KCl (open circles) or by a single-turnover light flash (closed circles), measured in the presence of 2 mmol/l $MgCl_2$ and 3 µmol/l valinomycin. The absorbance scale of the spectrum is plotted in relative absorbance units.

increase in absorbance caused by a single-turnover light flash, is independent on the presence of 2 mmol/l $MgCl_2$. $MgCl_2$ at concentrations above 10 mmol/l was found to suppress the magnitude of this response to a certain extent. The spectrum of the reversible absorbance changes caused by addition of 10 mmol/l KCl and the spectrum of the changes caused by single-turnover saturating light flashes appear to be similar (Fig. 14).

4.5 DISCUSSION

The spectrum of the reversible absorbance change in the 460-540 nm wavelength region upon addition of KCl to a chloroplast suspension containing $MgCl_2$ and valinomycin (Fig. 14) suggests that the change is closely associated with a reaction of P515.

Light-induced absorbance changes of P515 have been argued to be due to an electrochromic bandshift of these pigments, in response to the photo-electrical field (potential) across the membrane (Junge & Witt, 1968; Emrich et al., 1969; Witt & Zickler, 1973; Witt, 1971). The spectrum of the absorbance change caused by a single-turnover saturating light flash is identical with the difference spectrum of the salt-induced absorbance change (Fig. 14). A similar correspondence has been demonstrated for bacterial chromatophores with respect to the carotenoid bandshifts (Crofts et al., 1972; Jackson & Crofts, 1969a).

In strict analogy with these experiments, the data of Figs. 11 and 12 confirm that the absorbance changes induced by KCl addition in the presence of $MgCl_2$ occur in response to a membrane (diffusion) potential. (i) The absorbance change upon addition of 10 mmol/l KCl in the presence of valinomycin is much higher than upon addition of 10 mmol/l NaCl, as would be expected in the presence of this ionophore at which the membrane permeability to K^+ is high as compared to that of NaCl. (ii) The presence of NaCl in the suspending medium does not influence the extent of the absorbance change upon KCl addition, whereas the response upon KCl addition in the presence of KCl is significantly smaller. (iii) The extent of the 515 nm absorbance change is proportional to the logarithm of the concentration of added KCl (Fig. 12). Moreover, the decay kinetics of the absorbance changes closely resemble the kinetics of the KCl-induced changes in delayed light emission in broken chloroplasts (Barber, 1972a; 1972b). These changes indeed have been interpreted in terms of the decay of the membrane diffusion potential, due to the redistribution of K^+. The rate of this redistribution has been evidenced to be controlled by the rate of entry of Cl^- across the membrane into the thylakoid inner space (Barber, 1972a). It was found that the magnitude of the 515 nm absorbance change (Fig. 11), upon addition of a fixed amount of KCl (in the presence of $MgCl_2$ and valinomycin), is proportional to the chlorophyll concentration in the chloroplast suspension, for concentrations lower than 30 µg/ml chlorophyll. The same was found to be true for the magnitude of the light-induced absorbance change.

Fig. 12 shows that a 10-fold increase in K^+ concentration corresponds with a 515 nm absorbance change of approx. 4.9×10^{-3}. In the absence of permeating ions this increase would have corresponded with a change in the membrane diffusion potential of 58.5 mV (at 20°C), according to the Nernst equation. Correcting for the presence of Cl^- in the medium (2 mM $MgCl_2$) and substituting an estimated (Barber, 1972a) permeability ratio between Cl^- and K^+ of 0.04 in the presence of valinomycin, the Goldman constant field equation yields that, at the concentrations used, a 10-fold increase in K^+ concentration corresponds with a change in the membrane diffusion potential of about 35 mV. Thus the proportionality factor between 515 nm absorbance change and membrane potential is about 1.4×10^{-4} per mV. The data of Fig. 12 may need a correction factor for a small absorbance change that could have occurred at the reference wavelength (642 nm) used. According to spectral data of Reich et al. (1976) this correction factor is about 0.8. This would mean that the absorbance change at 515 nm has been 1.1×10^{-4} per mV at a chlorophyll concentration of 25

μg/ml. At the same concentration a single-turnover saturating light flash (e.g. Fig. 13) would cause an absorbance change of 2.2×10^{-3}, which corresponds with a membrane potential of 20 mV. Similar experiments, done with various samples, have indicated that a single-turnover saturating light flash causes the generation of a transmembrane potential (E_e) in the range between 15 and 35 mV with an average value of about 25 mV. This value is about equal to the estimated value of E_e associated with the charging of the membrane capacitance, due to one charge separation in all reaction centers present in the thylakoid membrane (Junge & Witt, 1968; Witt, 1971; Vredenberg, 1976), and falls within the range of flash-generated potentials measured with micro-electrodes in chloroplasts of *P. metallica* (Vredenberg & Tonk, 1975; Vredenberg, 1976). Values of about 100 mV have recently been concluded from analyses of the 515 nm absorption changes in chloroplasts in the presence of the voltage-dependent ionophore alamethicin (Zickler et al., 1976). The reason for the apparent discrepancy is unknown as yet. It might be that, contrary to the assumptions made (Zickler et al., 1976), the proportionality factor between concentration and the characteristic potential of the ionophore is different in artificial lipid bilayers and thylakoid membranes. The present calibration method has the important advantage that the light- and salt-induced 515 nm absorbance changes are measured in identical samples under equal conditions.

As can be concluded from the data of Fig. 10, the approach of setting K^+ diffusion potentials across the thylakoid membrane in order to measure its associated electrochromic shifts could only be applied if low concentrations of $MgCl_2$, or high concentrations of NaCl (not shown), were present in the suspending medium. In accordance with the results of others (Gross & Libbey, 1972), the time course of the irreversible absorbance changes in the 450-550 nm wavelength region upon addition of KCl in the absence of $MgCl_2$ (Fig. 10), indicates a change in light scattering, probably due to structural changes in the membrane, associated with cation binding at fixed negative charges. Subsequent addition of $MgCl_2$ results in an absorbance increase at wavelengths above 500 nm. This effect has been noticed by others (Gross & Prasher, 1974). It probably reflects the substitution of monovalent cations by divalent cations at the fixed charges, caused by changes of ionic concentrations near the electrical double layer (Barber et al., 1977; Chapter 8).

5 Analyses of the flash-induced P515 absorbance changes in isolated chloroplasts

5.1 INTRODUCTION

Recently it has been reported that the flash-induced ΔA515 in intact (Class I) chloroplasts (Horváth et al., 1978), like in intact algal cells (Joliot & Delosme, 1974), occurs with complex multi-phasic rise and decay kinetics. However, rise and decay of the flash-induced potential changes in coupled intact chloroplasts, measured with micro-electrodes, have been shown to follow single first-order reaction kinetics (Bulychev et al., 1972; Vredenberg & Tonk, 1974a; Bulychev & Vredenberg, 1976a). The decay rate of the potential change after a single flash was found to be dependent on the ionic permeability of the thylakoid membrane only (Bulychev & Vredenberg, 1976a). Apparent discrepancies on the magnitude and kinetics of the light-induced transmembrane potential changes, as measured with ΔA515 and micro-electrodes, respectively, have been a matter of discussion (Junge, 1977b; Rumberg, 1977; Vredenberg, 1976; Vredenberg & Tonk, 1975; Schapendonk & Vredenberg, 1977).

This chapter deals with analyses of the kinetics of flash-induced P515 absorbance changes and of the transmembrane potential, studied on thylakoid membranes of dark-adapted spinach and *Peperomia metallica* chloroplasts, respectively. The analyses suggest that the ΔA515 in intact and broken chloroplasts is the composite result of at least two different processes, which are called Reaction I and Reaction II. The kinetics of the ΔA515 caused by Reaction I, characterized by a rise time < 0.5 ms and a decay rate of 8.6-17.3 s^{-1}, show similarities with the kinetics of the transmembrane potential, measured by means of micro-capillary glass electrodes. The absorbance change of P515, associated with Reaction II and characterized by a rise time of 100-150 ms and a decay rate of 1.1-1.7 s^{-1} is presumed to be induced by a local intramembranal electric field associated with charge interaction between a compound at the reducing site of Photosystem 1 and cytochrome f or plastocyanin.

5.2 MATERIAL AND METHOD

Intact and broken chloroplasts were isolated from freshly grown spinach leaves and prepared by the method described in Chapter 2, except for the following modifications with respect to the incubation and assay medium. Intact chloroplasts were incubated and measured in a medium which contained 330 mmol/l sorbitol, 0.5 mmol/l K_2HPO_4, 10 mmol/l $NaHCO_3$, 2 mmol/l HEPES (pH 7.5) and 10 mmol/l $MgCl_2$. Broken chloroplasts were obtained by osmotic shock in H_2O containing either 10 mmol/l $MgCl_2$ or 10 mmol/l KCl. The assay medium contained 330 mmol/l sorbitol, 30 mmol/l tricine (pH 7.7), 20 μmol/l diquat and either 10 mmol/l $MgCl_2$ or 17.5 mmol/l KCl. Chloroplast concentration usually was equivalent to about 50 μg/ml chlorophyll. Flash-induced electric potential changes were measured on single chlo-

roplasts in intact leaves of *Peperomia metallica*. Experimental conditions and methods were as described in Chapter 2. Inhibition of the electron transport between Photosystem 2 and Photosystem 1 was achieved by pre-incubation of the sample in the presence of KCN, by the method described elsewhere (Ouitrakul & Izawa, 1973), or by addition of DCMU.

5.3 EVIDENCE FOR THE INDUCTION OF A FAST AND A SLOW P515 RESPONSE UPON SATURATING LIGHT FLASHES IN INTACT CHLOROPLASTS

A representative example of the time course of the ΔA515 upon a saturating light flash in intact spinach chloroplasts is shown in Figs 15a and 15b. Analyses of the semi-logarithmic plots of these changes reveal at least 5 different phases in the kinetics. After the initial fast absorbance increase, called phase a and completed within a time shorter than the resolution time of the measuring system (0.5 ms), a relatively slow increase in absorbance occurs in the first 10-20 ms up to an absorbance level which is more or less constant for the next 20-80 ms. According to the analysis, the subsequent decrease in absorbance appears to be composed of three different phases. A major phase c with a half-life time of 420 ms is preceded by a relatively fast phase c' with a half-life time of 75 ms, and followed by a small slow phase d decaying with a half-life time of 1.5 s. Phases c', and d are not resolved during the first 70-100 ms after the flash due to the slow absorbance rise or even constant level in this time period. However, extrapolation of the decay line of phase c' to time zero (Fig. 15b) results in an initial level which coincides reasonably well with the one reached in the fast phase a rise. This certainly is not true for phase c.

The data of Fig. 15b suggest that the phase c' decay is associated with the phase a rise. This suggestion is substantiated by the analyzed data of the absorbance response in a double flash (Fig. 15c). These show that the ΔA515 upon a second flash, characterized by a fast rise and a single decay phase equal to the c' component analyzed in the first flash, is superimposed upon the ΔA515 induced by the first flash. The break between the fast (c') and the slow (c) phase after the second flash is at the absorbance level determined by phase c and d of the first flash. The kinetics of the response upon third and following flashes, fired at time intervals in the range of 10-100 ms, were found to be similar to those induced by the second flash. Thus, we arrive at the conclusion that the rapid decay component (phase c') is associated with the fast rise (phase a). These components have been ascribed to a reaction, called Reaction I.

The deconvolution of the overall P515 response into Reaction I and II is shown in Fig. 16. The rise kinetics of Reaction II show a slow absorbance increase within 140 ms. This is called phase b. The decay of Reaction II after the flash is biphasic with rate constants determined by the characteristic relaxation times of phases c and d. For a large variety of preparations, the half-life time of phase c', c and d was found to be in the range of 40-80 ms, 400-600 ms and 1.5-3 s, respectively. Fig. 16, representative for intact spinach chloroplasts, shows that the phase a and b, associated with Reaction I and II, respectively, are about equal in magnitude. The spectra of these changes were found to be identical with the characteristic difference spectrum of P515 (e.g. Chapter 4). Therefore we suggest that Reactions I and II are due to electrochromic responses of the P515 pigment complex.

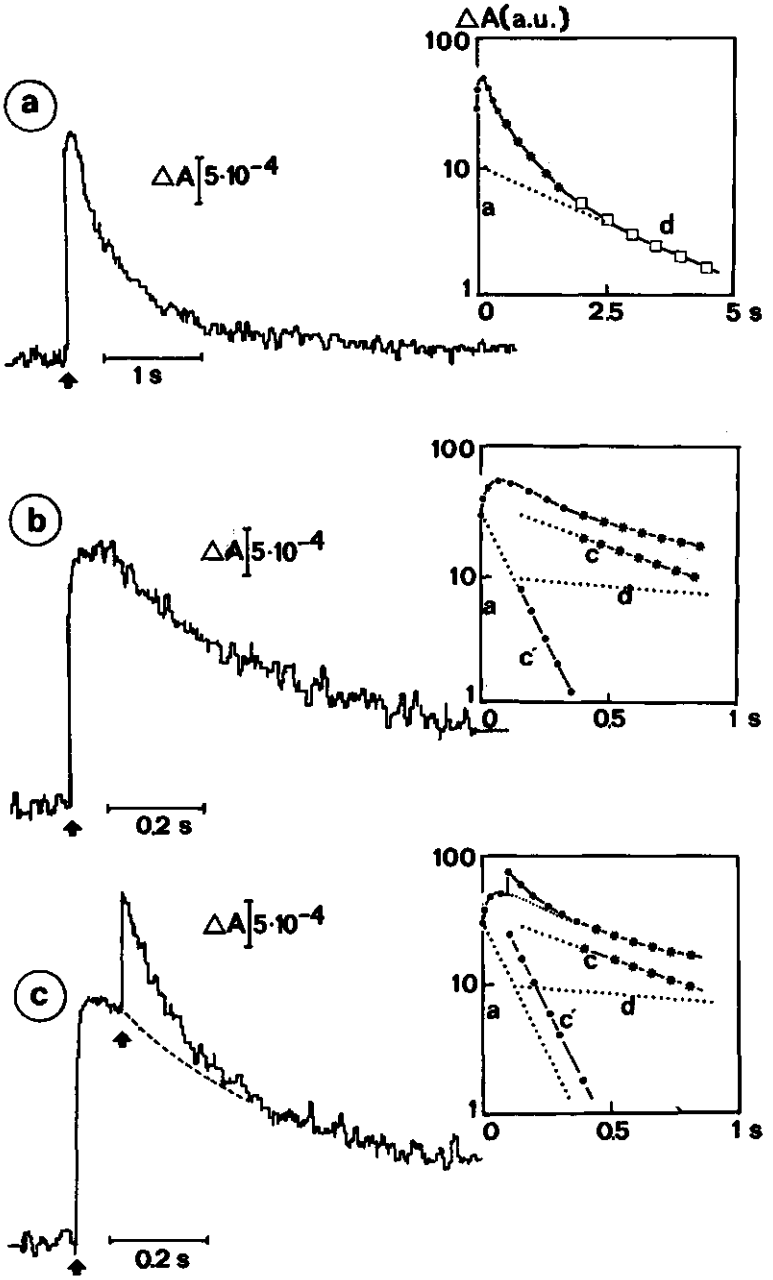

Fig. 15. Absorbance changes at 515 nm in dark-adapted intact spinach chloroplasts, induced by single (a,b) or double (c) flashes, recorded and displayed on 5 s (a) and 1 s time scales (b,c). Average of 32 single or double flashes, fired at a rate of 0.05 Hz. The arrows mark the moments at which the flashes were fired. On the right the corresponding semi-logarithmic plots of the experimental curves are shown and the curve analyses into exponential phase d (open squares), c (closed stars) and c' (closed circles), respectively, according to an adopted method (Atkins, 1969). (ΔA is given in arbitrary units (a.u.)). Extrapolation of phase d and subtraction of the absorbance of phase d from the experimental absorbance at corresponding times, gives the ΔA in the absence of phase d. This absorbance curve yields the measuring points of phase c. Analogously, phase c' is resolved (see text). The dotted line parallel with phase c' (lower right-hand figure) represents the fast phase c' induced by the first flash (middle right-hand figure). Other dotted lines represent the extrapolated parts of the respective phases.

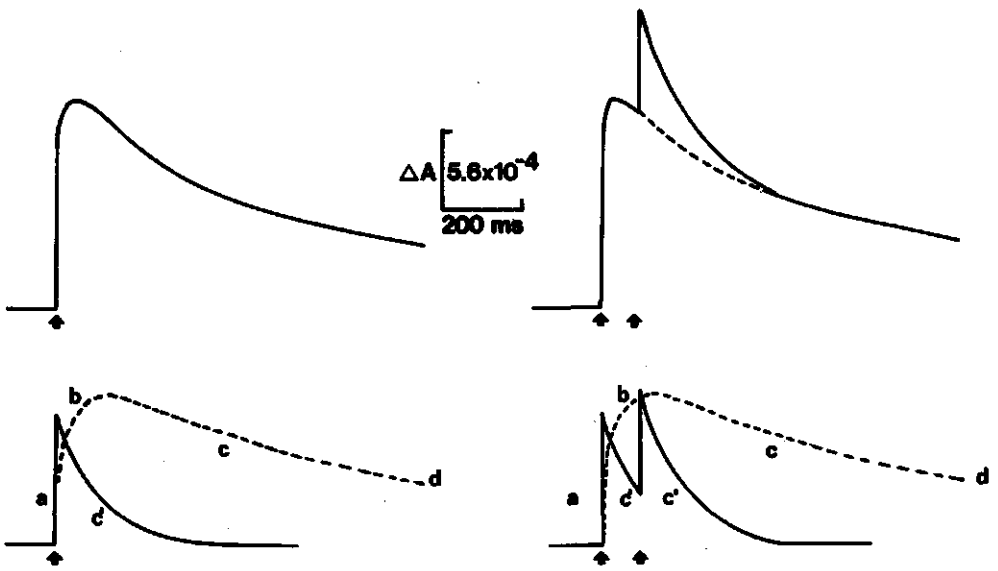

Fig. 16. Resolution (bottom part of figure) of the ΔA515 induced by a single flash and a series of two flashes (upper part of figure) into two components, Reaction I (solid curves) and Reaction II (broken curves). The curves were computed according to the data obtained from the curve analysis depicted in Fig. 15. Further explanations are in the text.

The spectrum of the relatively small phase d was found to be partially dissimilar to that of P515. Phase d, which is negligibly small in broken chloroplasts (see below), might represent the decay of a reaction different from Reaction I and II. Therefore, Reaction II in intact chloroplasts might need a small correction for the ΔA515 associated with phase d.

According to the data of Figs 15 and 16 one would expect for intact chloroplasts that, after one pre-illuminating flash, single flashes fired at a repetition rate of 4-5 Hz cause the ΔA515 of Reaction I only, i.e. ΔA515 with a fast rise (phase a) and a single exponential decay (phase c') with a rate constant of about 9 s^{-1}. This response is superimposed upon a constant absorbance level determined by Reaction II induced in the pre-illuminating flash(es). Experiments of this kind will be illustrated for broken chloroplasts in Section 5.4.

According to the analyses (not shown), the effect of a non-saturating second flash (Fig. 17b) is identical to that of a saturating second flash (Fig. 17a), except for the magnitude of phase c'. Fig. 17c shows the response upon a first non-saturating and a successive saturating flash. The absorbance increase induced by the second flash is enhanced as compared to the response after a saturating first flash. Its rise kinetics suggest a contribution of phase b in this case (not resolved here). The decay kinetics after the second flash appear to be identical to those of a second flash observed after a saturating first flash (compare Figs. 17b and 17c). The analyses of these time courses indicate that the second flash causes an absorbance increase that decays exponentially in a phase c', characterized by an intensity-independent half-life time of 60-80 ms. After completion of

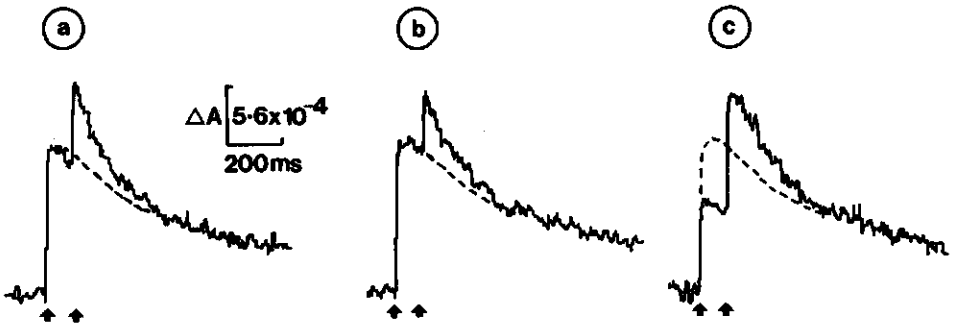

Fig. 17. Time courses of the 515 nm absorbance change in intact chloroplasts, induced by two successive flashes, both saturating (a), the second nonsaturating (b) and the first nonsaturating (c). The broken lines present the kinetics of a single saturating (first) flash.

