Chapter 24

Electrogenic Reactions and Proton Pumping in Green Plant Photosynthesis

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I. INTRODUCTION

Photosynthesis by green plants is the fundamental energy-providing process for terrestrial life. Energy from sunlight is used to synthesize carbohydrates from water and carbon dioxide, which serve as both fuel and substrates for plants, and indirectly for animals. The first relatively stable intermediates between light absorption and carbohydrate synthesis are re-

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duced nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP). The light-driven production of NADPH and of ATP, which is topologically separated from the subsequent "dark" reactions, occurs in the inner membrane system (thylakoids) of chloroplasts. Thylakoid membranes form disc-shaped vesicles (diameter ~500 nm and thickness ~10 nm) often arrayed in stacks (grana) with their internal phases extensively interconnected.

The reduction of $NADP^+$ occurs along a linear electron transport chain, driven by two photochemical reaction centers (in series), with water as the ultimate electron donor. Phosphorylation of ADP is indirectly coupled to electron transport, and—though the point was once highly disputed—protons are now generally accepted as the obligatory "high-energy" intermediate (see Boyer *et al.*, 1977). Figure 1 illustrates the sequence of events in the thylakoid membrane: Absorption of light by chlorophylls and

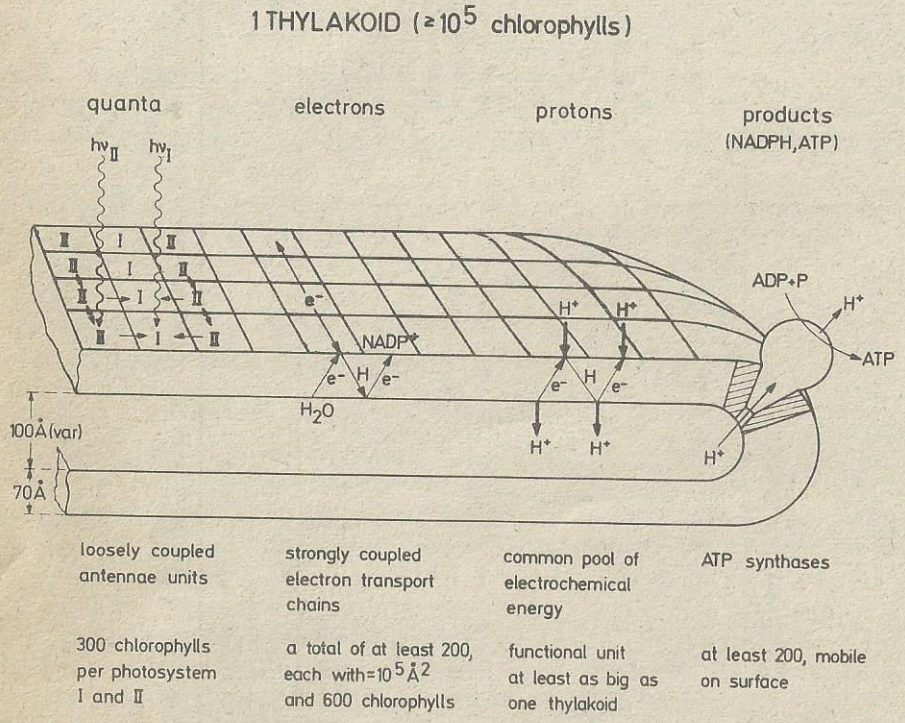

FIG. 1. Schematic representation of antennae function, electron transport, proton pumping, and phosphorylation in thylakoids from green plants. The rectangular arrangement of photosystems is arbitrary (Junge, 1977a).

carotenoids—acting as antennae for two types of photochemical reaction centers (photosystems I and II)—promotes the transfer of electrons from water to $NADP^+$. Some of the redox reactions (e.g., the oxidation of water) are followed by proton release; others (e.g., the reduction of quinones) are followed by proton uptake. With electron transfer directed across the membrane as indicated, the thylakoid membrane becomes electrically charged, and protons are pumped into the internal space. The stored electrochemical energy is then used by a proton-translocating ATP synthetase whose properties and mode of action are summarized by Gräber in this volume.

Under continuous illumination of thylakoid membranes, the linear electron transport chain gains approximately 1.2 eV as the free energy of the $NADPH/NADP^+$ couple and another 0.2 eV at each of the two sites where protons are pumped into the internal space of thylakoids. The total gain of 1.6 eV requires the input of two light quanta. If these are derived from red light, which is sufficient to drive these processes (e.g., at a wavelength of 690 nm equivalent to 1.8 eV), the efficiency for the conversion of light energy into useful forms of energy is approximately 40%, with one-quarter stored as the electrochemical potential of protons. (The normal practical efficiency, however, is an order of magnitude lower; Knox, 1979).

The main purpose of this article is to review the molecular events—as they are currently understood—which connect redox reactions in the thylakoid membrane with the generation of an electrochemical potential difference for protons across this membrane. The subject has been reviewed previously by numerous authors, and the interested reader may refer, for example, to Witt (1975, 1979) and to Hauska and Trebst (1977).