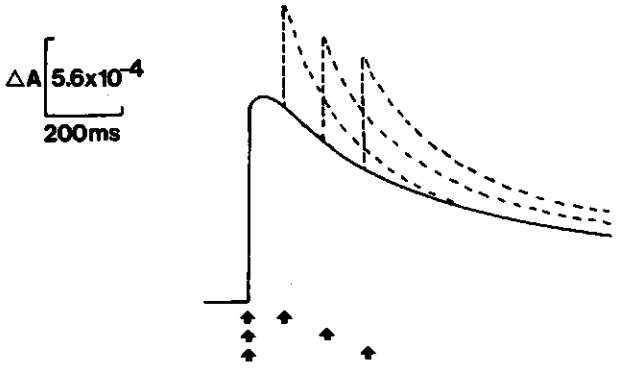

Fig. 18. Time course of the 515 nm absorbance changes induced by two successive flashes. The time interval between both flashes was varied as indicated by the arrows.

phase c', the slow phases c and d are observed that are solely due to the effect of the first flash (if saturating).

The effect of the second flash was found to be invariable when the dark time between the flashes was varied from 1 to 100 ms. At dark times above 100 ms a gradual increase in the phases c and d, induced by the second flash, was observed. Representative curves are shown in Fig. 18. At dark times shorter than 1 ms the absorbance change upon the second flash decreased significantly (without an effect upon the half-time of phase c' and was found to become zero at dark times below 400 to 500 μs (not shown).

5.4 ANALYSES OF THE P515 RESPONSE IN BROKEN CHLOROPLASTS

Experiments on broken spinach chloroplasts were performed to study the effect of various additions on the P515 kinetics in the absence of the "barrier function" exerted by the chloroplast envelope and stroma phase. Fig. 19 illustrates that the P515 response in fresh broken chloroplasts also is composed of a Reaction I- and Reaction II-type. In general the

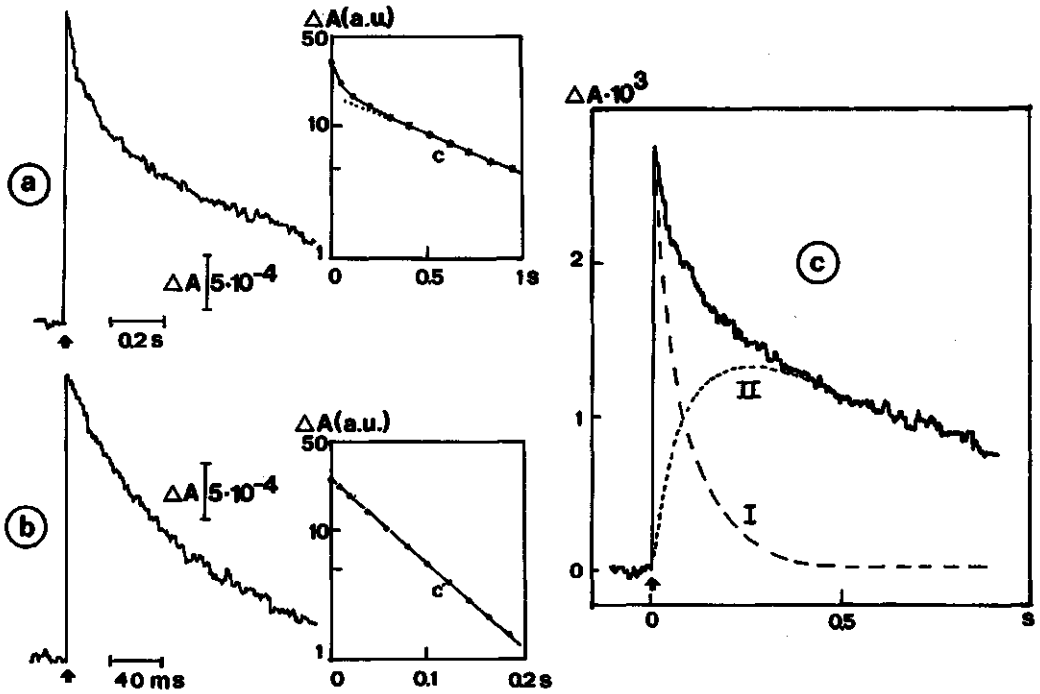

Fig. 19. Time courses of the 515 nm absorbance changes in broken spinach chloroplasts (in the presence of 10 mmol/l $MgCl_2$), induced by repetitive single flashes fired at a repetition rate of 0.05 Hz (a and c) and 4 Hz (b). Curve b was measured immediately after pre-illumination with 6 flashes, fired at a rate of 4 Hz. On the right in a and b: semi-logarithmic plot of the absorbance change against time. Note the different time scales in a and b. The broken curve (I) in c is identical to the response measured in b, i.e. characterized by an exponential decay phase c'; curve II is the difference between the experimental curve (identical to the one shown in a) and curve I.

occurrences in broken chloroplasts were found to be similar to those in intact chloroplasts provided that a gentle isolation procedure was applied, including osmotic shocking in the presence of cations.

Fig. 19a shows the P515 response in dark-adapted broken chloroplasts, suspended in a Mg^{++}-containing medium, illuminated by flashes, fired at a repetition rate of 0.05 Hz. The semi-logarithmic plot shows that the decay does not follow first-order reaction kinetics. A slow and a fast phase can be resolved which, in analogy with the situation in intact chloroplasts, are called phase c and phase c', respectively. Phase c' can be resolved after saturation of Reaction II with seven pre-illuminating flashes (see below). Subsequent flashes, fired at a repetition rate of 4 Hz cause a P515 response which exhibits a single-exponential decay with a rate constant of 12 s^{-1} (Fig. 19b). Because of the clear similarities with the occurrences in intact chloroplasts, this response is attributed to Reaction I. Subtraction of Reaction I from the overall response then gives the response of Reaction II, as illustrated in Fig. 19c. The increase in absorbance caused by Reaction II is about 50% of the absorbance increase associated with Reaction I.

The relative magnitude of Reaction II in broken chloroplasts appears to be dependent

on the ionic composition of the medium. Figs 20c and 20d show that the magnitude of Reaction II in chloroplasts, suspended in a Mg^{++}-containing medium, is lower than that of Reaction II in chloroplasts suspended in K^{+}-containing medium. As can be concluded from the semi-logarithmic plots (inserts Figs 20a and 20b), the rate constant of the decay of

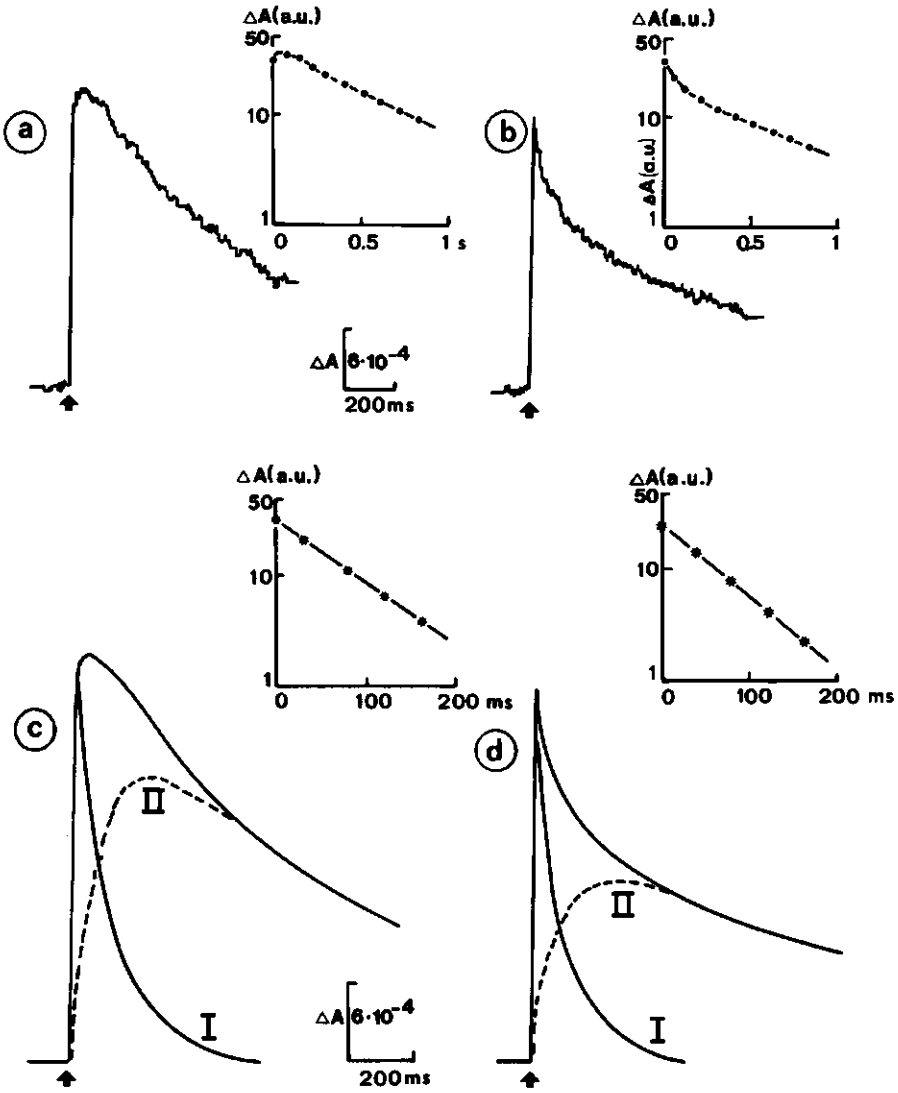

Fig. 20. Time courses of the 515 nm absorbance changes in broken chloroplasts induced by repetitive single flashes fired at a repetition rate of 0.05 Hz in the presence of 10 mmol/l KCl (a) or 10 mmol/l $MgCl_2$ (b). Resolution (bottom part of figure) of the ΔA515 responses into two components: Reaction I and Reaction II. Curves I (c and d) were measured by firing repetitive single flashes at a repetition rate of 4 Hz, after pre-illumination with 6 flashes. Curves II (c and d; broken lines) were obtained by subtracting curves I from the overall ΔA515 response (a and b). The inserts present semi-logarithmic plots of the overall responses (a and b) and of the Reaction I decay kinetics (c and d), respectively.

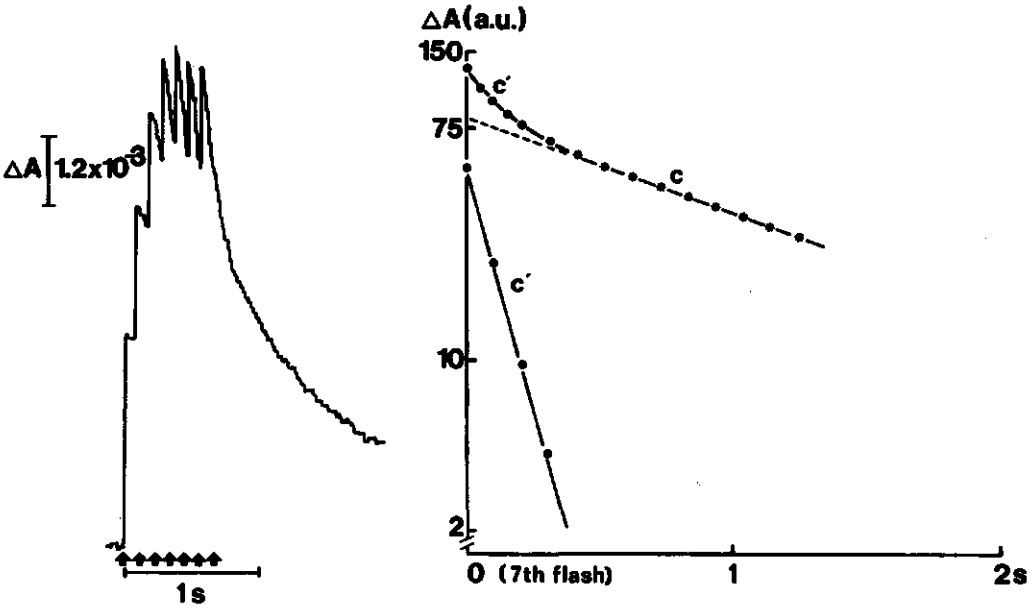

Fig. 21. Time course of the absorbance changes at 515 nm in broken chloroplasts, in the presence of 10 mmol/l KCl, induced by a series of 7 flashes, fired at 100 ms time intervals. Reaction I (phase c', closed circles), was obtained after extrapolating the slow phase c (closed stars) to the time at which the seventh flash was fired (semi-logarithmic plot), and subsequent subtracting from the overall decay.

Reaction II is independent on the ionic composition of the medium. The semi-logarithmic plots of Reaction I (inserts Fig. 20c and 20d), indicate that the decay of Reaction I is about 25% faster in the presence of 10 mmol/l Mg^{++}, in comparison with its decay in the presence of 10 mmol/l K^{+}. More data with respect to this ionic effect are in Section 6.2.

In broken chloroplasts, a group of several flashes causes a cumulative increase of the absorbance up to a saturation level which is reached after 4-6 flashes, fired at time intervals of 100 ms (Fig. 21). The increase of the absorbance which appears to be due to an increase of Reaction II is about 1.9 times the extent of Reaction II after one flash (compare Fig. 20 and Fig. 21). Because Reaction II reaches a saturation level after five successive flashes, the decay after the seventh flash can be analyzed by extrapolating the slow phase, associated with Reaction II, to the time at which the 7th flash had been fired. Subtracting the extrapolated part from the experimental curve gives rise to the single-exponential decay of Reaction I with a half-life time that equals the half-life time of Reaction I after a single flash i.e. approximately 100 ms.

5.5 KINETICS OF THE LIGHT-GENERATED TRANSMEMBRANE POTENTIAL

Fig. 22 shows the single and double flash response of the potential across the chloroplast (thylakoid) membrane of a *Peperomia* chloroplast *in situ* measured with a micro-electrode. The analyses indicate a similar single-exponential potential decay both after the first and the second flash with a half-life time of 35 ms. In a few instances the relaxation af-

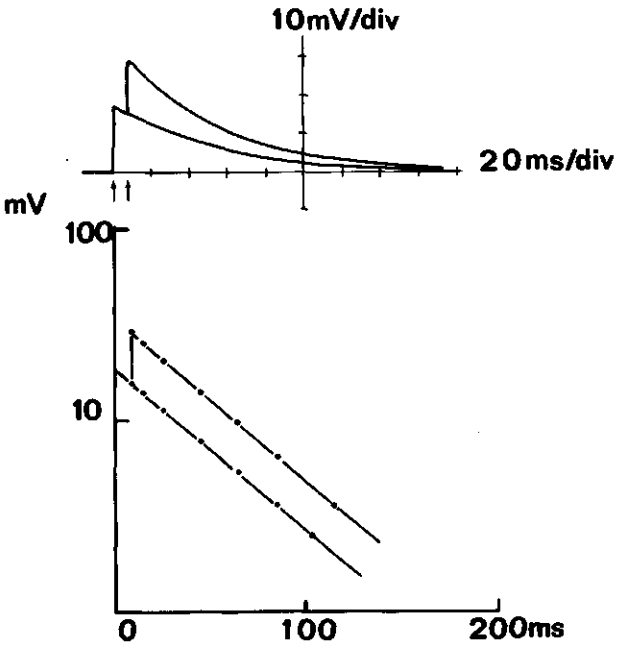

Fig. 22. Response and semi-logarithmic plot of the photo-electric potential of a *Peperomia metallica* chloroplast to a saturating light flash or to a series of two saturating light flashes separated by a dark time of 20 ms. Measurements were done in a cross section of a *Peperomia metallica* leaf with a micro-capillary glass electrode inserted into a single chloroplast.

ter the second flash was slightly (≈ 10%) faster than after the first flash. The half-life time of the potential decay for a large number of different chloroplasts was 30-60 ms. The potential increase measured in the first flash was in the range of 15-40 mV; the ratio between the potential change in the second and first flash was 0.6-0.8. The similarities between the kinetics of Reaction I (Fig. 16) and the kinetics of the transmembrane potential changes, as measured with micro-capillary glass electrodes (Fig. 22), suggest that the ΔA_{515} associated with Reaction I, is due to the transmembrane electric field. A more detailed study on the Reaction I kinetics will be given in Chapter 6.

5.6 EFFECT OF VALINOMYCIN

It seems unlikely, that the slow decay kinetics of Reaction II, which mainly determine the decay of the overall P515 response after a flash in the 0.1-1 s time period (c.f. Figs 15 and 16), are associated with the decay of a delocalized electric field. This suggestion is substantiated by the results depicted in Fig.23, which show the half-life times of the decay of Reaction I and the magnitude of Reaction II, after a single flash, as a function of the valinomycin concentration. The magnitude of Reaction I and the decay rate of Reaction II were found to be independent on the valinomycin concentration (not shown). The magnitude of Reaction II appears to be extremely sensitive to low concentration of the ionophore. Reaction II is completely inhibited at a valinomycin concentration, equal or less than 250

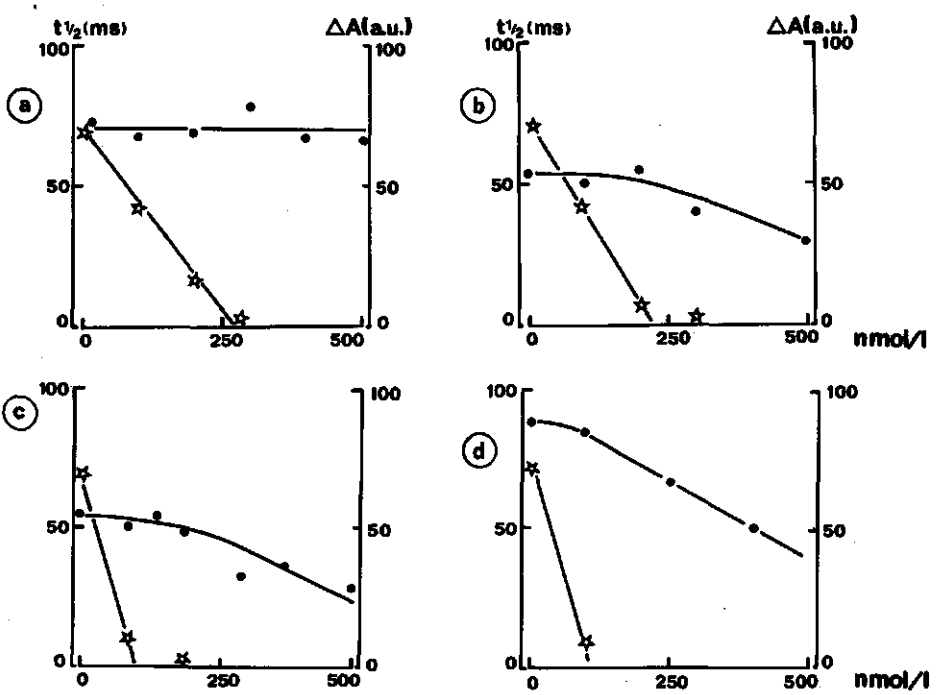

Fig. 23. The magnitude of Reaction II in broken spinach chloroplasts (open stars, right hand scale) and the half-life time of Reaction I (closed circles, left hand scale) plotted as a function of the amount of valinomycin in the suspending medium, in the presence of 10 mmol/l $MgCl_2$ and 1 mmol/l KCl (a), 10 mmol/l $MgCl_2$ and 6 mmol/l KCl (b), 10 mmol/l $MgCl_2$ and 10 mmol/l KCl (c), or 6 mmol/l KCl (d).

mmol/l. A 50% inhibition of Reaction II, measured in the presence of Mg^{++}, is obtained at 140 mmol/l and 60 mmol/l valinomycin in the presence of 1 mmol/l and 10 mmol/l KCl (Fig. 23a and 23c), respectively. Reaction I is only accelerated when Reaction II has been completely inhibited. In the presence of Mg^{++} at a valinomycin concentration below 200 nmol/l, the half-life time of the decay of Reaction I is hardly dependent on the K^+ concentration. This indicates that Mg^{++} is a more permeant ion than K^+, even in the presence of a low amount valinomycin. This is substantiated by the observation that, Reaction I, in the presence of 6 mmol/l K^+, is equally accelerated by 450 nmol/l valinomycin (Fig. 23d) and 10 mmol/l $MgCl_2$ (Fig. 23b). See also Section 6.2.

The observed acceleration of the overall decay of P515 in the presence of the ionophore apparently is not due to an enhanced rate constant of the decay of the transmembrane electric field but to a decrease of the magnitude of Reaction II. In general, the magnitude of Reaction II in broken chloroplasts unlike that of Reaction I was found to be strongly dependent on the physiological condition of the chloroplasts and to be substantially suppressed by membrane-modifying substances (Fig. 23), divalent cations (Fig. 20) , ageing (Fig. 24) and freezing (not shown). See Chapter 7 for a more detailed discussion.

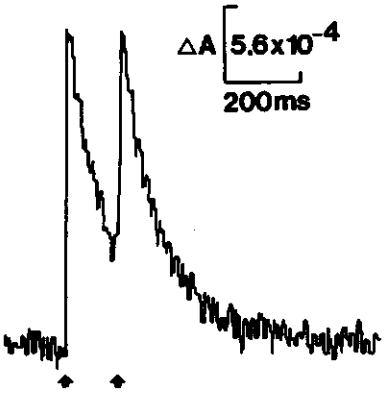

Fig. 24. Time courses of the 515 nm absorbance changes in an aged preparation of ion-free prepared broken chloroplasts upon double-flash excitation. Repetition rate of separate flash sequences was 0.05 Hz.

5.7 ACTIVATION OF REACTION II BY PHOTOSYSTEM 1

Figs 25a and 25b show representative examples of the ΔA515 in intact chloroplasts upon saturating single-turnover light flashes, which excite both photosystems with excitation light of wavelengths above 665 nm (Fig. 25a), or preferentially Photosystem 1 by light of a narrow wavelength band around 717 nm (Fig. 25b), respectively. According to the analyses, the magnitudes of Reaction I and of Reaction II in 717 nm light are about 50% and about 75%, respectively, of the magnitude caused by activating both photosystems. Figs 26a and 26b show the ΔA515 upon illumination with 3 successive light flashes of wavelengths above 665 nm and of 717 nm, respectively, in intact chloroplasts. The time intervals between the

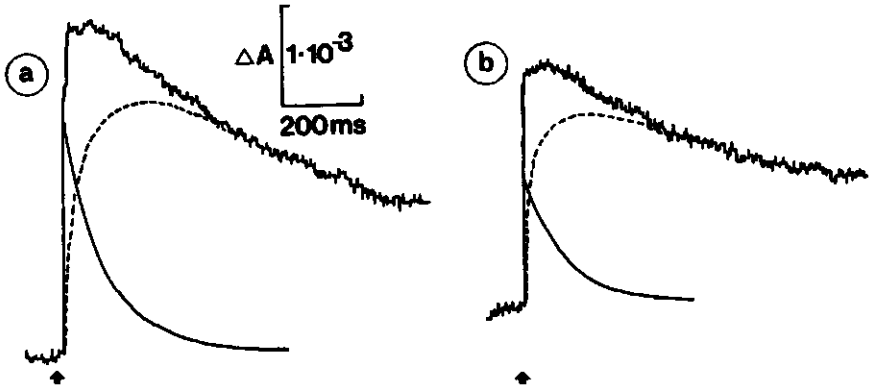

Fig. 25. Time courses of the 515 nm absorbance changes in intact chloroplasts upon illumination with single-turnover light flashes of wavelengths above 665 nm (a) and of a narrow wavelength band around 717 nm (b). Flash frequency was 0.05 Hz (noisy curves) or 3 Hz (solid curves). In case of the latter, 3 pre-illuminating flashes were fired (see text). The dotted lines represent the resultant kinetic pattern of Reaction II obtained after subtraction of the solid curves (Reaction I) from the overall responses.

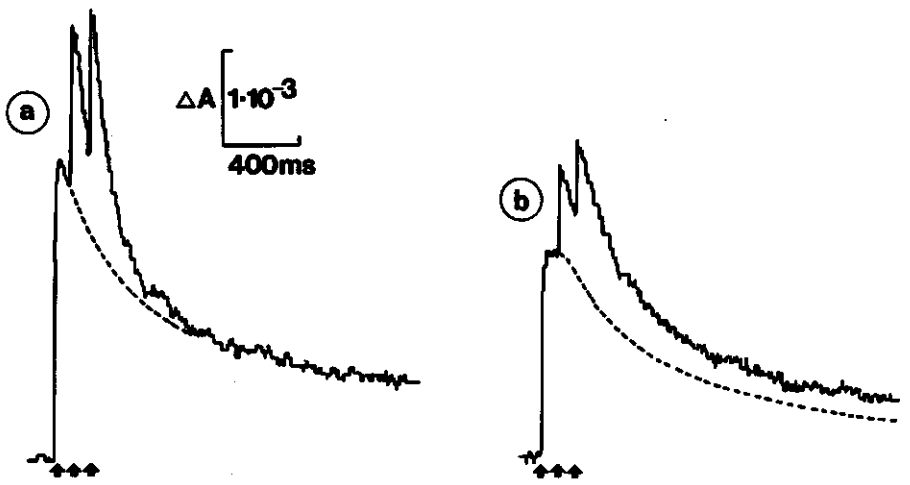

Fig. 26. Time courses of the 515 nm absorbance changes in intact chloroplasts upon illumination with triple flashes of wavelengths above 665 nm (a) and of a narrow wavelength band around 717 nm (b). Time span between separate flashes was 80 ms. Series of 3 flashes were fired at a frequency of 0.05 Hz. The dotted lines represent the responses to single flashes fired at 0.05 Hz.

flashes was 80 ms. The dotted curves represent the P515 absorbance responses upon a single flash. As the absorbance decay in the dark, 300 ms after a flash, is exclusively due to that of Reaction II (c.f. Fig. 25), it appears, according to Fig. 26a, and conclusive with results obtained with double flash experiments, that Reaction II is saturated by a single light flash of wavelengths above 665 nm. This apparently is not the case when light of 717 nm wavelength is used. Repetitive excitation of Photosystem 1 gives rise to a further increase of Reaction II in the second and third flash (Fig. 26b). It should be mentioned that, with 717 nm light, the third and the second flash were not completely saturating. Therefore, it might be that the saturation level of Reaction II can be reached after two successive saturating excitations with Photosystem 1 light.

Fig. 27 shows the responses in broken chloroplasts, in the absence and presence of the electron transport inhibitors DCMU and KCN. In the presence of DCMU (b), the extent of the P515 response is about 50% of the one associated with Reaction I in the absence of DCMU (a). The ΔA515 in the presence of DCMU can not be resolved into its components because Reaction I can only be measured accurately in fast-repetitive flash experiments. However, as would be expected in the presence of DCMU, no signal is observed under that condition (see also Renger & Wolff, 1975).

Addition of DCPIP/ascorbate creates a condition in which a total recovery of the P515 response is measured (Fig. 27c). A similar response has been measured after addition of 70 µmol/l PMS to DCMU-poisoned chloroplasts (not shown). The response after pre-incubation of the chloroplasts in the presence of 30 mmol/l KCN is shown in Fig. 27d. The magnitude of the signal is about 40% of that of the Reaction I response in the control experiment. The decay of the response in the presence of KCN appears to be faster than the decay of the signal in DCMU-poisoned chloroplasts and suggests the complete absence of Reaction II

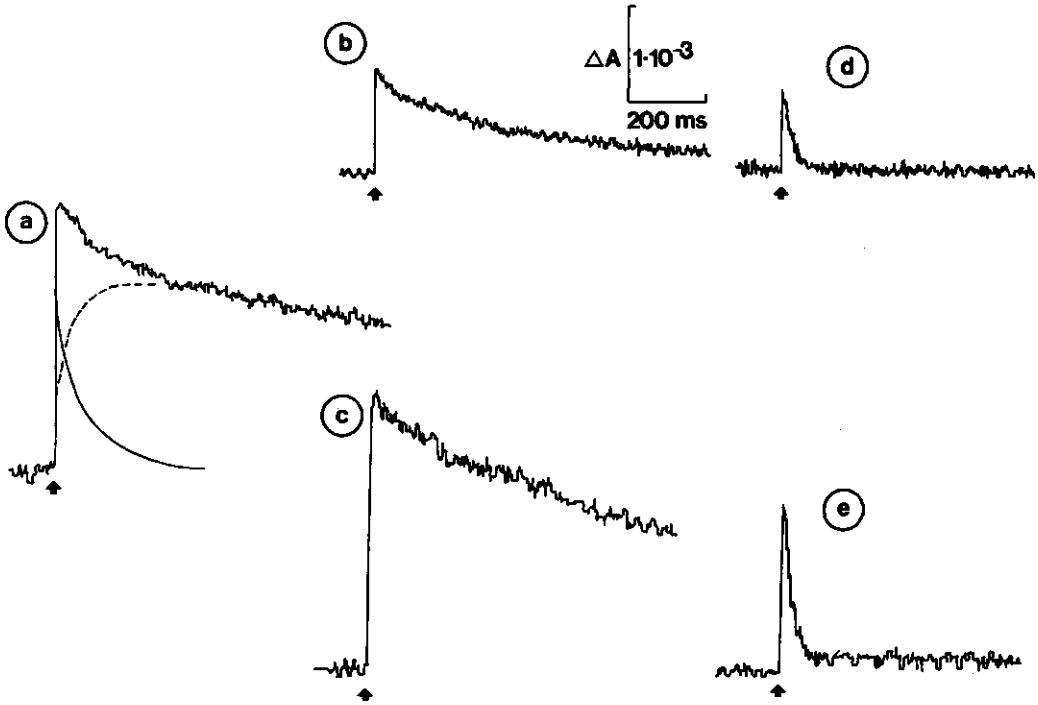

Fig. 27. Time course of the 515 nm absorbance changes upon illumination with single-turnover light flashes of wavelengths above 665 nm fired at a frequency of 0.05 Hz in broken chloroplasts. Chloroplasts were suspended in 330 mmol/l sorbitol, 30 mmol/l tricine (pH 7.6) and either 17.5 mmol/l KCl (a,b and c) or 17.5 mmol/l KCN (d and e). a) Control experiment; b) in the presence of 2 µmol/l DCMU after pre-illumination with 4 flashes; c) in the presence of 2 µmol/l DCMU, 3.3 mmol/l ascorbate and 48 µmol/l DCPIP; d) after pre-incubation in 30 mmol/l KCN; e) after pre-incubation in KCN and subsequent addition of 3.3 mmol/l ascorbate and 48 µmol/l DCPIP.

and a somewhat faster decay rate of Reaction I. Addition of DCPIP/ascorbate (Fig. 27e) or 70 µmol/l PMS (not shown), in this case, creates a condition in which the flash-induced ΔA515 equals the Reaction I response of the control, except for the somewhat faster decay. The dark kinetics suggest the absence of Reaction II in KCN-inhibited chloroplasts in the presence of DCPIP/ascorbate.

Apparently, reduced DCPIP and PMS can react with oxydized P700 in a direct way, independent of plastocyanin. Ouitrakul & Izawa (1975) reported a recovery of 18% and 20% of the electron transport rate in KCN-treated chloroplasts by 50 µmol/l DCPIP and 100 µmol/l PMS, respectively. Thus, a direct reaction of these mediators with P700 seems plausible. A 20% recovery of the electron transport rate is sufficient to reduce $P700^+$ completely in the dark periods between the repetitive flashes.

5.8 DISCUSSION

The magnitude of the absorbance increase at 515 nm has been used as a linear measure of the electric transmembrane potential of the thylakoids (Witt, 1971; Junge, 1977a; Gräber &

Witt, 1975). A single-exponential decay of the 515 nm response after a single-turnover light flash is usually observed in aged, or uncoupled ion-free prepared broken chloroplasts (Fig. 24). This would indicate a voltage-independent membrane conductance which determines the rate constant of field-dissipating passive ion movements (see also Chapter 6). Biphasic decay kinetics have been shown to occur in broken chloroplast under phosphorylating conditions. These are believed to be due to changes in the proton conductance through the ATPase above a critical potential (Junge & Witt, 1968; Boeck & Witt, 1972; Schliephake et al., 1968; Rumberg & Siggel, 1968; Junge et al., 1970; Schmid et al., 1976; Girault & Galmiche, 1978). This hypothesis has led to conclusions about a correlation between ATP synthesis and P515 kinetics. However, it may be doubted if protons contribute significantly to the membrane conductance, whether under phosphorylating conditions or not (Section 6.3). Moreover, the experiments with broken chloroplasts described in this chapter were carried out under non-phosphorylating conditions and therefore no multi-phasic decay kinetics would be expected to occur in view of this hypothesis. Furthermore, as can be concluded from Fig. 21 and Fig. 20a, the transition from a fast to a slow decay phase after the last flash of a series of 7 flashes, occurs at a higher level than the transition after a single flash. It is difficult to reconcile with this observation in terms of the existence of a distinct critical potential for the activation of the ATP-synthesizing complex.

On the basis of results depicted in Figs 15-21, we arrive at the conclusion that the flash-induced 515 nm absorbance change in dark-adapted intact chloroplasts is the consequence of at least two different processes. One process (Reaction I) is characterized by a fast rise (phase a) and a decay (phase c') with a half-life time of 60-100 ms, the other (Reaction II) is characterized by a relatively slow rise and a decay (phases c and d) with a half-life times of 400-800 ms and 1-3 s, respectively.

The spectrum of the slow decay of phase d was found to be different from that of P515. This phase might represent the decay of a reaction different from Reaction I and II and probably corresponds to changes in scattering level (see also Chapter 8). The estimated magnitude of phase d in a single flash is about 10% of the total P515 response.

The observed similarity between Reaction I kinetics and the kinetics of the transmembrane potential changes as measured with micro-capillary glass electrodes, provides reasonable good evidence for the conclusion that the 515 nm absorbance change associated with Reaction I is due to the transmembrane electric field. This field, measured with microelectrodes is equally activated by both pigment systems (Vredenberg, 1974). According to the data of Fig. 25, it appears that the magnitude of the fast rise of Reaction I in Photosystem 1 (717 nm) light is about 50% of the magnitude measured in light absorbed by both photosystems. Moreover, the magnitude of the P515 response measured in DCMU- or KCN-treated chloroplasts (Fig. 27b and 27d, respectively) upon flash light absorbed by both systems is about 50% of the Reaction I response in the absence of the inhibitors (Fig. 27a). Illumination of DCMU-poisoned broken chloroplasts with 717 nm light flashes under these conditions did not result in an absorbance response in the absence of electron donors for Photosystem 1. We therefore conclude, in agreement with the interpretation of Renger & Wolff (1975), that the response measured in the presence of these electron-transport inhibitors is brought about by charge separation in Photosystem 2 only. According to Figs 27a, 27c and 27e, Reaction I can be restored completely in DCMU- or KCN-poisoned chloro-

plasts by the addition of electron donors for Photosystem 1. Thus, it seems reasonable to conclude that Reaction I is equally activated by Photosystem 1 and Photosystem 2.

The decay of the overall P515 response after a flash in the 0.1-1 s time period (c.f. Figs 15-21), which is mainly due to Reaction II, is difficult to interpret in terms of the decay of the transmembrane electric field. This is nicely illustrated by the inhibiting effect of low concentrations of valinomycin on the magnitude of Reaction II. In this respect it is of interest to recall the 200 to 600 fold increase in P515 decay rate, usually observed in intact chloroplasts in the presence of 1 μmol/l valinomycin. If we assume that in the absence of valinomycin the K^+ concentration in the chloroplast stroma is at least 30 mmol/l (Nobel, 1969; Gimmler et al., 1974; Vredenberg, 1976) and will decrease considerably in the presence of the ionophore at a low outside concentration of K^+ (Bulychev & Vredenberg, 1976a), and further assume that the K^+ permeability coefficient of the thylakoid membrane is increased by a factor of 10 in the presence of valinomycin (Barber, 1972a), one would not expect that the membrane conductance changes to such a large extent. It has been reported (Bulychev & Vredenberg, 1976a) that the decay of the transmembrane potential under similar but not identical conditions is accelerated by a factor of no more than 5.

Reaction II depends quite differently on Photosystem 1 and Photosystem 2 illumination. We found for a variety of preparations that excitation of only Photosystem 1, by single light flashes, caused an activation of Reaction II, that appeared to be 60-80% of the response after excitation of both photosystems. As Fig. 26 shows, Reaction II is saturated after double or triple excitation of Photosystem 1. This suggests that the redox poise at the reducing side of Photosystem 2 partly controls Reaction II, possibly by its influence on the redox state of cytochrome f (Mitchell, 1975). The observation, that Reaction II is mainly dependent on a process activated by Photosystem 1 excitation, is in agreement with the observation (Fig. 22) that Reaction II is relatively small, or even absent in DCMU- (Fig. 27b) and KCN-treated (Fig. 27d) chloroplasts, respectively, and can be completely restored in the presence of DCMU after addition of electron donors for Photosystem 1.

The decay of the P515 response in the presence of DCMU (Fig. 27b) suggests a contribution of Reaction II, which in this case is about 20% of the maximal response under conditions at which electron transfer through Photosystem 1 is possible (Figs 27a and 27c). Whether this small contribution of Reaction II has to be attributed to Photosystem 1 activity cannot be said with certainty. Photosystem 1 excitation by 717 nm light under these conditions was found not to result in a P515 response. But it is possible that, upon excitation of both Photosystems, the reduced acceptor of Photosystem 2 is oxidized in the dark by a component which in its reduced form serves as a (weak) donor for oxidized carriers at the donor side of Photosystem 1. Thus, upon excitation, some electron transport through Photosystem 1 would be possible with the consequent appearance of a small Reaction II (and Reaction I). In intact chloroplasts (in the presence of DCMU), PQ might be come re-reduced via the pathway: ferredoxin → cyt b564 → PQ (Mills et al., 1979). Subsequently, the reduced plastoquinone might reduce the primary donor of Photosystem 2. Such a mechanism, however, is not to be expected in broken chloroplasts, which easily have released ferredoxin.

Our results do not permit definite conclusions about the process which is responsible for the appearance of Reaction II. A slow flash-induced ΔA515 increase has been reported

to occur in pre-illuminated chloroplasts (Velthuys, 1978), which has been suggested to be caused by a proton translocation at the oxidizing side of the plastoquinone pool. The results of our experiments, however, still show a slow ΔA515 associated with Reaction II, under conditions at which plastoquinone is in the oxidized form (Photosystem 1 illumination). However, a relation between electrogenic cyclic electrontransport and the slow P515 response (Arnon & Chain, 1975; Crowther et al., 1979; Mills et al., 1979) cannot be excluded as yet, provided that the reaction rate of the electrogenic cyclic electron transport turns out to be compatible with Reaction II rise and decay kinetics. This suggestion is difficult to reconcile with the fact (Fig. 23) that the decay of Reaction II is unaltered whereas its magnitude is suppressed in the presence of low concentrations of valinomycin.

The kinetics and spectral characteristics of Reaction II suggest that its manifestation is a reflection of physico-chemical processes in the membrane near the P515 pigment complex. Several workers showed that the decay of the P515 response in broken chloroplasts has relatively rapid single first-order kinetics (see also Fig. 13). This suggests that Reaction II in these preparations does not result in an electrochromic response of the P515 pigment complex, or alternatively does not occur at all. The reason for this is unknown as yet. I assume that the structural organization of membrane constituents and specifically the sensitivity of (part of) the P515 pigments to trans- and innermembrane electrical fields are affected by some, as yet unknown, critical steps in the procedure of chloroplast preparation.

The absence of Reaction II in KCN-poisoned chloroplasts, in the presence of electron donors for Photosystem 1 (Fig. 27e) would suggest a functional role of plastocyanin or cytochrome f in the reaction process. This suggestion is in agreement with the observation (Malkin, 1978) that a distinct component of the P515 response is dependent on the redox condition of a component with a midpoint potential of +385 mV, which is about equal to the midpoint potentials of cytochrome f and plastocyanine.

The reason for the observed difference in saturation of Reaction II in intact and broken chloroplasts is not clear as yet but it should be recalled that differences in the P515 spectrum of broken- and intact chloroplast were suggested to be due to cation-dependent changes in the position of electron carriers and other pigments in the membrane (Chapter 3). It has been observed (Haehnel, 1977), that oxidized P700 can be reduced completely after one saturating flash, whereas only part of PC is oxidized. Successive flashes increase the oxidation degree of the PC pool. Similar results with respect to cytochrome f were obtained by Koike et al. (1977). An electric interaction between oxidized PC and P700, proposed to be part of one complex (Ke, 1975), might explain the observed increase of Reaction II in successive flashes in broken chloroplasts. Therefore it might be interesting to study the oxidation of PC and the reduction of P700 in intact chloroplasts. This, according to the observed saturation of Reaction II in a single flash, should have a one--to-one stoichiometry under these conditions.

It is unlikely that some, relatively fast, electrogenic process, mediated by either oxidized cytochrome f or plastocyanin, may account in a direct way for the slow rise and decay kinetics of Reaction II. However, such an electrogenic process may well cause conformational changes in the membrane. A charge separation between either oxidized plasto-

cyanin or cytochrome f and a catalist at the reducing side of Photosystem 1 may induce changes in the structural assembly of lipid and protein molecules. Such conformational changes are supposed to alter the mutual orientation of fixed charged groups of the molecular structures within the membrane. We propose that this may result in (a change of) the intrinsic electric field, associated with these charge displacements. As a consequence this may result in ΔA515 of the Reaction II type. It is interesting to note that the rise and decay kinetics of the P515 absorbance changes associated with Reaction II closely resemble the optical response of the potential probe oxonol VI (Schuurmans, 1978; Bashford, 1978). The magnitude of the oxonol absorbance shift has been shown to be inhibited by valinomycin (Schuurmans et al., 1978). This inhibition appears to be similar to the inhibition of Reaction II. The action of valinomycin can be understood by discerning the chaotropic effect of this ionophore. A direct effect of valinomycin on the phosphorylating system has been reported (Trebst & Reimer, 1977) and the changes in lipid aggregation caused by the antibiotic (Walz, 1977) might hamper the conformational change of the activated coupling factor.