II. THE MEMBRANE

A. Structure

In intact mature chloroplasts, the larger part of each thylakoid membrane is a disc-shaped bag having a typical diameter of 500 nm, an apparent membrane thickness of about 7 nm, and an internal aqueous phase which measures 10–20 nm thick in the electron microscope (see, e.g., Muehlethaler, 1977) but appears smaller—perhaps as thin as 5 nm—from distribution studies (Heldt *et al.*, 1973) based on the surface density of chlorophyll (see Wolken and Schwertz, 1953; Thomas *et al.*, 1956). In isolated broken chloroplasts the shape and internal volume of thylakoids are highly variable, depending on the salt composition and osmolarity of the suspending medium. Thicknesses of 15–120 nm can be calculated from the data of Gaensslen and McCarty (1971),

Rottenberg *et al.* (1972), and Ort *et al.* (1976), assuming the basic disc shape to be preserved.

With vesicles of such dimensions, the extent to which internal events can be treated as (averaged) bulk phase events depends upon the reach of electrostatic effects from the membrane surface into the inner aqueous phase. This reach diminishes in absolute size as the ionic strength of the solution increases and as the fixed charge density of the membrane falls; it diminishes in importance as the volume (thickness) of the inner phase increases. With chloroplasts suspended, for example, in 10 m*M* KCl as the only salt and having a surface charge density of 1 μC/cm^2 (Itoh, 1979a,b), the surface potential would be 39 mV and the Debye length would be 3 nm. If the internal phase were 15 nm thick, the average internal pH would be 0.2 units more acid than that of the medium, and the average internal K^+ concentration would be 14 m*M*. Thus, the *average* ion concentrations in the internal phase would be reasonably close to those of the medium; but near the membrane–water interface much stronger deviations would occur.

The thylakoid membrane, like the cristae membrane of mitochondria, has a relatively high protein content. Forty-seven percent of the total dry weight is ether-soluble (Wintermans, 1967), but this includes 10% pigments (chlorophylls and carotenoids), so the lipid content in a narrower sense must be less than 40%. And it could be much less, since only 10–15% of the total area (outer surface) was found susceptible to lipid antibodies (Radunz, 1979). This has the following consequences for the electrochemical behavior of the membrane: (1) Proteins will contribute to the surface charge density and to the proton-buffering capacity. (2) The membrane dielectric as well as the surface charge density will be highly granular. (3) The granularity will probably not be blurred by rapid lateral diffusion of the charge carriers, because the apparent microviscosity of the thylakoid membrane appears quite high, possibly caused by protein aggregation (see Wagner and Junge, 1980).

The thylakoid membrane also appears complex in the direction normal to the plane, as indicated by two different compartmentation experiments. Ausländer and Junge (1974) observed proton uptake from a region near the outer side of the membrane, which was shielded from the outer bulk phase by a proteinaceous barrier. And Quintanilha and Packer (1978) observed accumulation of an impermeant amphiphilic spin probe in a region of membrane only weakly influenced by the diffuse ionic double layer.

Despite such complexities, we shall use the following simplified model of the thylakoid membrane throughout most of this article: A more-or-less homogeneous dielectric core (electric capacitance of 0.5–1.0 μF/cm^2) is bounded by fixed negative charges (density 1–3 μC/cm^2) which create a negative surface potential and a diffuse ionic double layer reaching into the

adjacent water phases. There are five main compartments in such a model: bulk water, interface, membrane core, interface, and bulk water. In certain experiments, further subcompartments may need to be considered, as indicated above (see also Kell, 1979).

B. Surface Charge Density and Buffering Capacity

Because of their potential influence on surface reaction rates and on total energy storage in ionic gradients, two physical parameters which must be kept in mind during the discussions of electrogenesis in thylakoid membranes are the membrane surface charge and the proton-buffering capacity of the thylakoids. Table I summarizes recent determinations of surface charge density, calculated mostly via the Gouy–Chapman theory, from data on the influence of salt concentrations (in the internal or external bulk phases) on the apparent rates of reactions involving charged species [$Fe(CN)_6^{3-}$, $Fe(CN)_6^{4-}$, and H^+]. For a variety of reasons, the results must at present be taken as semiquantitative estimates, but they do make clear that the two surfaces of the thylakoid membrane are not widely different, both having values of 1–3 μC/cm^2, or one negative charge per 5–16 nm^2 of surface. (The average area per chlorophyll molecule is 2.2 nm^2). A comprehensive review of the role of surface potentials in photosynthesis has been presented by Barber (1981).