In general, it appears that the rise of Reaction II occurs in the same time range as the flash-induced response of external probes which are indicators of either changes in the membrane surface potential (Kraayenhof & Arents, 1977), or of conformational changes of the chloroplast ATPase (Kraayenhof & Slater, 1975). It has been discussed (Ort et al., 1976; Vinkler et al., 1978) that some mode of membrane energization or conformation is needed for the onset of ATP synthesis in the light. This activation step appears to be completed in the same time range as the rise of Reaction II (see also Chapter 7).

6 Reaction kinetics of transmembrane potential changes in relation to ion permeability

6.1 INTRODUCTION

The relationship between electron transport and proton pumping into the thylakoid inner space has been the subject of several studies. Net uptake of protons was observed by Neumann & Jagendorf (1964) in broken chloroplasts and by Heldt et al. (1973) in intact chloroplasts. Using phenol red as a pH indicator or the fluorescence quenching of fluorescent amines, the steady-state rates of proton translocation (Chow & Hope, 1977) and the kinetics of onset and decay of ΔpH (Chow & Hope, 1976) have been studied extensively.

To maintain electrical neutrality in the bulk phases, the H^+ uptake is balanced by either anion influx or cation efflux. Unfortunately, the knowledge of passive ion fluxes across the thylakoid membrane and its conductance is rather unsatisfactory. Sofar, often conflicting results have been reported. The efflux of K^+ and Mg^{++} has been reported to be the main charge balancing ion movement (Dilley & Vernon, 1965). Bulychev & Vredenberg (1976b) have given support for this suggestion and have emphasized the consequence of the relatively high concentration of both ions available in the intact chloroplast. Deamer & Packer (1969) reported Cl^- to be a major co-ion for proton pumping. The importance of Cl^- in this respect was also discerned by Hind et al. (1974).

Nakatani et al. (1978) emphasized the importance of surface potentials of thylakoid membranes in terms of ion distribution between the membrane boundary and the bulk phase and the effects thereof on the membrane conductance. These results, confirmed by later findings (Nakatani et al., 1979), have given support to the proposal that Mg^{++} may act as the main counterion for proton transport. Alternatively, Gräber & Witt (1975, 1976) concluded that the establishment of a proton gradient caused an enhancement of the specific proton conductance, to such an extent, that the active proton uptake under steady-state conditions is balanced both by passive proton leakage and proton efflux through the ATP-synthesizing complex. Junge (1977a) and Harris & Crofts (1978) discussed an electrically gated ATP-synthesizing complex that would enlarge the membrane conductance due to a higher proton permeability coefficient (see however Chapter 5 and 7).

The search for an effect of the membrane potential on the ion conductivity remains a challenge for a better understanding of ion-controlled processes, including ATP synthesis. Reversible changes of the membrane conductance caused by light-dependent processes have been observed by Vredenberg & Tonk (1973) at the plasmalemma of *Nitella translucens*.

In this chapter, the decay rate of Reaction I (c.f. Chapter 5), proposed to be proportional to the thylakoid membrane conductance, is studied in relation to the ionic environment of thylakoid membranes of spinach chloroplasts. From this study conclusions are drawn with respect to the relative contribution of different ions to the passive membrane conductance. Analysis of Reaction I kinetics induced by a flash, fired immediately after

the pre-illuminating periods, leads to the conclusion that the proton conductance of the thylakoid membrane is relatively low and suggests evidence for the existence of a Mg^{++}-H^{+} exchange mechanism. Additional information on the effect of energization and associated changes of the electrical resistance of the membrane, is derived from electrical stimulation of thylakoid membranes of *Peperomia metallica* by means of micro-capillary glass electrodes.

6.2 EFFECT OF ELECTROLYTE CONCENTRATION ON THE DARK KINETICS OF REACTION I

Fig. 28a shows the reciproke half-life time of Reaction I plotted as a function of the amount of KCl present in the assay medium at different pH values. At pH 7 and 8, the decay appears to be hardly dependent on the concentration of KCl, indicative for a low membrane permeability of both K^{+} and Cl^{-} under these conditions. Similar results were obtained by Schuldiner & Avron (1971) and Yakovleva & Molotkovskii (1979) who measured dark swelling of thylakoids upon addition of KCl. Lowering the pH of the assay medium apparently results in an increase in the membrane conductance as may be concluded from the pH-dependent decay rate of Reaction I in the absence of ions (Fig. 28b). Moreover, at lower pH the decay rate of Reaction I becomes more dependent on salt concentration, according to the altered slopes in Fig. 28a. If Cl^{-} is replaced by aspartate, no appreciable effect

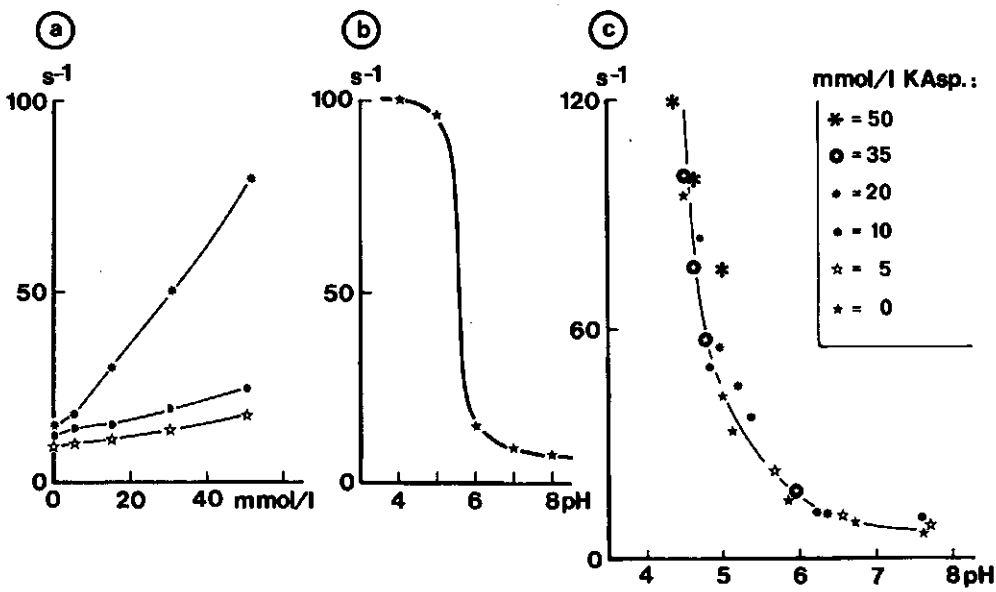

Fig. 28. a) Reciproke of the half-life time of the Reaction I decay after a single-turnover flash, measured at different pH values in broken spinach chloroplasts plotted as a function of KCl concentration in the suspending medium: pH 8, open stars; pH 7, closed crescents; pH 6, closed stars.
b) Effect of pH on the reciproke half-life time of Reaction I in broken chloroplasts, suspended in an ion-free assay medium. c) Effect of pH on the reciproke half-life time of Reaction I in broken chloroplasts, suspended in a medium containing 5 mmol/l KCl and in addition varying amounts of potassium aspartate (see insert).

of salt concentration on the Reaction I decay kinetics was found at different pH. The pH-dependent changes of the decay rate are similar to those in the absence of ions (compare Figs 28b and 28c). Thus, I conclude that the thylakoid membrane is impermeable to both aspartate and K^+. From these results it may be derived that, between pH 8 and 6, the membrane conductance is determined by the amount of Cl^-. The transport of Cl^- apparently is facilitated by low pH as can be concluded from its effect on the slope of the concentration dependent Reaction I decay rate (Fig. 28a).

Fig. 29 shows the half-life time of Reaction I as a function of Cl^- concentration at different pH. Cl^- was added either as potassium salt or as magnesium salt. Because K^+ is rather impermeant (Fig. 28c), the apparent differences in decay rate at equal Cl^- concentrations are suggestive for a contribution of a Mg^{++} flux to the membrane conductance. The reason for the initial inclination in some of the curves is not known as yet but it might reflect secondary effects of Mg^{++} addition associated with ion redistribution processes in the electrical double layer adjacent to the plane of shear (c.f. Chapter 7 and 8). Conclusions about the passive membrane conductance, however, can be derived from the linear part of the curves. From the differences in the slopes of the concentration lines, an estimation can be made of the Mg^{++}- and the Cl^--conductivity, respectively. The membrane conductance at pH 7, in the presence of 40 mmol/l Cl^- added as a Mg salt, exceeds the one in the presence of the same amount of Cl^- added as K^+ salt by a factor of about 4. At this pH, the Mg^{++} conductivity apparently is four times the one of Cl^-. This factor was about 2 both at pH 6 and pH 8. The Cl^- conductivity has no pH optimum but rather increases constantly upon lowering the pH to a value of 5 (not shown).

Fig. 30 shows the anion-dependent half-life times of Reaction I. All salts were added as Mg^{++} salts. Therefore, the observed differences in concentration curves may be attributed solely to the anion effect on the membrane conductance. The plots show a more or

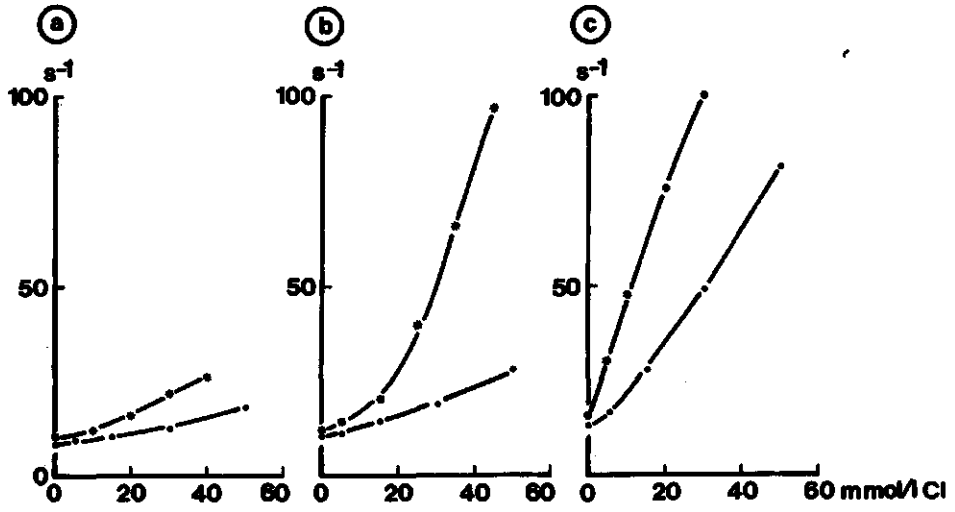

Fig. 29. Reciproke of the half-life time of Reaction I decay, measured at pH 8 (a), pH 7 (b) and pH 6 (c), plotted as a function of Cl^- concentration. Cl^- was added as $MgCl_2$ (closed stars) or as KCl (closed circles), respectively.

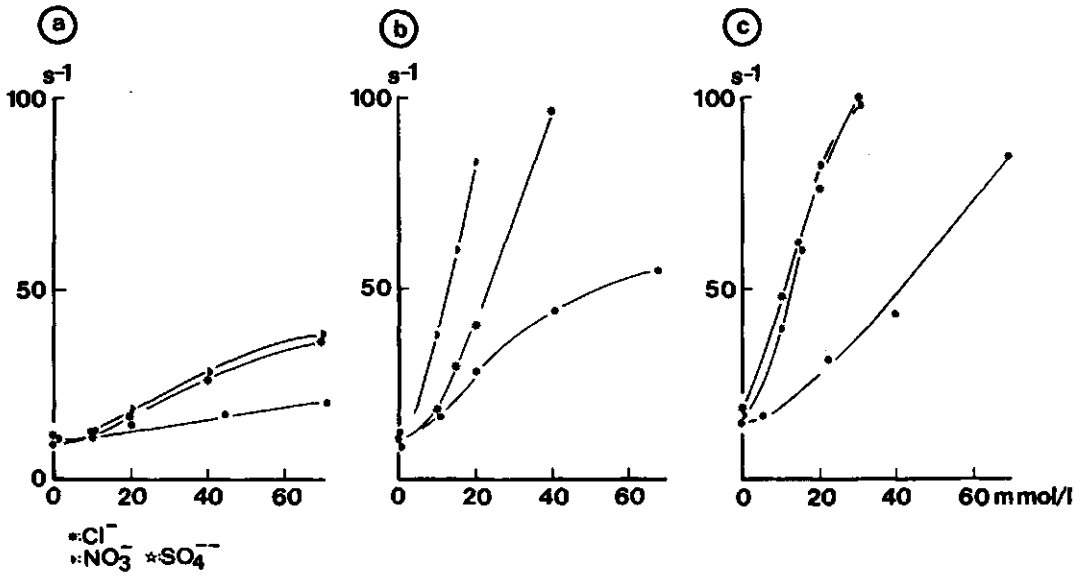

Fig. 30. Reciproke of the half-life time of Reaction I at pH 8 (a), pH 7 (b) and pH 6 (c) plotted as a function of negative charge equivalents: Cl^- (closed stars), SO_4^{--} (open stars) and NO_3^- (closed crescents). All anions were added as Mg-salts.

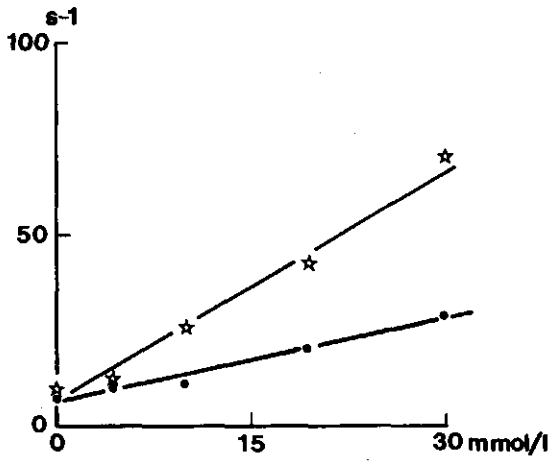

Fig. 31. Reciproke of the half-life time of the decay of Reaction I, measured by fast repetitive (3 Hz) flash firing, after pre-illumination with 6 flashes, plotted as a function of the $MgCl_2$ concentration in the suspending medium. Closed circles present the results of experiments performed on broken chloroplasts without further manipulation; open stars: broken chloroplasts after CF_1 extraction by mild EDTA treatment.

less sigmoidal concentration dependence indicative for a more complex mechanism than only a passive ion diffusion. The permeability coefficient of NO_3^- and Cl^- appear to be equal except at pH 7, as may be concluded from the more pronounced effect of NO_3^- addition at that pH. The results of SO_4^{--} addition show, in accordance with results reported by Yakovleva & Molotkovskii (1979), that this anion is relatively impermeant.

Extraction of CF_1 by EDTA treatment has been reported to create a conductance channel through CF_0 that retains its selectivity for protons (O'Keefe & Dilley, 1977). Results depicted in Fig. 31 are in conflict with this interpretation. Extraction of CF_1 by mild EDTA treatment (chloroplasts were incubated for 1 min in 0.5 mmol/l EDTA, 2 mmol/l tricine pH 8.0), results in a 10% increase in the Reaction I decay rate in the absence of salts (not shown). To investigate wether this small acceleration was caused by an increased proton conductance through CF_0, the reciproke of the half-life times of Reaction I in control- and extracted chloroplasts were plotted as a function of $MgCl_2$ concentration (Fig. 31). The difference between the slopes of the lines, depicted in this figure, indicates that the Cl^- and Mg^{++} conductivity in extracted chloroplasts is enhanced. The acceleration of Reaction I decay could be reversed by DCCD substitution (not shown). DCCD is a chemical effector that blocks the ion conducting channel through CF_0. Thus, I conclude that the relative high membrane conductance in extracted chloroplasts is not exclusively caused by a selective increase of the proton permeability.

6.3 DECAY OF REACTION I AFTER PRE-ILLUMINATION

Table 1 shows the half-life times of the decay of Reaction I as a function of pre-illumination. If pre-illumination causes a proton accumulation in the thylakoid inner space this will lead to an increase of the specific proton conductance of the membrane. Short pre-illumination of 200 to 500 ms indeed results in an enhancement of the Reaction I decay kinetics but extension of this pre-illumination period up to 32 s does not result in a further acceleration of the decay of Reaction I. It has been established that a maximum ΔpH across the thylakoid membrane is achieved after 5-30 s of illumination (Hind et al., 1974; Karlish & Avron, 1971). Thus it appears that the maximum acceleration of Reaction I decay after 0.25 s of pre-illumination is not directly caused by an increased proton conductance due to light-dependent proton accumulation in the thylakoid inner space.

Ausländer & Junge (1974) and Fowler & Kok (1974a) suggested that there might exist localized proton reservoirs on both sides of the thylakoid membrane. It is plausible that such reservoirs, seperated from the bulk phase, may lead to a fast rise of proton concentration in the light, causing a significant increase in proton conductivity within a time span observed above. Addition of DCCD, that is expected to decrease the specific proton permeability through CF_0 (under phosphorylating conditions), however, did not result in a different effect of pre-illumination compared with the situation in the absence of DCCD.

Table 1. Effect of pre-illumination on the half-life time of Reaction I.

Pre-illumination time (s)	Half-life time (ms)
0.1	67
0.25	39
0.5	42
1.0	43
2.0	41
4.0	50
32	48

Furthermore I observed that the effect of pre-illumination was about similar under both phosphorylating conditions and non-phosphorylating conditions whereas the proton permeability is expected to be distinctly different. Therefore it seems unlikely that the observed effects of pre-illumination are due to a proton gradient whether localized or not. I suggest that the enhancement of Reaction I decay after short pre-illumination is due to a fast competitive release of cations from H^+ binding sites at the inside thylakoid surface. A local increase of free Mg^{++} concentration at the membrane boundary may cause the observed increase of membrane conductance. Accordingly, I observed that the effect of pre-illumination on Reaction I kinetics is considerably less in the presence of $MgCl_2$ concentrations above 4 mmol/l (not shown). In this case, the membrane conductance is less affected by proton-induced cation release from the membrane because of the existence of a high concentration of free moving ions at the boundary of the membrane (see also Section 8.6).

6.4 MEASUREMENT OF THE ELECTRICAL RESISTANCE OF THYLAKOID MEMBRANES

The single-electrode pulse method (Lettvin et al., 1958) opens perspectives to obtain information on the magnitude of the resistance of the thylakoid membrane. However, the method has some shortcomings mainly due to the fact that there are no means for a test of the electrode quality after impalement. Therefore the method gives only qualitative information on the resistance of the thylakoid membrane. But hitherto, it is the only direct method available. After impalement of the electrode, the total resistance of the circuit, detected by the potential response upon a 400 ms hyperpolarizing current injection of 1 nA, was found to be 50-60 MΩ. The response upon such a current pulse is biphasic (Fig. 32b). The fast phase is attributed to the potential difference across the electrode tip and is determined by the electrode resistance. The slow phase is a reflection of the transmembrane potential difference generated by the charging current passing across the membrane. Extrapolation of the semi-logarithmic plot (Fig. 32b) of the slow phase to time zero shows that the potential across the membrane is about 2 mV at a loading current of 1 nA indicating a membrane resistance of 2 MΩ. Similarly, the response upon depolarizing pulses could be analyzed (not shown). The relaxation time of the current-induced potential change (Fig. 32b) agrees fairly well with that of the potential decay after a single-turnover flash (Fig. 32a). The membrane resistance derived from such experiments in general varied between 2 and 40 MΩ, depending on the magnitude of the pulse and the state of the chloroplast. I observed a maximum value of 60 MΩ at a depolarizing pulse of 0.25 nA (not shown). High current injection above 1-1.5 nA gave rise to a potential overshoot indicative for the "punch-through" effect (Coster, 1965) that is caused by a sharp decline of the membrane resistance above a certain critical potential. The validity of the pulse method is supported by the observed disappearance of the slow potential kinetics in the presence of the ionophore valinomycin (Fig. 32c) that is known to increase the specific membrane conductance for K^+. It should be noted that the amount of K^+ in the chloroplast may increase due to KCl leakage out of the electrode tip.

The membrane conductance is determined by the permeability of ions and the amount of ions available for charge transport across the membrane. The electrode measurements

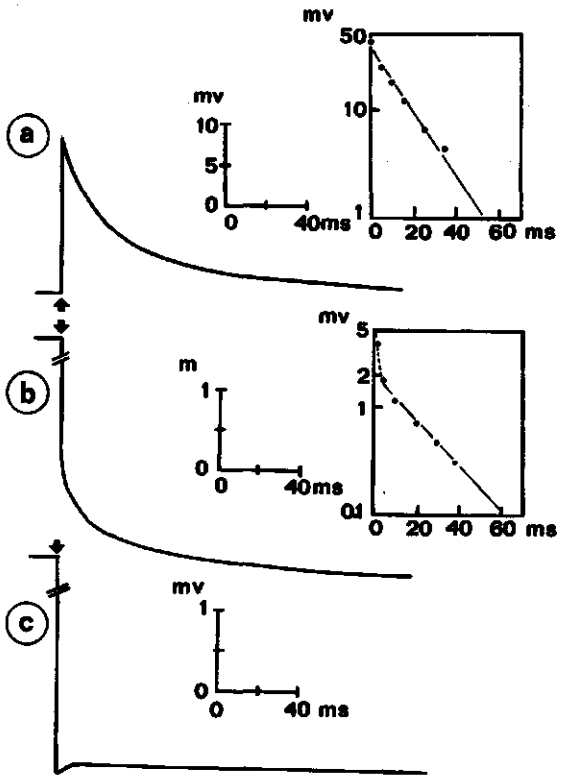

Fig. 32. a) Time course of a flash-induced electric potential and the semi-logarithmic plot of its decay kinetics. The arrow indicates the moment at which the flash was fired. b,c) Electrical responses of the electrode and the thylakoid membrane upon a hyperpolarizing current injection in the absence (b) and the presence (c) of 30 µmol/l valinomycin. The arrows indicate the moment of pulse injection. Extrapolation of the slow phase in the semi-logarithmic plot to time zero gives the voltage, generated across the thylakoid membrane.

are not suitable for a quantitative study of the selective conductance of the thylakoid membrane because variation of ion concentration in the inside of the chloroplast is hampered by the envelope barrier. Therefore this sort of experiments were performed on broken chloroplasts using the Reaction I kinetics of P515 as the determinant probe (Section 6.2).

Fig. 33 gives the I-V characteristic of the thylakoid membrane measured with a single electrode with reference to an external electrode. Before inserting the electrode, its current-voltage linearity was checked. This check is important to prevent a falsified interpretation of results due to artifacts caused by a non-linear current-voltage relationship of the electrode. It is discerned, however, that this control is not a definite precaution against possible artifacts because the condition of the electrode tip may be different before and after impalement. The different ionic composition of the thylakoid interior and contaminating substances clinging to the electrode tip may cause changes in electrode resistance. However, the typical bended shape of the membrane current-voltage relation, depicted in Fig. 33, was only observed if reproducable light-dependent potential responses could be evoked. If the latter were relatively small, or even absent, the cur-

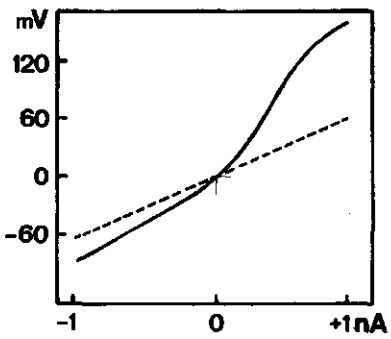

Fig. 33. Current-voltage characteristic of the thylakoid membrane. The curves were measured by applying a current, from -1 nA to +1 nA changing at a uniform rate of 0.66 nA s^{-1}. The broken line represents the current-voltage characteristic of the electrode before impalement.

rent-voltage characteristic usually was straight although a considerable increase of resistance was observed after electrode impalement.

After a rather constant value, a depolarizing current across the thylakoid membrane was found to cause an increase of the apparent resistance up to a certain level. Beyond this level the resistance was found to decline and might even become zero. At this high current flow spontaneous firing of action potentials was observed indicating a dramatic change of the functional condition of the membrane.

6.5 DISCUSSION

The data depicted in Fig. 33 show that the electrical resistance of the thylakoid membrane is not a constant but rather depends on the direction and magnitude of a polarizing current flux through the membrane. Non-linear current-voltage characteristics have been observed in the plasmamembrane of *Nitella translucens* (Vredenberg & Tonk, 1973) and also in thylakoid membranes of *Peperomia* chloroplasts (Bulychev et al., 1976). Although the latter authors do not specify the rectifying property of the electrode, they conclude about the same resistance for the thylakoid membrane at zero current (i.e. 20-30 MΩ).

Assuming a specific conductance of the thylakoid membrane of 30 $\mu S.cm^{-2}$ which can be calculated from the data in Fig. 32a (see also Vredenberg, 1976) and a total resistance of 20 MΩ (Fig. 33) it can be calculated that the membrane surface involved is about 15×10^4 μm^2. The surface area of one thylakoid is about 3.5 μm^2. Therefore it seems that about 4.3×10^4 thylakoids are interconnected, corresponding to an internal volume of about 110 μm^3. The total volume of a *Peperomia* chloroplast is about 700 μm^3. The amount of thylakoids being interconnected might be even higher because in my calculation the whole thylakoid surface was assumed to participate in the ion exchange mechanism. However, only part of the thylakoid surface (i.e. the margin) is exposed to the stroma; the major part is situated in the partition zone. The resistance measurements support the evidence that granum stacks are no seperate entities but rather form a unity, interconnected by stroma lamellae. Paollilo (1970) points to the existence of a spiral fretwork, interconnecting individual grana and granum stacks. Even several parallel frets may be similarly associ-

ated with a single granum. For a review see Coombs & Greenwood (1976).

The observed non-linear current-voltage relationship of the thylakoid membrane suggests evidence for an asymmetrical double-lattice model of the thylakoid membrane. Such a model has been described for other biological membranes (Coster, 1965). In an asymmetrical fixed-charge lattice model, an abrupt transition of the electrostatical potential exists at the so-called depletion zone. The concentration of mobile ions in the vicinity of this zone is dependent on the sign and the magnitude of the applied voltage. Fluctuation of the mobile ion concentration at the depletion zone by an applied external field will cause concentration gradients in the neighbouring fixed charge regions and consequently a diffusion of ions down the concentration gradient will take place. The current density due to ion diffusion decreases if the junction potential is magnified by the external applied voltage. Alternatively, the current density increases if the applied voltage is opposite to the junction potential. As may be concluded from Fig. 33, the current density through the thylakoid membrane increases if the sign of the applied voltage is negative. This would suggest that the fixed negative charge-density at the inner thylakoid surface is less than at the outer surface.

Analyses of the relative contribution of different ions to the thylakoid membrane conductance (Figs. 28-32) pertain to four conclusions:

1. The proton conductance of the thylakoid membrane at pH 5-8 is negligible as compared to its conductance to other ions, both after a dark period and a pre-illumination period, long enough to establish a maximum proton gradient across the membrane. The small acceleration of Reaction I after a short period of light apparently is not due to a direct effect of proton accumulation. I propose that this acceleration is the result of an increase in the permeability of other ions or an increase of the cation concentration in the membrane boundary due to expulsion of cations from the membrane or inner membrane double layer by proton-binding. A competitive Mg^{++} extrusion in the dark upon H^{+} addition has been observed by Bose & Hoch (1978).

2. A similar conclusion is drawn from the observation that lowering pH apparently results in an increased membrane conductance which is not due to the higher amount of available protons. The mechanism of the proton-induced permeability increase is not known. From the effect of surface-charge neutralisation on the ion distribution at low pH, the Mg^{++} concentration at the boundary may be expected to decrease which consequently may cause a decrease of the Mg^{++} conductivity. However, the changes in surface charge density in the traject from pH 6 to pH 8 are only minor (c.f. Chapter 7). A possible mechanistic explanation may be given by pH-dependent opening and closing of specific Mg^{++}-conductance channels or alternatively a H^{+} - Mg^{++} antiport mechanism (Karlish & Avron, 1968) facilitated by low pH (see Section 8.6). The observed competition of Mg^{++} and H^{+} for binding sites (Bose & Hoch, 1978) makes the antiport mechanism acceptable. The existence of specific ion-conductance channels however, may not be excluded. Such channels may fulfill an important regulating role, expecially when the function of that specific ion is determined by its distribution over different compartments. Therefore, a selective proton channel through the ATP-synthesizing complex is indispensable, but even then the selectivity apparently is not determined by the membrane component (CF_0) but it is rather a consequence of the CF_1 binding at the outside of the channel (Fig. 31).

3. In this respect the observed increase of selective Cl^- conductivity upon lowering pH from 8 to 5 might be caused by a decrease of negative surface charges favouring an increase of Cl^- ions at the membrane boundary.

4. The observed differences in permeability of a number of anions agree with observations of others using an independent method (Schuldiner & Avron, 1971).

7 Light-dependent conformational changes in the thylakoid membrane

7.1 INTRODUCTION

Based on available kinetic data, at least two categories of light-dependent structural changes can be distinguished:

- Rather slow conformational changes occurring in the second to minute time range due to changes in the ionic micro-environment and dehydration of components within the membranal core that are probably correlated with ion fluxes and water movement during illumination (Packer, 1963; Dilley & Vernon, 1965; Murakami & Packer, 1970; Torres-Pereira et al., 1974).
- Relatively fast conformational changes with a half-life time of 20-100 ms (Kraayenhof & Slater, 1975; Kraayenhof, 1975) that were found to be correlated with an energy dependent exposure of negative sites towards the outside thylakoid membrane surface or an occlusion of positive sites on the ATPase (Giaquinta et al., 1973, 1974; Kraayenhof, 1975).

Boyer (1965), proposed a mechanism of sequentional conformational-energy transduction in which electron-transporting proteins are involved. These conformational changes, which are probably accompanied by changes in pK_a of protein groups on the oxidation-reduction components (Papa, 1976) are likely to result in an altered charge density.

The occurrence of conformational changes in the ATP synthetizing complex was first observed by Ryrie & Jagendorf (1972) and their findings were confirmed by others (McCarty & Fagan, 1973; Lutz et al., 1974; Kraayenhof & Slater, 1975; Harris & Slater, 1975).

I suggested (Chapter 5) that the slow component of the flash-induced absorbance change at 515 nm, was a reflection of an altered conformation of proteins or lipids in the membrane, induced by an electrogenic effect near Photosystem 1. This was proposed to lead to a change in strength and orientation of internal electric fields near the P515 pigment complex. Therefore I investigated whether there was some common mechanistic basis for fast conformational changes of the ATP-synthetizing complex, Reaction II and the proposed changes in electrical charge density of the membrane, in the course of photosynthetic energy transduction.

Most biological membranes including chloroplast membranes (Dilley & Rothstein, 1967; Gross & Hess, 1974; Berg et al., 1974; Gitler, 1971) bear a net negative surface charge in the physiological pH-domain. The corresponding negative electric surface potential may be considered as an entity that controls the ionic composition in the diffuse double layer adjacent to the membrane surface and thereby intrinsic metabolic and structural membrane properties, including the translocation of ions (Gross & Prasher, 1974; Barber et al., 1977; Theuvenet & Borst-Pauwels, 1976a, 1976b). A number of surface-related phenomena, such as the electrostatic absorption of dye molecules (Montal & Gitler, 1973; Searle et al., 1977) and uncouplers (Bakker et al., 1975) seem to be in good harmony with the theory of

the diffuse double layer developed by Gouy and Chapman (Davies & Rideal, 1963; Aveyard & Haydon, 1973). McLaughlin (1977) has reviewed general aspects of this theory with special emphasis to electrostatic phenomena of surfaces of biological membranes.

A 15% increase of the (negative) surface charge has been observed upon illumination of intact spinach chloroplasts (Nobel & Mel, 1966). In a recent electrophoretic study on membranes of intact chloroplasts and of divalent cation-depleted thylakoids membranes, Nakatani et al. (1978) did not observe any light-induced increase of surface charge density.

In this chapter a characterization of the electrokinetic behavior of chloroplasts in response to energization by light will be given. The results are discussed in relation to the processes that give rise to the Reaction II response of P515. See also Schapendonk et al. (1980).

7.2 MATERIAL

Absorbance changes were measured in chloroplasts, prepared as described in Chapter 2. All other measurements were carried out with chloroplasts prepared as described below. "Intact" (outer membrane-containing) chloroplasts were made from freshly grown or market spinach by homogenization of pre-cut leaves (10 g) with a cavitation disperser (IKA Ultra-Turrax, equiped with 18N shaft) for 2 x 1 s at maximum speed, followed by filtration through 8 layers of perlon net (40 μm mesh width) and 1 min centrifugation at 2000 *g*. The isolation and resuspension medium (usually 50 ml and 2 ml, respectively) contained 330 mmol/l sorbitol and 2 mmol/l tricine, pH 7.2 and, where indicated, 5 mmol/l $MgCl_2$. Broken chloroplasts were obtained by suspending the pellets in 1 ml H_2O or 10 mmol/l $MgCl_2$ for 1 min, after which 1 ml of double-strength medium was added. The electrophoresis media were as indicated in the legends to the tables and figure. The method for measuring electrophoretic mobility of chloroplast particles is described in Chapter 2.

7.3 SOME ELECTROKINETIC DATA OF DIFFERENT CHLOROPLAST PREPARATIONS

The electrophoretic mobility (u) of chloroplasts and the effect of illumination were found to depend on chloroplast integrity. Broken chloroplasts showed a slightly larger dark-mobility and the light-stimulation ($\Delta u_{D \to L}$) was found to be roughly 2 to 5 times that of intact ones.

The screening of negative charges on the thylakoid membranes by the chloroplast envelope is probably the reason for the relatively low increase in mobility in intact chloroplasts. The effect of $MgCl_2$ on the mobility of broken chloroplasts was investigated and the results are summarized in Table 2. Chloroplasts isolated and broken in the absence of $MgCl_2$, but without drastic depletion of bivalent cations (Nakatani et al., 1978), show a decrease of electrophoretic mobilities and of light-stimulation upon increasing the $MgCl_2$ concentration in the electrophoresis medium (Table 2). The obvious advantage of using broken chloroplasts isolated in the absence of cations, is that they provide a less complicated membrane system more suitable for comparing the cation effects with theoretical models as predicted by the Gouy-Chapman theory. The results of these tests indeed are found

Table 2. Effect of $MgCl_2$ on the electrophoretic mobility of broken chloroplasts prepared in the absence or presence of $MgCl_2$[a].

Experiment	$MgCl_2$ (mmol/l)	$-u \cdot 10^5$ ($cm^2 \cdot V^{-1} \cdot s^{-1}$)		$\Delta u_{D \to L}$ (%)
		dark	light	
A	0	19.9	28.5	43
	0.05	19.0	24.5	29
	1.0	13.0	14.6	12
B	0.05[b]	13.8	26.9	95
	1.0	13.6	18.2	34

a. The broken chloroplasts were isolated in the absence (expt. A) or presence (expt. B) of 5 mmol/l $MgCl_2$. In both experiments electrophoresis was carried out in isolation medium plus 10 µmol/l diquat. Chlorophyll content was 10 µg/ml.
b. This concentration of $MgCl_2$ was introduced into the medium with the chloroplast suspension.

to be in good harmony with the theory (Searle et al., 1977; Nakatani et al., 1978). If chloroplast membranes are kept under a more physiological condition by isolation in the presence of Mg^{++} ions, they show a smaller electrophoretic mobility in the dark, but the stimulatory effect of light at low Mg^{++} concentration is considerably increased. Further experiments to be reported here are performed with broken chloroplasts only.

7.4 THE RESPONSIVENESS OF ELECTROPHORETIC MOBILITY AND REACTION II UPON CHEMICAL TREATMENT

The pH-dependence of the dark- and light-mobilities of broken chloroplasts is shown in Fig. 34. Both mobility curves sharply decline below pH 6 and indicate a charge neutralization point at pH 4.8, roughly confirming the results of Nakatani et al. (1978) on non-

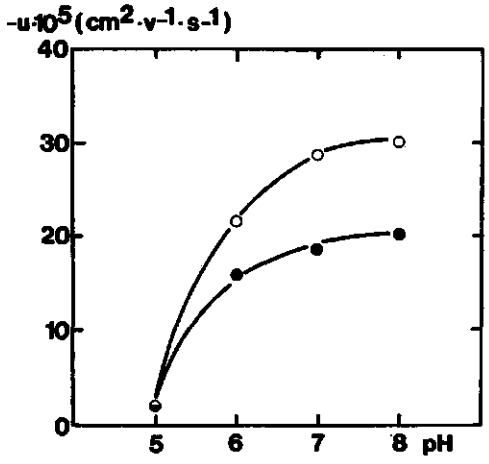

Fig. 34. The electrophoretic mobility of broken chloroplasts in dark (closed circles) and light (open circles) as a function of pH, measured in the absence of $MgCl_2$. Experimental conditions were as in Table 2A.

energized chloroplast membranes. It appears that illumination causes exposure of additional negative groups with identical pK_a as the groups already exposed in the dark.

Fig. 35 shows single-flash induced absorbance changes, measured at pH 6.7 and pH 5.2. The slow decay component representative for Reaction II, which is clearly observed at pH 6.7 appears to be absent at pH 5.2. At this pH only Reaction I is observed. A plot of the Reaction II/Reaction I ratio, as a function of pH, is depicted in Fig. 35. There appears to be a maximum value for this ratio at pH 6.7 and a sharp decline between pH 6 and pH 5.2. The magnitudes of Reaction I and Reaction II were analyzed as described in Chapter 5.

Table 3 lists the effects of chemical and physical treatments on the electrophoretic mobility of broken chloroplasts. The electron transfer inhibitor DCMU does not affect the

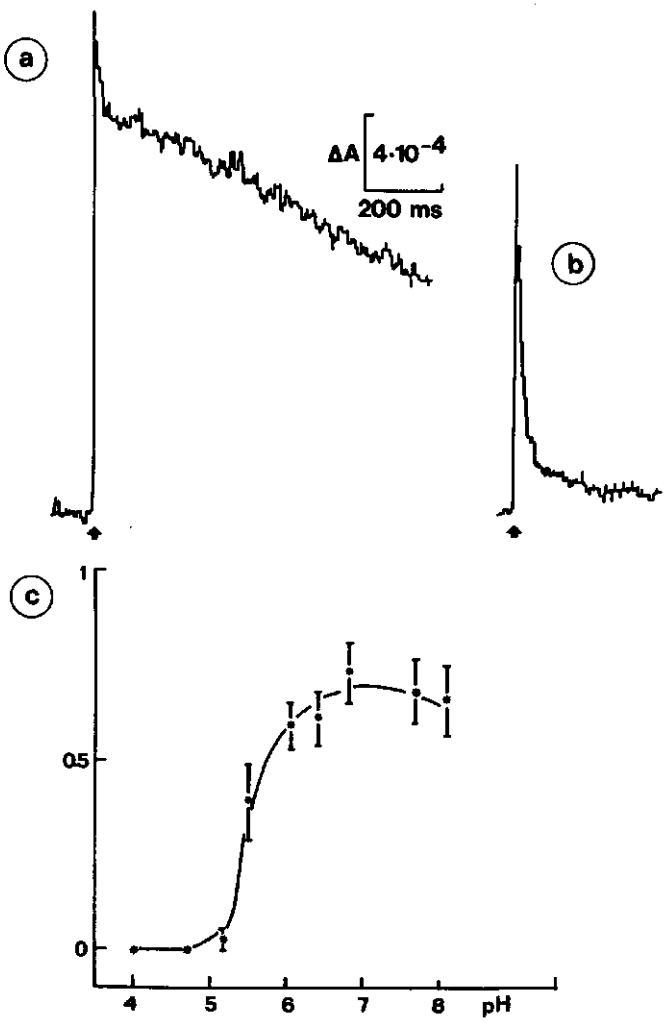

Fig. 35. Upper part: Response of P515 to flashes fired at a repetition rate of 0.05 Hz, at pH 6.7 (a) and pH 5.2 (b), respectively. Lower part: the ratio Reaction II/Reaction I, plotted as a function of pH (c).

Table 3. Effects of electron transfer inhibitor, uncouplers and physical treatments on the electrophoretic mobility of broken chloroplasts[a].

Addition or treatment	$-u \cdot 10^5$ ($cm^2 \cdot V^{-1} \cdot s^{-1}$)		$\Delta u_{D \to L}$ (%)
	dark	light	
None	14.0	27.0	93
DCMU (0.5 µmol/l)	14.0	15.0	7
DCMU (0.5 µmol/l) + PMS (6 µmol/l) - diquat	14.9	27.1	82
CCCP (5 µmol/l)	17.4	14.8	-15
S-13 (1µmol/l)	13.5	14.1	4
ACMA (15 µmol/l)	12.1	14.0	16
1.5 min 60°C	5.3	5.1	- 4
2 min 2.5% glutaraldehyde	6.7	6.9	3
valinomycin (1 µmol/l)	12.6	20.7	64
valinomycin (5 µmol/l)	12.1	12.5	3

a. The broken chloroplasts were prepared in the presence of 5 mmol/l $MgCl_2$. The electrophoresis medium contained 330 mmol/l sorbitol, 10 mmol/l KCl, 2 mmol/l tricine buffer, pH 7.2, 10 µmol/l diquat (0.05 mmol/l $MgCl_2$ introduced with chloroplast suspension). Chlorophyll content 10 µg/ml.

dark-mobility but prevents the light stimulation. Addition of PMS completely restores the light effect. A similar observation has been described in Chapter 5 for Reaction II.

The effect of valinomycin was tested under a variety of conditions. We found that this antibiotic invariably eliminates the light-stimulation both in the presence (Table 3) and absence (not shown) of added K^+ ions, which is in complete agreement with the observed inhibition of Reaction II by the ionophore (c.f. Chapter 5), exept for the fact that Reaction II appears to be inhibited at somewhat lower concentrations. This discrepancy might be due to the difference in the isolation procedures used. Treatments which inhibit Reaction II, are also found to diminish the light-induced increase of negative surface charges: A short heat-treatment (1.5 min at 60°C) of a chloroplast suspension drastically lowers the dark-mobility and abolishes the light-effect. Ageing of the chloroplasts for 3 h at 25°C did not affect the dark-mobility significantly but gradually diminished the light-stimulation (not shown). Treatment of the chloroplasts for 2 min with 2.5% glutaraldehyde results in a decreased dark-mobility and an inhibition of the light-stimulation. West & Packer (1970) reported that in the latter case ATP synthesis is impaired, whereas electron transfer and H^+ uptake still proceeds.

7.5 REACTION II AS A POSSIBLE INTRINSIC MARKER FOR THE CONFORMATIONAL STATE OF THE COUPLING FACTOR

Fig. 36 shows the semi-logarithmic plot of the 515 nm absorbance decay after a series of 7 flashes in the absence and the presence of $MgCl_2$ (Fig. 36a and 36b, respectively). The absorbance level in the 0.4-1 s time domain which is due to Reaction II (c.f. Chapter 5) was found to be appreciably lower in the presence than in the absence of ADP and K_2HPO_4, both in the absence (Fig. 36a) and the presence (Fig. 36b) of Mg^{++}. The ionic strength of the assay medium in the absence of K_2HPO_4 was adjusted to an equivalent molarity with KCl. The decay kinetics of Reaction II appear to be similar, although in the absence of Mg^{++},

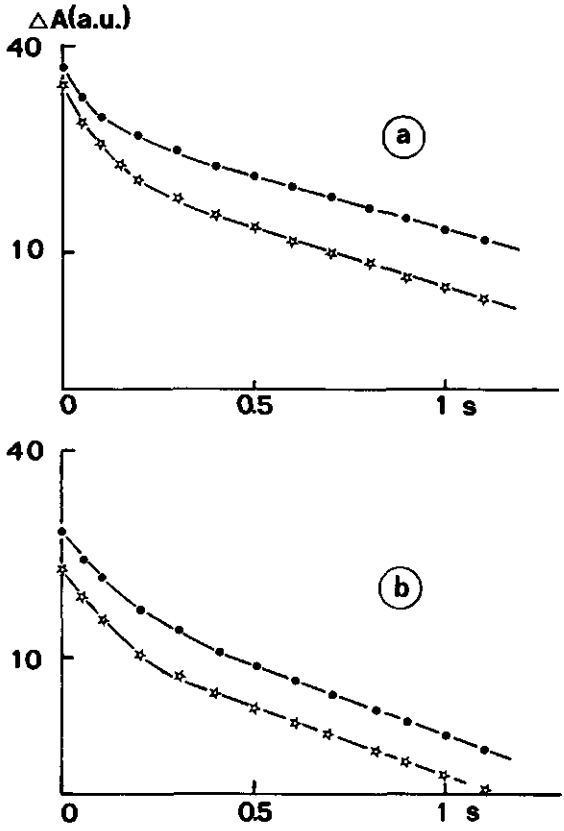

Fig. 36. Semi-logarithmic plots of the absorbance decay at 515 nm after a series of 7 flashes fired at 100 ms time intervals in the presence of either 10 mmol/l KCl (a) or 10 mmol/l $MgCl_2$ (b). The P515 decay under phosphorylating-(+0.8 mmol/l ADP and 1 mmol/l KH_2PO_4) and non-phosphorylating conditions is indicated by open stars and closed circles, respectively. The logarithms of the absorbance changes are given in arbitrary units (a.u.).

the decay is slightly faster in the presence of ADP and K_2HPO_4. The decay of Reaction I was found to be unaffected by ADP and K_2HPO_4 (Section 6.3). Conclusive with results of Girault & Galmiche (1976) we found that addition of ATP had qualitatively the same effect on Reaction II as ADP and K_2HPO_4. Accordingly, the apparent acceleration of the overall response in the presence of ADP or ATP must be due to a decreased contribution of Reaction II to the overall response. These data suggest that the induction of changes in the local electric field (Reaction II), is partly affected by an altered conformation of a membrane component, possibly the ATPase complex, due to binding of ADP or ATP.

Fig. 37a and 37b show the analyses of the P515 response in the absence and the presence of 4 μmol/l DCCD per mg chlorophyll. In the presence of DCCD, Reaction II appears to be completely inhibited. The slow process that is responsible for the DCCD insensitive part of the absorbance change (Fig. 37b) is not yet known but its slow rise and decay kinetics which are different from the kinetics of Reaction II, lead to the suggestion that some other as yet unknown effect might be involved. A similar absorbance component which we called phase d (c.f. Chapter 5), was observed in the absence of DCCD. We did not correct

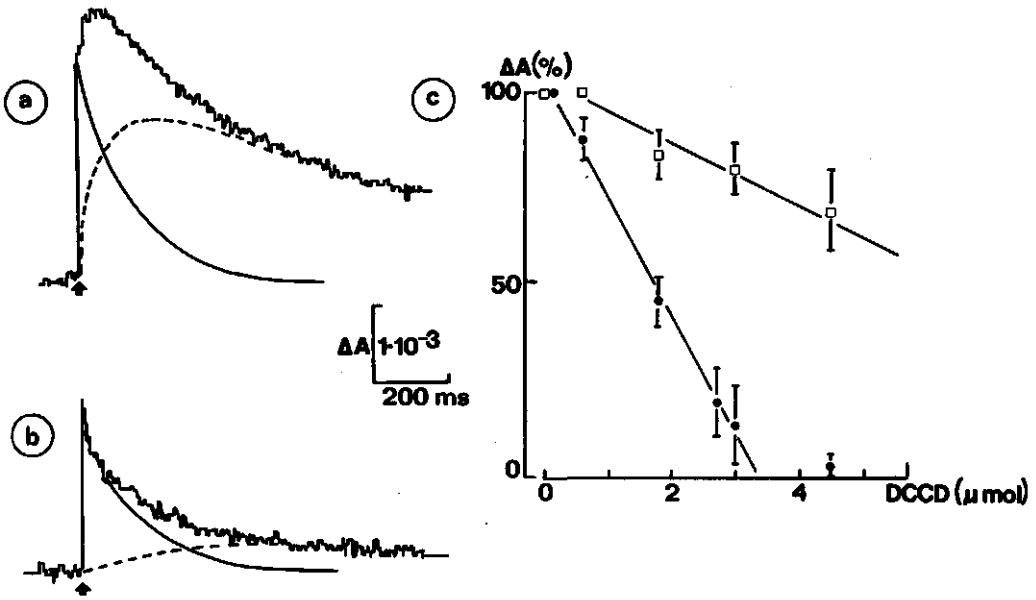

Fig. 37. Time course of the P515 absorbance changes in broken chloroplasts in the absence (a) and the presence (b) of 4 μmol DCCD per mg chlorophyll, respectively, induced by repetitive single-turnover flashes, fired at a repetition rate of 0.05 Hz. Reaction I kinetics (solid curves) were measured immediately after pre-illumination with 6 flashes, fired at a repetition rate of 3 Hz. In (c) the relative contribution of Reaction I (open squares) and Reaction II (closed circles) is plotted as a function of the added amount of DCCD per mg chlorophyll.

for this phase because of its relatively small contribution. Fig. 37c shows the maximum extent of Reaction I and Reaction II, respectively, as a function of the amount of DCCD. Reaction II is completely inhibited at a DCCD concentration at which the magnitude of Reaction I corresponds to 75% of the control. The observed decrease of Reaction I upon addition of DCCD probably is caused by a secondary effect due to inhibition of electron transport (Uribe, 1971). This effect might also be responsible for a partial inhibition of Reaction II, because the onset of the conformational change associated with Reaction II is suggested to be primarily caused by an electrogenic mechanism located at the Photosystem 1 site (Section 5.7). However, the different concentration levels of DCCD at which Reaction I and Reaction II are inhibited (Fig. 37c), indicate that Reaction II inhibition can not exclusively be caused by inhibition of electron transport, even when DCCD would have a predominant effect on electron transport activated by Photosystem 1 only. Moreover it has been observed that restoration of electron transport in DCCD poisoned chloroplasts by addition of 6 μmol/l PMS results in a complete recovery of Reaction I but without a concomitant recovery of Reaction II (not shown).

Extraction of CF_1 by mild EDTA treatment (chloroplasts were incubated in 0.5 mmol/l EDTA and 2 mmol/l tricine (pH 8) resulted in a partial inhibition of Reaction II (Fig. 38). The ability of chloroplasts to phosphorylate or to carry out light-dependent H^+ uptake is known to be lost under these conditions (Avron, 1963; McCarty & Racker, 1966). This again gives an indication that the processes reflected by Reaction II are connected with

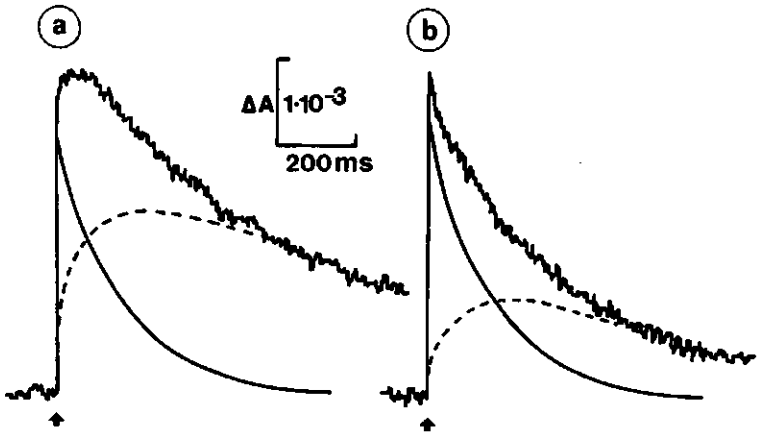

Fig. 38. Time course of the P515 absorbance change in broken chloroplasts before (a) and after EDTA treatment (b), induced by repetitive single-turnover flashes fired at a repetition rate of 0.05 Hz. The resolved responses are obtained according to the analysis discussed in Chapter 5, Fig. 15.

the functional integrity of the ATP-synthesizing system. An attempt to inhibit the conformational changes in CF_1 and concomitantly Reaction II more specifically by treatment with anti-serum against CF_1, was unsuccesfull. The isolated CF_1 fraction was found to be highly pure according to the electrophoretic band pattern in a SDS-polyacrylamide gel, but the antibodies inhibited neither the Ca^{++}-dependent ATPase (J. Verheyen, personal communication), nor the light-dependent ATP synthesis, nor the electron transport rate. Although the antibodies did precipitate with antiserum and chloroplast agglutination was observed, no inhibition was found to occur, even when the antibody was titrated over a wide concentration range. These results are in agreement with those of others (Nelson et al., 1973; Shoshan et al., 1975) who showed that antibody functioning in relation to ATP synthesis is confined to very specific parts of the CF_1 rather then to an indispensable unity of the total CF_1.

7.6 DISCUSSION

The present experiments show that illumination of chloroplasts in the presence of suitable electron transfer mediators causes a marked enhancement of particle mobility, the effect being most pronounced in broken (envelope-free) chloroplasts. The smaller light-stimulation (15-30%) in "intact" chloroplasts would confirm earlier observations of others (Nobel & Mel, 1966). Obviously, a more or less intact outer membrane shields the thylakoid surface charge effects (and changes thereof). On the other hand, the structural and functional integrity of the thylakoid membranes themselves is a prerequisite for the light-effect. Extensive depletion of divalent cations and $tris^+$-washing prior to the electrophoresis experiments (Nakatani et al., 1978) leads to a modification of gross chloroplast structure (grana stacking) as well as of the molecular structure of the thylakoid membrane; the lipid bilayer is expected to become less ordered and membrane-bound proteins, including the ATPase, are removed. Earlier work on divalent cation binding (Gross et al., 1969;

Gross, 1972) shows that divalent cation depletion leads to uncoupling of energy-linked functions. This may explain the lack of light-stimulation of electrophoretic mobility in the thylakoid preparations used by Nakatani et al. (1978) and the low extent or even absence of Reaction II in chloroplast that are broken and incubated in absence of salts.

In contrast to the observations of Nobel & Mel (1966) the light-induced increase of the negative membrane surface charge density, like the occurrence of Reaction II, is shown to be uncoupler-sensitive (Table 3) and seems to go hand in hand with the light-induced conformational changes detected as an electrostatic interaction of chloroplasts with cationic probes, such as aminoacridines (Kraayenhof, 1973), ethyl red (Heath, 1973) and PMS (Homann, 1976). However, it should be remembered that the Reaction II kinetics were studied in flash illumination and the electrophoretic experiments were studied in continuous illumination. With this in mind, one is left with the notion that addition of valinomycin, acidification, addition of DCMU and PMS and various physical treatments, have qualitatively the same effect on both the extent of Reaction II and the light-stimulated increase in surface charge density.

The magnitude of the charge density corresponds to a zeta (ζ) potential of -18 mV in the dark and -37 mV in the light (Schapendonk et al., 1980). It seems very unlikely that the light-induced increase of negative surface charges is a direct result of charge reorientation at the photosynthetic reaction centers, since these charge changes are expected to be too small to be detected by electrophoresis (Nakatani et al., 1978). From chemical modification studies, Nakatani et al. (1978) deduced that the thylakoid surface charge is mainly determined by carboxyl groups of aspartic and glumatic acid which suggests that membrane proteins are most likely involved in the altered charge density upon illumination, described here.

The obvious similarity between the effect of pH on the extent of Reaction II (Fig. 35) and on the decrease of the light-induced change of the negative charge density (Fig. 34) respectively, expecially the narrow transition phase between pH 6 and pH 5, is in good agreement with the suggestion that both Reaction II and the observed negative charge exposure are an expression of a reorientation of fixed negative charges associated with conformational changes in the membranal core.

The fact that both Reaction II and the light-stimulated generation of fixed negative charges can be catalysed by Photosystem 1 only, also suggests the existence of a common basic principle (compare Table 3 and Fig. 27). If Reaction II and also aminoacridine binding are believed to originate from the same electrogenic process as the changes in surface charge density, then the kinetics of the former processes show that the generation of surface charges is much faster than the energy-dependent increase of chloroplast light-scattering and bulk ion translocation. The generation of negative charges and the rise-time of Reaction II appear to coincide with the light-induced conformational change in the membrane-bound ATPase, monitored with a covalent fluorescent label (Kraayenhof, 1977b).

I want to pay some attention to the functional aspects of both Reaction II and the electrophoretically detected charge exposure in the light, in particular with respect to the activity of the ATP-synthesizing complex. It should be noticed that the inner side of the thylakoid membrane also has an excess of fixed negative charges, which will decrease upon illumination in conjunction with proton uptake (Witt & Zickler, 1973; Fowler & Kok,

1974b). The resultant effect may be a localized electric field of significant strength, which might contribute to the protonmotive force (Rumberg, 1977) and which might be detected by the P515 pigment system, resulting in the appearance of Reaction II.

Alternatively there is no direct evidence that the proposed change in membrane conformation, reflected by the increase in surface charge density and the appearance of Reaction II, is a measure of the energized state of the thylakoid membrane, although, the observed inhibitory effects of uncouplers like S13 and CCCP on these processes (not shown for Reaction II) would confirm such an interpretation. If the magnitude of Reaction II would reflect such energized state, or even would be quantitatively linked with the intermediary high-energy state of the ATP-synthesizing complex, then its decrease in the presence of added ATP cannot be easily understood. Moreover, the decay rate of Reaction II would be enhanced under phosphorylating conditions. According to the results shown in Fig. 36, this appears not to be the case. However, it might be that the regulation of the capacity of the ATP-synthesizing complex, governed by a change in its conformation, goes hand in hand with membrane conformational changes, that find their expression in the appearance of Reaction II and the change in surface charge density. The inhibition of Reaction II by EDTA extraction of CF_1, or by addition of DCCD even incline to the idea that Reaction II is associated with a conformational change of the coupling factor itself. An attempt to fix the conformation of the ATPase more specifically by antibody complexation unfortunately was unsuccessful.

Harris & Crofts (1978) suggested that the activation of ATP production is controlled by a distortion of a local electric field in the vicinity of the ATPase complex. The characteristics of Reaction II suggest that under some conditions the P515 complex responds similarly, to these distorted fields. However, modification of either the ATPase or the P515 complex might result in an altered and mutually different responsiveness. Nevertheless, the manifestation of these dynamic membrane phenomena points to the functional importance of a mechanism that is highly accessible to influences of pH and ion movements and provides a possibility for regulation of the ATP-synthesizing capacity. In this context it is of interest to recall (Chapter 5) that Reaction II is activated by Photosystem 1 and as may be concluded from data of Crowther et al. (1979) its rate of appearance is substantially enhanced under conditions at which cyclic electron transport occurs. This would mean, if our interpretation is correct, that the light-activation of the ATPase is mainly, if not exclusively, dependent on (cyclic) electron transport around Photosystem 1. In agreement with this hypothesis, electron-microscopy studies of thylakoid membranes revealed that the margins, stroma lamellae and end membranes contain the ATP-synthesizing complex and only Photosystem 1, whereas the partition regions of the grana contain both Photosystem 1 and Photosystem 2 but no ATPase complex (Sane et al., 1970; McCarty et al., 1971). Further experiments have to be designed to clarify the very details of the membrane processes that control the efficiency of the energy transduction reactions that lead to ATP production and ion displacements.

8 The P515 response and the photo-electrical response during prolonged illumination

8.1 INTRODUCTION

The kinetics and magnitude of the electrical component of the electrochemical proton gradient in continuous light, resulting from capacitive membrane charging, followed by H^+ uptake and field dissipating ion fluxes, are still a matter of discussion. From changes in delayed light emission in chloroplast membranes, Barber (1972b) concluded that the steady-state potential in the light is 75-105 mV. From the distribution of permeant ions, equilibrating in the light according to a Nernst potential equal to the steady-state potential, a value of about 10 mV has been deduced (Rottenberg et al., 1972). This value was found to be similar to the one obtained by direct measurements with a micro-electrode inserted into a stack of thylakoids (Vredenberg et al., 1973).

The kinetics of the absorbance changes at 515 nm in continuous light have been proposed to reflect changes of the transmembrane electric field. The kinetics and magnitude of these optical changes in continuous light, as well as in flash light (Chapter 5) were found to be different from the transmembrane potential as measured by micro-electrodes (Larkum & Bonner, 1972; Vredenberg et al., 1973; Thorne et al., 1975; Krendeleva et al., 1977). Optical measurements, however, have been criticized because of the occurrence of scattering changes that may contribute to the observed P515 changes (Thorne et al., 1975; Deamer et al., 1967). To test the effect of scattering changes, absorbance and scattering changes in the light were recorded simultaneously at 515 nm under various conditions and an attempt was made to correct the apparent absorbance changes for the scattering changes. The results are presented in this chapter and corrected optical changes are compared with the potential responses measured by micro-electrodes.

8.2 MATERIAL

Chloroplasts were prepared according to the method described in Chapter 2 except for the following modifications. After homogenization in a Omni Mixer for 5-10 s, the chloroplasts were centrifuged at 1650 *g* for 1 min. It should be mentioned that under these conditions the chloroplast envelope might be broken prior to osmotic shocking. The measuring systems, to detect absorbance changes, scattering changes and light-dependent Mg^{++} efflux are described in Chapter 2. Although the chloroplasts, used in electrode and absorbance (difference) measurements, are of different origin, the P515 responses of intact spinach chloroplasts were found to be qualitatively similar to those in intact leaves of *Peperomia* and spinach. *Peperomia* chloroplasts were difficult to isolate, therefore spinach chloroplasts have been used in the P515 measurements.

8.3 P515 ABSORBANCE CHANGES AND CHANGES IN LIGHT SCATTERING

The time courses of the electrical potential in the light, measured in *Peperomia* chloroplasts with micro-electrodes (Fig. 39b), is obviously different from the time course of the absorbance change at 515 nm (Fig. 39a), measured in isolated spinach chloroplasts. The most prominent detail is the observed high steady-state absorbance level reached in the latter. In order to investigate to what extent this high absorbance level was caused by scattering changes, a phenomenon that is well established in thylakoid preparations (Itoh et al., 1963; Packer et al., 1965; Crofts et al., 1967), both absorbance changes (ΔA) and 90^{o}-scattering changes (ΔSc) were recorded simultaneously (Fig. 40a). 90^{o}-Scattering changes of thylakoid membranes, measured at 515 nm, are most likely a manifestation of selective dispersion due to a change of the refractive index near the absorption peak (Latimer & Rabinowitch, 1956, 1957), leading to scattering maxima at the long-wavelength sides of the absorption maxima. If the refractive index of the medium is increased towards that of the thylakoid membranes, by addition of 30% bovine serum albumen, the scattering maximum shifts towards the absorbance maximum and this, as expected, results in a decrease of the light-induced 90^{o}-scattering changes (compare Figs 40a and 40b). Thus, in continuous light, using a low chloroplast density, a significant part of the apparent absorbance changes at 515 nm may be caused by 90^{o}-scattering.

If scattering changes are suppressed (Fig. 40b) the kinetics of the absorbance changes are qualitatively similar to the potential response as probed with an electrode (e.g. compare Fig. 39b and 40b).

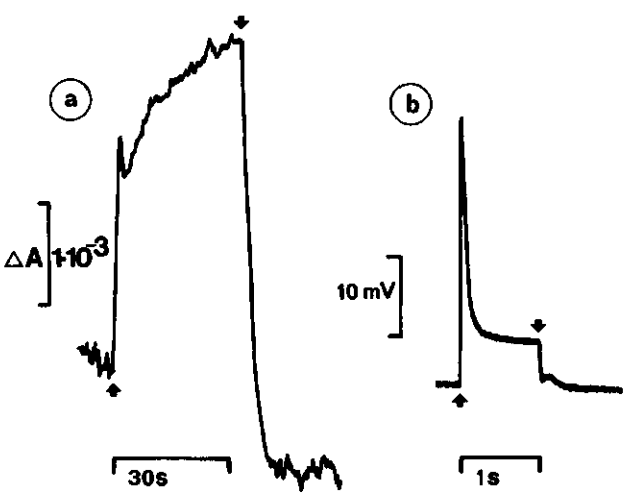

Fig. 39. Time course of the light-induced absorbance changes at 515 nm of broken spinach chloroplasts (a) and the time course of the electric potential across the thylakoids of a *Peperomia metallica* chloroplast measured by micro-electrodes (b). Upward and downward pointing arrows mark the onset and end of the illumination, respectively.

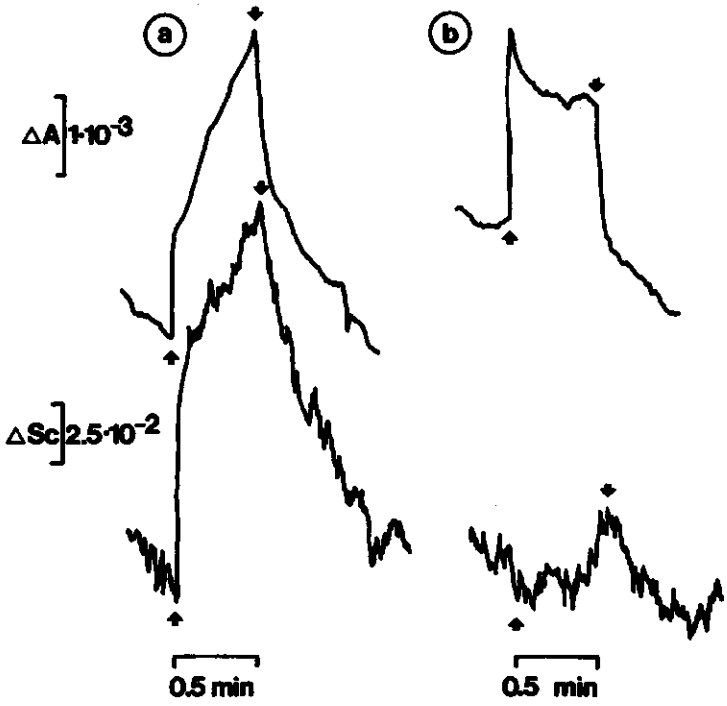

Fig. 40. Simultaneous recordings of the light-induced 515 nm absorbance changes (upper part) and 90°-scattering changes (lower part). Chloroplasts are suspended in 0.33 mol/l sorbitol. 5 mmol/l $MgCl_2$, 5 mmol/l HEPES, pH 6.5, in the absence (a) and the presence of 30% bovine serum albumen (b). Chlorophyll concentration was equivalent to 25 µg/ml chlorophyll (optical path 1 cm). Measurements were performed in the double-beam mode with a dark reference. The upward and downward pointing arrows mark the start and the end of an illumination period, respectively.

8.4 PROTON TRANSLOCATION

Convincing arguments have been given by others (Deamer et al., 1967; Thorne et al., 1975), favouring the idea that the 90°-scattering changes reflect small alterations of the index of refraction due to thylakoid loading of protons and conformational changes in the thylakoid membrane. Fig. 41a shows the effect of pH, both on the 515 nm absorbance and on the 90°-scattering. A lowering of pH results in an increase of both 90°-scattering and absorbance. The linear relation between the absorbance increase and the scattering increase indicates that the former is a direct reflection of the latter which in turn may be associated with protonation of the thylakoid membrane. There is a deviation from linearity at high scattering changes (below pH 4.5). The reason for this is unknown as yet. The $\Delta A/\Delta Sc$ ratio (in the linear region) was found to depend on the chloroplast density.

Figs 42a and 42b show the time courses of light-induced absorbance and scattering changes in a thylakoid suspension isolated in the presence of $MgCl_2$ and incubated in a low salt (0.5 mmol/l $MgCl_2$) assay medium. The $\Delta A/\Delta Sc$ ratio under these conditions was determined according to the method depicted in Fig. 41. Assuming that the light-dependent processes, and the lowering of the pH in the dark, give rise to the same $\Delta A/\Delta Sc$ ratio, a

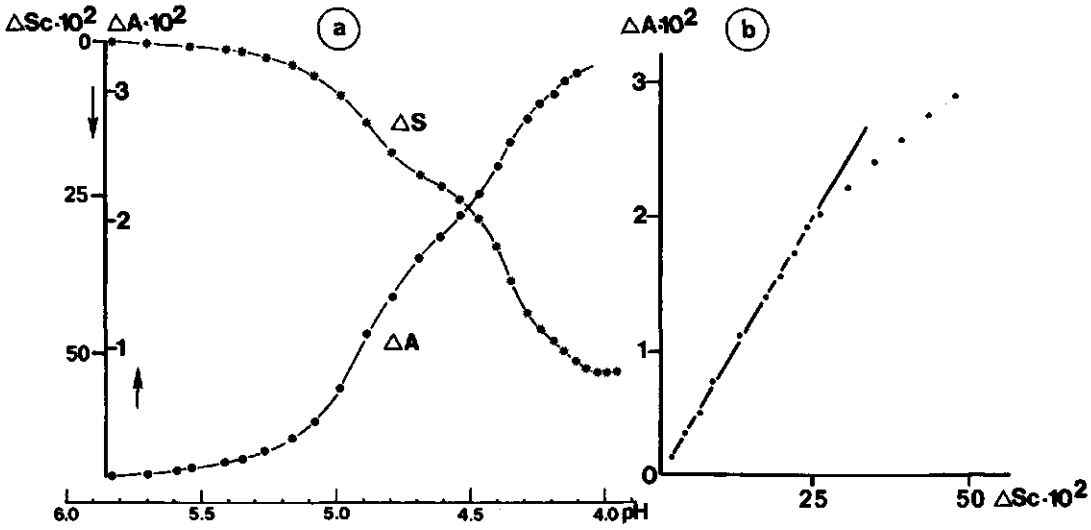

Fig. 41. Simultaneous recordings of the absorbance (closed circles) and 90°-scattering changes (closed stars) measured at 515 nm, plotted as a function of pH (a). An upward inflection of the absorbance curve and a downward inflection of the scattering curve indicate an absorbance increase and a scattering increase, respectively. b) Plot of ΔA as a function of ΔSc, derived from the recording in which the chloroplast concentration was equivalent to 130 µg/ml chlorophyll (optical path 1 cm).

correction for the apparent absorbance increase in the light can be made by subtracting the light-induced scattering change, multiplied by the estimated ΔA/ΔSc ratio (Fig. 42c). The resultant corrected kinetic pattern (Fig. 42d) shows a time course of the absorbance change much more, if not completely, compatible with the time course of the electrode-detected electric potential (Fig. 39b).

Different and complicated results were obtained in chloroplasts that were isolated and broken in ion-free medium. These chloroplasts showed a light-induced scattering decrease that appeared to be most pronounced at pH 5.9-6.3 and to be negligible at pH 8 (Fig. 43). In the latter situation the kinetic pattern of the P515 response was qualitatively similar to the response measured in the presence of ions, but corrected for scattering changes (c.f. Fig. 42d). The correction method could not be applied for the scattering decrease at low pH. In this case, the contribution of an increase of forward scattering, is probably appreciable, due to shrinkage of the unstacked thylakoids. Therefore, a different analysis and correction procedure should be applied in this case.

8.5 EFFECT OF Mg^{++} ON LIGHT-INDUCED 90°-SCATTERING CHANGES

Mg^{++} extrusion in ion-free prepared broken chloroplasts in the light was found to be 60-80 nmol/l Mg^{++} per mg chlorophyll in 20 s (Fig. 44). I assume that, in the light, Mg^{++} is not released from binding sites at the outer thylakoid surface but it is extruded either from the thylakoid interior volume or from the inner thylakoid surface, dissipating the electrochemical potential gradient. With a chloroplast volume of 26 µl/mg chlorophyll

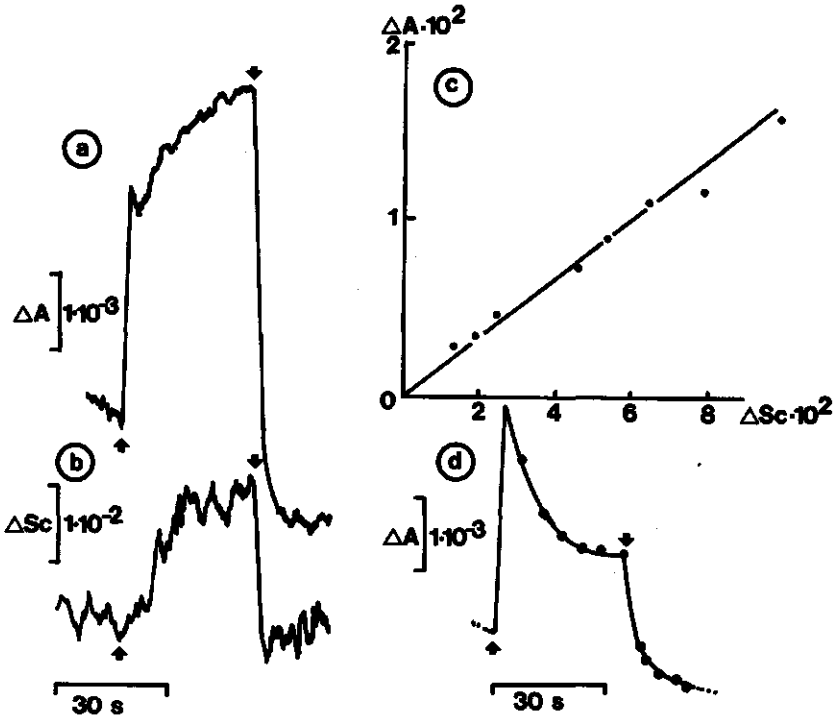

Fig. 42. Simultaneous recordings of light-induced absorbance changes at 515 nm (a) and scattering changes (b) in a suspension of broken chloroplasts containing 0.33 mol/l sorbitol, 2 mmol/l MES, pH 6.3, and in addition 0.5 mmol/l $MgCl_2$. The relation between the absorbance and scattering increase (c) is determined according to the method depicted in Fig. 41. The resolved light-induced absorbance change is depicted in d. Chloroplast concentration was equivalent to 25 µg/ml chlorophyll (optical path 1 cm). Further details are in the text.

(Heldt et al., 1973), extrusion from the thylakoid interior volume during illumination would give a calculated decrease in Mg^{++} concentration of 2-5 mmol/l. However, only 0.24 mmol/l were available at the start of the experiment indicating that the major part of Mg^{++} is membrane-bound. The release of bound Mg^{++} in the light may account for the scattering decrease depicted in Fig. 43. The amount of light-dependent Mg^{++} efflux across the thylakoid membrane has been reported to be high at low pH and to be negligible at pH 8.6 (Ben-Hayim, 1978; Portis & Heldt, 1976). The pH dependence of the 90^{o}-scattering decrease is fully compatible with these findings (Fig. 43).

Fig. 45 shows the kinetics of light-induced absorbance and 90^{o}-scattering changes at pH 6.3 in either the absence or presence of 5 mmol/l $MgCl_2$. The presence of this relatively high $MgCl_2$ concentration apparently creates a condition that favours the light-stimulated 90^{o}-scattering increase (compare Figs. 45a and 45b). This strongly suggests that light-driven Mg^{++} movement and consequent changes in Mg^{++} binding at the thylakoid surface cause 90^{o}-scattering changes similar to H^{+}-induced 90^{o}-scattering changes. Under circumstances that sufficient Mg^{++} is present in the diffuse double layer next to the inner thylakoid surface to meet the demand for counterions, the competition between the tightly-

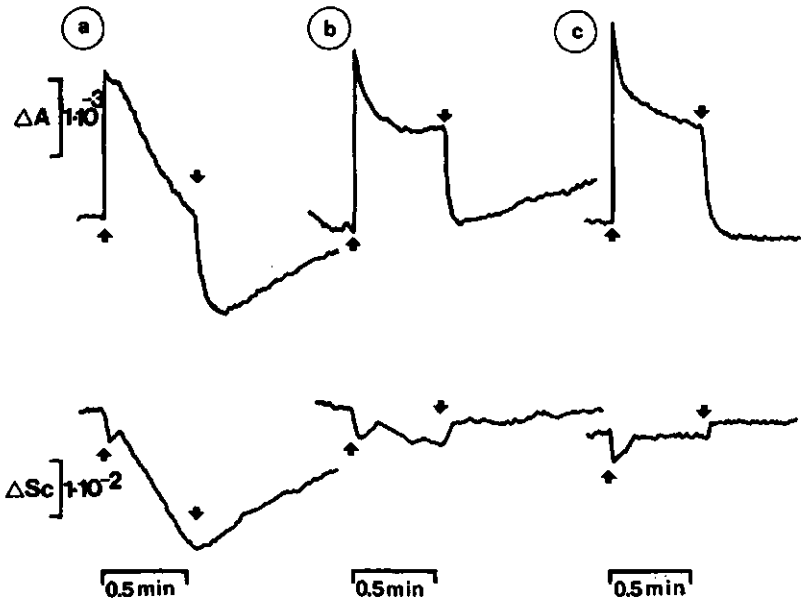

Fig. 43. Simultaneous recordings of light-induced 515 nm absorbance changes (upper part) and 90^{o}-scattering changes (lower part) at different pH: 6 (a); 7 (b); 8 (c). Chloroplasts were isolated and suspended in 0.33 mol/l sorbitol and 2 mmol/l HEPES. Chloroplast concentration was equivalent to 15 µg/ml chlorophyll (optical path 1 cm).

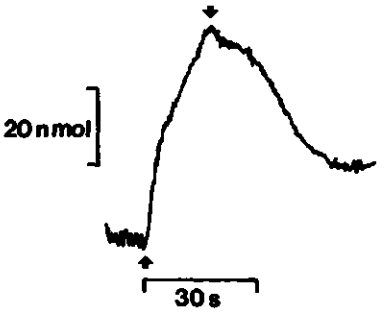

Fig. 44. Light-induced Mg^{++} extrusion from chloroplast membranes incubated in the presence of 0.24 mmol/l $MgCl_2$ measured with a Mg-sensitive electrode. The bar corresponds with 20 nmol Mg^{++} per mg chlorophyll. Arrows mark the start and end of illumination.

bound Mg^{++} and H^{+} may stop and consequently the scattering decrease (Fig. 43a) will be inhibited. The reason for the relatively high light-induced 90^{o}-scattering increase in the presence of 5 mmol/l $MgCl_2$ (Fig. 45b) as compared to the one in the presence of 0.5 mmol/l $MgCl_2$ (Fig. 42b) is not clear as yet. Recent studies (Krause, 1974; Ben-Hayim, 1978), however, support the idea that the released Mg^{++} is not diluted in the medium but remains in the diffuse double layer next to the membrane. This may lead to a high local concentration of Mg^{++} and an associated shift of the equilibrium towards complex formation of Mg^{++} and fixed negative charges at the outside surface of the thylakoid membrane. Subse-

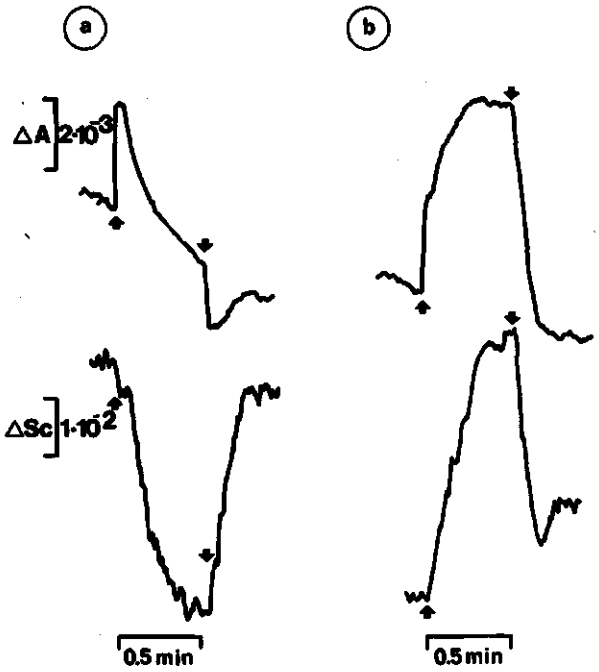

Fig. 45. Simultaneous recordings of 515 nm absorbance changes (upper part) and 90°-scattering changes (lower part) in the light. Chloroplasts were prepared and incubated, either in the absence (a) or in the presence (b) of 5 mmol/l $MgCl_2$. The chloroplast concentration was equivalent to 27 µg/ml chlorophyll (optical path 1 cm).

quent dehydration of membranal components could then lead to an increase of 90°-scattering. Our results suggest that this effect occurs at rather high concentrations of free Mg^{++} (> 0.5 mmol/l), which is seemingly conflicting with a dissociation constant of 51 µmol/l for complex formation of Mg^{++} and negative charges on the thylakoid surface as has been proposed by Prochaska & Gross (1975). However, it is difficult to predict the actual ion concentrations in the diffuse double layer, which are known to differ from those present in the bulk phase. Moreover, the net increase of the outside surface charge density (Section 7.3) upon illumination will shift the equilibrium towards complex formation.

Unfortunately, we were not able to correct for Mg^{++}-induced scattering changes in the same way as was done for H^+-induced 90°-scattering changes. The main reason for this is the effect of Mg^{++} on other processes then those under survey here, which in addition to 90°-scattering give rise to changes in forward scattering. Furthermore the effect of addition of Mg^{++} on 90°-scattering and 515 nm absorbance changes was found to be pH-dependent (Fig. 46). Because the pH of the thylakoid interior is known to decrease upon energization, associated Mg^{++} movements and binding processes will cause 90°-scattering changes that are dependent on the prevailing pH conditions. This in fact creates a rather complicated situation that cannot be analyzed by the method depicted in Fig. 42.

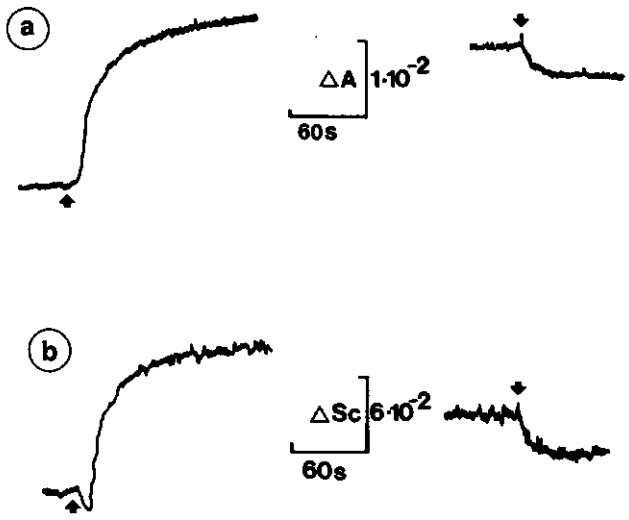

Fig. 46. Effect of addition of 10 mmol/l $MgCl_2$ (indicated by the arrows) on the 515 nm absorbance (a) and the 90°-scattering (b) of a chloroplast suspension at pH 7.6 (left-hand part) and pH 4.2 (right-hand part), respectively. Chloroplast concentration was equivalent to 80 µg/ml chlorophyll. An upward inflection means an increase in absorbance and scattering, respectively.

8.6 DISCUSSION

The results presented in this chapter indicate that the 515 nm absorbance changes in continuous light, conclusively with the interpretation of others (Thorne et al., 1975; Deamer et al., 1967), may be attributed at least partly, to light-induced 90°-scattering changes. In the past, several authors did not discern the influence of scattering phenomena on the 515 nm absorbance in the light and consequently considered the kinetic data of these changes as a direct reflection of electrogenic processes monitored by the P515 pigment system (Larkum & Bonner, 1972; Larkum & Boardman, 1974; Krendeleva et al., 1977; Satoh & Katoh, 1977, 1979; Yamamoto & Nishimura, 1977). It is not my intention to discuss their results in detail here. However, as may be concluded from the overall responses of the 515 nm absorbance changes such an interpretation will lead to an overestimation of the membrane potential (c.f. Figs 39,42 and 45). The observed 90°-scattering changes are proposed to be the result of alterations in refractive index of the thylakoid membranes due to both H^+- and Mg^{++}-binding effects on the hydration of the thylakoid membranes, and conformational changes in the membrane caused by Mg^{++}-binding.

Fig. 47 presents a possible mechanism for light-induced 90°-scattering changes in the absence (a) and presence (b) of $MgCl_2$. In the absence of Mg^{++} in the bulk phase (Fig. 47a), still a small amount of Mg^{++} is retained at special binding sites. Light-driven proton accumulation in the thylakoid interior is proposed to result in a dissociation and subsequent extrusion of bound Mg^{++} in order to reach a steady-state condition. The observed light-dependent Mg^{++} extrusion from thylakoid membranes that were prepared in the absence of Mg^{++} (Fig. 44), supports this mechanism. In addition, part of the protons pumped into the thy-

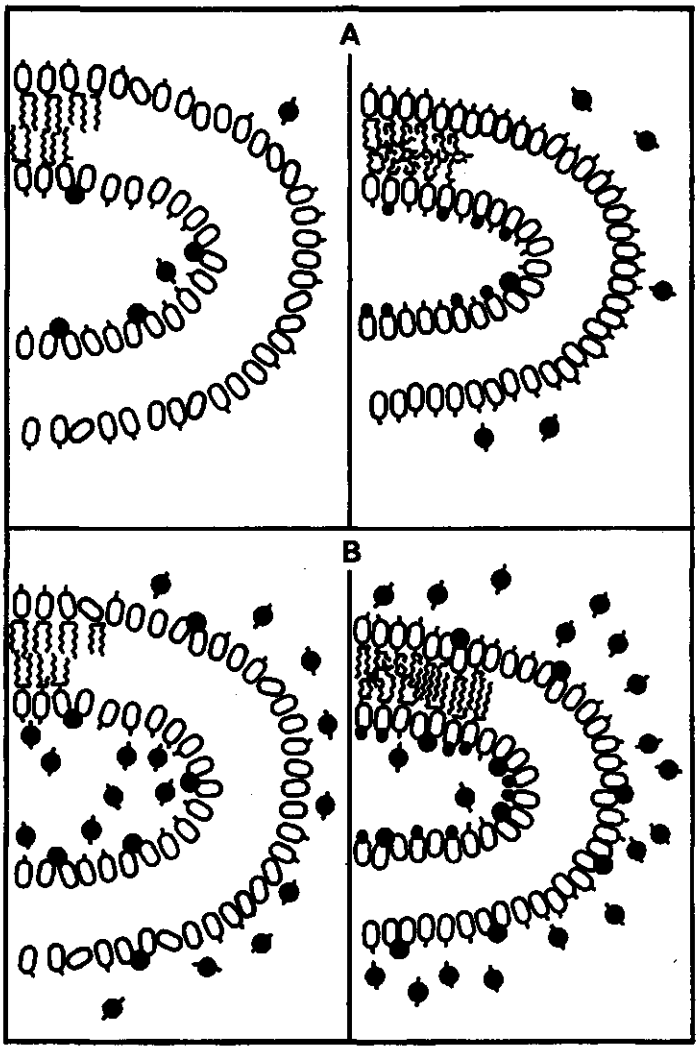

Fig. 47. Schematic visualisation of light-induced changes of binding properties of H^+ (closed oval symbols) and Mg^{++} (closed circles) in chloroplast membranes, in the absence (A) and presence (B) of Mg^{++} in the bulk phase. Negative charges at the membrane are indicated by small dashes. Dark- and light-conditions are presented in the left- and right-hand part of the figure, respectively.

lakoid bind at the inner side of the membrane. The resultant effect apparently is a decrease of 90°-scattering mainly caused by conformational changes associated with Mg^{++} dissociation. In the presence of relatively high Mg^{++} concentration (Fig. 47b) the amount of free Mg^{++} in the boundary layer is proposed to be sufficient for counterion transport and therefore no bound Mg^{++} will be exchanged to balance inward proton pumping. Protonation of the thylakoid interior will lead to dehydration, increase in hydrophobicity and an increase in 90°-scattering. Under these conditions the extruded Mg^{++} may bind at the outer surface (Ben-Hayim, 1978), driven by the increased negative charge density at the outer surface

(Section 7.3). This process may lead to an additional increase of 90^{o}-scattering. It is assumed that binding of Mg^{++} at the outer surface is negligibly small at a Mg^{++} concentration equal or less than 0.5 mmol/l in the bulk phase.

The contribution of the 90^{o}-scattering changes to the 515 nm absorbance change is clearly demonstrated in Figs 40 and 45. However, an elimination of all artifacts in such a complicated system as a thylakoid membrane suspension cannot be attained due to the heterogenity of the absorbing compounds, and the great number of processes that take place upon illumination, probably affecting the P515 absorbance changes. Multiple scattering and consequently alterations of the effective pathlength of the measuring light caused by membrane particles cannot be excluded, neither the distortion of absorbance changes by mutual shading of pigments associated with an altered ionic environment of the membrane during illumination (Duysens, 1956), see also Chapter 4. Osmotic swelling or shrinkage is likely to occur in energized ion-free prepared chloroplasts (Fig. 43) but may be small in chloroplasts suspended in the presence of salts with a permeable anion (Deamer & Packer, 1969; Thorne, 1975).

This leads me to the conclusion that the P515 absorbance change in chloroplasts cannot be taken as a measure for the transmembrane potential in continuous light. A quantitative correction for other processes that affect the real electrochromic absorbance change can only be made under special conditions.

Summary

The main subject of this publication is a comparative study of electrical phenomena and associated processes in chloroplasts measured by means of spectroscopic and electrophysiological techniques. The processes under survey are directly linked with the primary photo-acts and the associated conversion of light energy into chemical energy.

A general introduction to the subject of the energy-conserving system in photosynthetic membranes is given in Chapter 1. The methods and complementary techniques used to study light-dependent reactions in chloroplasts are described in Chapter 2. A spectroscopic method to characterize electric phenomena in chloroplast (thylakoid) membranes, is based on an absorbance bandshift of an intrinsic pigment complex (P515) due to light-induced changes in the membrane electric field. Flash-induced absorbance difference spectra are shown to be different, dependent on the type of chloroplasts used (Chapter 3). The difference spectrum, characteristic for the bandshift of intact chloroplasts in the 460-540 nm wavelength region, could not, in contrast to the one of ion-free prepared broken chloroplasts, be explained by a single bandshift. Computer simulation of a combined red bandshift of a single gaussian band at 497 nm and a blue bandshift of a single gaussian band at 495.5 nm, showed a good similarity with the observed difference spectrum in intact chloroplasts. This led to the conclusion that the field-indicating pigments in intact chloroplasts are not homogeneously distributed in the membrane core. Alternatively, the blue bandshift might be caused by an energy-dependent exposure of part of the pigments to an oppositely orientated electric field.

A method for the voltage calibration of the P515 absorbance changes caused by single-turnover saturating light flashes is discussed in Chapter 4. Absorbance changes, caused by adding KCl to a suspension of broken chloroplasts in the presence of valinomycin and a low concentration of $MgCl_2$, were measured in the wavelength region 460-540 nm. The magnitude of the KCl-induced absorbance changes was shown to be proportional to the logarithm of the KCl concentration. The spectrum of these absorbance changes was found to be identical with the difference spectrum of the light-induced absorbance changes, which was attributed to the electrochromic absorbance bandshift of P515. This was interpreted as evidence that under these conditions absorbance changes of P515 respond exclusively to a membrane diffusion potential induced by salt addition. The results indicate that the electric potential across the thylakoid membrane, generated by a single-turnover light flash, is in the range between 15 and 35 mV.

Analyses of the kinetics of flash-induced absorbance changes of P515 and of the transmembrane potential, as measured by a micro-electrode inserted in a single chloroplast of *Peperomia metallica* (Chapter 5), suggested evidence that the P515 absorbance changes are the composite result of at least two processes: Reaction I and Reaction II. Absorbance changes, accompanying Reaction I, are proposed to be related with a change of the trans-

membrane potential. They showed a kinetic pattern, similar to the photo-electric response measured with micro-electrodes. Both Photosystem 1 and Photosystem 2 were found to contribute equally to the flash-induced absorbance change of P515 associated with Reaction I. Absorbance changes accompanying Reaction II are attributed to changes in innermembrane electric fields due to alterations in the conformation of membrane constituents. Experiments carried out both with broken and intact chloroplasts showed that the induction of Reaction II is exclusively dependent on Photosystem 1 activity. The data suggest evidence that the oxidation-reduction state of cytochrome f or plastocyanin is a determinant factor for the induction of the conformational changes in the membrane. These changes are suggested to result in an altered exposure of negative fixed charges to the P515 pigment complex.

Some electrical characteristics of the thylakoid membrane are considered in Chapter 6. The current-voltage relation of the thylakoid membrane was found to be non-linear. It is concluded that the resistance of the innermembrane system is about 20 MΩ, which increases by a depolarizing current injection to a value of 60 MΩ and decreases to a value of 2 MΩ by a hyperpolarizing current injection.

The half-life time of the decay of Reaction I after a single flash was taken as a measure for the passive membrane conductance. It is concluded that protons hardly attribute to the membrane conductance. A small acceleration of Reaction I after a short preillumination period could not be attributed to a direct effect of an increased proton conductance, associated with proton accumulation in the thylakoid volume. The selective conductance of the thylakoid membrane to a variety of ions was found to be pH-dependent, exept for K^+, which was found to be rather impermeant in the pH range from 8 to 6. The conductivity of Mg^{++} appeared to be of significant importance at pH 7. All anions tested, were found to cross the thylakoid membrane at increasing rates, when the pH was lowered from 8 to 6. It is argued that this effect is due to the reduction of the fixed negative-charge density, favouring a high anion concentration in the diffuse double-layer adjacent to the membrane.

Chapter 7 deals with a closer study of processes that affect Reaction II. The extent of Reaction II was found to be highly sensitive to physical and chemical treatments of the chloroplasts and to the ionic environment of the thylakoid membrane. Light-induced exposure of negative charges at the thylakoid outer surface, detected by particle electrophoresis, showed a similar sensitivity to various physical and chemical treatments as Reaction II. This observation led to the conclusion that both phenomena might be a reflection of the same process. The rise time of Reaction II absorbance changes of P515 coincides with the activation time for the onset of ATP synthesis, which led to a search for a possible participation of CF_1 structural changes in the process that underlies Reaction II.

It was found indeed that Reaction II is inhibited by treatments that modify the structure and operation of the ATP-synthesizing complex such as extraction of CF_1, addition of adenine nucleotides and DCCD binding on CF_0. It is suggested, however, that the conformational changes, as reflected by Reaction II, are probably not the driving force for photophosphorylation but they rather create a condition in which photophosphorylation can proceed efficiently. However, further acquisition of insight in the mechanism by which structural changes in the membrane might be correlated with conformational changes of the

coupling factor will need more detailed experimentation.

Results of a comparitive examination of the kinetics of the transmembrane potential change and of the P515 absorbance change during prolonged illumination are given in Chapter 8. It is shown that part of the P515 absorbance changes in continuous light are due to simultaneously occurring scattering changes. It is suggested that H^+ accumulation and subsequent H^+ binding on the inside of the thylakoid membrane cause these scattering changes. A proportionality factor, relating the scattering change to an associated absorbance change, was obtained by acid titration of these changes in a chloroplast suspension. This factor was taken to correct light-induced P515 changes for the simultaneously recorded scattering changes. It is shown that under certain conditions, the resultant time course of P515 is qualitatively similar to the time course of the electrical potential, measured with microelectrodes. The limitations of the method are discussed, in particular in view of the occurrence of conformational changes which are caused by a rather complicated mechanism of Mg^{++} - H^+ exchange, and of Mg^{++} binding on the outside of the thylakoid surface after light-induced extrusion from the thylakoid interior.

Samenvatting

Deze publikatie geeft het resultaat van een vergelijkend onderzoek in chloroplasten naar elektrische verschijnselen en daarmee geassocieerde processen, gemeten met spectroscopische en elektrofysiologische technieken. De onderzochte processen zijn direkt verbonden met de omzetting van lichtenergie in chemische energie.

Hoofdstuk 1 geeft een algemene inleiding over het energie conserverende systeem. De methodes die bij het onderzoek naar lichtafhankelijke reacties in chloroplasten gebruikt zijn, alsmede verscheidene aanvullende technieken, worden beschreven in hoofdstuk 2. Een spectroscopische methode om elektrische verschijnselen in thylakoïdmembranen te karakteriseren is gebaseerd op een absorptieband verschuiving van een intrinsiek pigmentcomplex (P515), tengevolge van door licht geïnduceerde veranderingen van het elektrische veld. Verschilspectra van flits-geïnduceerde absorptieveranderingen blijken afhankelijk te zijn van de intactheid van de chloroplasten (hoofdstuk 3). In tegenstelling tot het verschilspectrum, waargenomen in gebroken chloroplasten in het golflengtegebied tussen 460 en 540 nm, kan het verschilspectrum in intacte chloroplasten in dat gebied niet worden verklaard met de verschuiving van één enkel absorptieband. Computersimulatie van een gecombineerde roodverschuiving van een band bij 497 nm en een verschuiving naar blauw van een band bij 495,5 nm, geeft wel een verschilspectrum overeenstemmend met dat van intacte chloroplasten. Dit leidt tot de conclusie dat de pigmenten, die de grootte van het veld aangeven, in intacte chloroplasten niet homogeen over de membraan verdeeld zijn. Anderzijds zou de blauwverschuiving veroorzaakt kunnen zijn door energie-afhankelijke blootstelling van een deel van de pigmenten aan een tegenovergesteld georiënteerd elektrisch veld.

Een kalibratie van de P515-absorptieveranderingen, als afspiegeling van de kinetiek van de elektrische transmembraanpotentiaal, is beschreven in hoofdstuk 4. Absorptieveranderingen veroorzaakt door KCl-toevoeging aan een suspensie van gebroken chloroplasten, in aanwezigheid van valinomycine en een lage $MgCl_2$ concentratie, werden gemeten in het golflengtegebied tussen 460 en 540 nm. Aangetoond wordt dat de door KCl geïnduceerde absorptieveranderingen evenredig zijn met de logaritme van de KCl-gradiënt over de membraan. Het verschilspectrum van deze absorptieveranderingen is identiek aan het verschilspectrum van de door licht geïnduceerde absorptieveranderingen. Dit wordt geïnterpreteerd als een bewijs dat, onder die omstandigheden, de optredende P515-absorptieveranderingen het gevolg zijn van een diffusiepotentiaal over de membraan. De resultaten tonen aan dat door een enkele lichtflits een potentiaal over het membraan gegenereerd wordt, die ligt tussen 15 and 35 mV.

Analyses van de kinetiek van de flits-geïnduceerde P515-absorptieveranderingen en de kinetiek van de transmembraan potentiaal, gemeten met een micro-elektrode in één enkele chloroplast van *Peperomia metallica* (hoofdstuk 5), tonen aan dat de P515-absorptieverandering het samengestelde resultaat is van tenminste twee processen: reactie I en reactie II genaamd. Absorptieveranderingen gerelateerd aan reactie I, zijn het gevolg van de

transmembraanpotentiaal en hebben een vergelijkbaar kinetisch patroon als de foto-elektrische respons gemeten met micro-elektrodes. Fotosysteem 1 en 2 dragen in dezelfde mate bij tot de initiële, met reactie I geassocieerde, absorptieverandering. De absorptieveranderingen die aan reactie II gerelateerd zijn worden toegeschreven aan veranderingen van lokale elektrische velden in de membraan tengevolge van conformatieveranderingen van bepaalde membraanstructuren. Experimenten met zowel gebroken als intacte chloroplasten tonen aan, dat reactie II uitsluitend afhankelijk is van de aktiviteit van Fotosysteem 1. De mate, waarin plastocyanine of cytochroom f geoxydeerd zijn, is bepalend voor de aanzet van conformatieveranderingen in de membraan en een veranderde blootstelling van het P515-pigmentcomplex aan geladen groepen.

Hoofdstuk 6 handelt over enkele elektrische eigenschappen van de thylakoïdmembraan. De relatie tussen de grootte van een kunstmatig-geïnduceerde stroom I en de gemeten spanning V is niet lineair. Daaruit kan worden geconcludeerd dat de weerstand R van de thylakoïdmembraan afhankelijk is van de grootte en de richting van de stroom I. In afwezigheid van een stroom I, is R ongeveer 20 MΩ. Een depolarizerende stroom veroorzaakt een stijging van R tot een maximum waarde van 60 MΩ en een hyperpolarizerende stroom veroorzaakt een daling van R tot een waarde van 2 MΩ.

De halfwaardetijd van het verval van reactie I na een flits wordt als maat genomen voor de passieve membraangeleiding. Het blijkt dat protonen nauwelijks bijdragen tot deze geleiding. De kleine versnelling van reactie I na voorbelichting kan niet worden verklaard met een verhoogde protongeleiding tengevolge van protonopname in de thylakoïden. De selektieve geleiding van de thylakoïdmembraan voor andere ionen is pH afhankelijk, behalve voor K^+, dat tamelijk slecht de membraan passeert in het traject tussen pH 8 en pH 6. De permeabiliteit voor Mg^{++} daarentegen is hoog met een maximum bij pH 7. De permeatiesnelheid van alle anionen die werden getest, neemt toe bij een pH-daling van 8 naar 6. Dit is waarschijnlijk het gevolg van een verhoogde anionconcentratie in de elektrische dubbellaag grenzend aan het membraanoppervlak, tengevolge van neutralisatie van de negatief geladen membraangroepen door protonen.

Hoofdstuk 7 behandelt de basisverschijnselen die ten grondslag liggen aan het optreden van reactie II. De mate, waarin reactie II optreedt blijkt zeer gevoelig te zijn voor fysisch-chemisch ingrijpen in de structuur van de thylakoïdmembraan en voor de ionensamenstelling van het suspensiemedium. Toevoeging van Mg^{++} en H^+ heeft een sterk remmend effect op reactie II. Vergroting van de negatieve oppervlaktelading van de thylakoïd (buitenzijde) in het licht, zoals die wordt gemeten in een elektroforese-experiment, heeft eenzelfde gevoeligheid voor verscheidene fysisch-chemische behandelingen als reactie II. Daarnaast is de vergroting van de negatieve oppervlaktelading, evenals reactie II, alleen afhankelijk van de aktiviteit van Fotosysteem 1. Deze observaties leiden tot de conclusie, dat beide verschijnselen een afspiegeling zijn van hetzelfde proces. De stijgtijd van absorptieveranderingen tengevolge van reactie II, is vergelijkbaar met de tijd, die wordt gemeten alvorens ATP-synthese een aanvang neemt. Derhalve is gezocht naar een mogelijke betrokkenheid van reactie II bij conformatieveranderingen in het ATP-synthetiserende enzymcomplex.

Overeenkomstig deze suggestie blijkt reactie II geremd te worden door structurele veranderingen van dit enzym, veroorzaakt door: extractie van CF_1, toevoeging van adenine-

nucleotiden en DCCD-binding aan CF_0; maar een direct bewijs voor een causaal verband tussen de grootte van reactie II en het ATP-synthese proces is niet gevonden (hoofdstuk 7). Op basis van de kinetiek van reactie II wordt geconcludeerd dat de processen die aan reactie II ten grondslag liggen, geen drijvende kracht vormen voor de fotofosforylering, maar dat zij mogelijkerwijs wel een voorwaarde zijn voor een efficiënte fotofosforyleringsactiviteit. Er is echter meer onderzoek vereist om inzicht te verkrijgen in het mechanisme, waardoor structurele veranderingen in de membraan (reactie II) zijn gekoppeld aan conformatieveranderingen van het ATP-synthetiserend enzym.

De resultaten van een vergelijkend onderzoek naar de kinetiek van de transmembraanpotentiaal en de P515-absorptieveranderingen tijdens langere belichtingsperioden zijn vervat in hoofdstuk 8. Een deel van de P515-absorptieveranderingen in continue belichting wordt veroorzaakt door gelijktijdige veranderingen in strooiing. Verondersteld wordt dat deze strooiingsveranderingen het gevolg zijn van protonopname en protonbinding aan de binnenzijde van de thylakoïdmembraan. De evenredigheidsfactor tussen strooiings- en absorptieveranderingen wordt verkregen door zuurtitratie van beide veranderingen. De strooiingsveranderingen worden gemeten onder een hoek van 90^{o} met het meetlicht. De waargenomen verhouding tussen absorptie- en strooiingsverandering wordt gebruikt om door licht-geïnduceerde P515-absorptieveranderingen te corrigeren voor gelijktijdig optredende strooiingsveranderingen. Het resulterende tijdsverloop van de P515-absorptieverandering blijkt dan, onder bepaalde omstandigheden, vergelijkbaar te zijn met de transmembraanpotentiaal, gemeten met micro-elektrodes. De methode heeft echter een geringe toepasbaarheid. De strooiingsveranderingen, tengevolge van protonbinding aan de binnenzijde van de thylakoïdmembraan lijken namelijk vergezeld te gaan van nog andere structurele veranderingen, die worden veroorzaakt door een uitwisseling van Mg^{++} tegen protonen. De mate, waarin dit in strooiingsveranderingen tot uitdrukking komt, lijkt afhankelijk van de relatieve hoeveelheid Mg^{++} die aanwezig is in de oplossing en de elektrische dubbellaag, en de hoeveelheid Mg^{++}, die gebonden is aan gefixeerde ladingen op het membraan.

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