Recent values of buffering capacity are summarized in Fig. 2. Curve 1, obtained from a slow dark titration, represents the internal and external phases taken together and agrees well with data obtained by other authors on a different chloroplast preparation (Junge and Ausländer, 1974; Saphon and Crofts, 1977b). The lower three curves present results for the inner phase only, but obtained by three different laboratories using different methods and covering different pH ranges. There is no consensus yet as to whether curve 2 or 4 is the most satisfactory in the acid range. It is clear, however, that at all pH values the buffering capacity of the external phase dominates that of the internal phase. It is also clear that the buffer capacity in both phases is essentially constant above pH 6.5, which is most easily explained by the involvement of a large number of buffering groups with different p*K* values (i.e., protein buffering). The steep increase in buffering capacity at low pH indicates the existence of dissociable groups (lipids?) with p*K* values of about 5 (Walz *et al.*, 1974; Mercer *et al.*, 1955; Akerlund *et al.*, 1979).

It may be worthwhile to compare the absolute magnitudes of the electric and chemical storage capacities of the thylakoid membrane. In a single turnover the two proton pumps in the linear electron transport chain translocate two charge equivalents per 10^3 nm^2. Under the assumption of an electric capacitance of 0.5 μF/cm^2 the generated voltage is 64 mV. How many turn-

TABLE I

SURVEY OF DATA ON THE SURFACE CHARGE DENSITY ON BOTH SIDES OF THE THYLAKOID MEMBRANE[a]

Surface charge density ($\mu C/cm^2$)		Locus	Experimental technique	Reference
Outer Side	Inner Side			
−1.1, −1.5	—	PS I, reducing side	Rate of electron flow from PS I to ferricyanide	Itoh (1979b)
−1.3	—	PS II, reducing side	Rate of electron flow from PS II to ferricyanide in the presence of DCMU	Itoh (1978)
−2.5	—	PS II, P680-Q	Chlorophyll fluorescence	Barber (1977)
—	−0.77, −0.88	PS I, oxidizing side	Rate of electron flow from ferrocyanide to PS I in sonicated chloroplasts	Itoh (1979b)
—	−0.59	Same as above	Same, but in PS I particles	Itoh (1979b)
—	−1.3	Average inside	Indirect argument, based on dark–light redistribution of K^+ and Cl^- between inside and outside	Rumberg and Muhle (1976)
—	−3.2	Between PS II and PS I	Reaction rate between PQH_2 and P700	Huber and Rumberg (1979)

[a] PS I, Photosystem I; PS II, photosystem II.

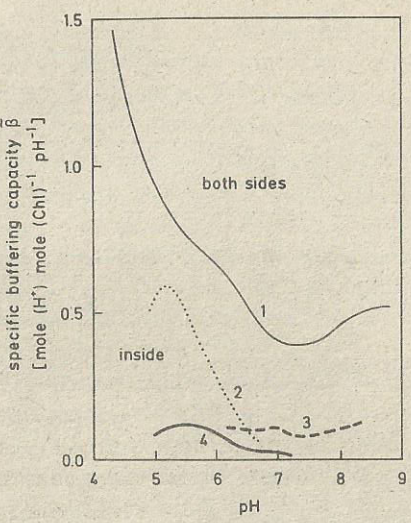

FIG. 2. Proton-buffering capacity of thylakoids as functions of the pH. 1, Lettuce chloroplasts, dark titration experiment, both phases (Walz *et al.*, 1974); 2, lettuce chloroplasts, internal phase only (Walz *et al.*, 1974); 3, spinach chloroplasts, internal phase only (Junge *et al.*, 1979); 4, spinach chloroplasts, internal phase only (Reinwald, 1970).

overs are required to create the energetically equivalent pH difference? With a specific internal buffering capacity of 0.18 mole H^+/mole chlorophyll per pH unit (Junge *et al.*, 1979) and 500 chlorophylls per electron transport chain, a minimum of 49 turnovers is calculated. Hence, in dynamic situations, transients of the voltage occur much faster than those of the pH difference. As thylakoids operate their ATP synthesis mainly on the pH difference, they are well buffered and protected against fluctuations of the illumination level in a time domain of several seconds.

III. ELECTROGENIC REACTION STEPS

A. Survey

Although electron and proton transfer reactions may be intrinsically electrogenic, only under three conditions will they noticeably contribute to the electric potential difference across the thylakoid membrane: (1) The reaction path must not be directed parallel to the plane of the membrane; (2) the reaction must cross a considerable distance of the dielectric core of the membrane; and (3) the transported charge must not be locally compensated (as occurs, for example, when electrons and protons are transported together on plastoquinone).

Of the three electrogenic reaction steps (see below) for which there is evidence in the thylakoid membrane, two are linked to the primary

photochemical reactions in photosystems I and II, respectively, and the other seems to be linked to thermally activated electron transfer in the neighborhood of photosystem I. Rise of the membrane potential via the first two steps (or sites) is extremely rapid (at least nanoseconds) following brief flash excitation, but is much slower (milliseconds) via the third site. In a single turnover, each site contributes about 25 mV to the membrane potential. While the voltage is primarily generated by electron transfer, under steady operation the interplay of redox reactions with protolytic reactions makes it appear as if inwardly directed proton transfer were electrogenic.

Most of the information available on these electrogenic reactions in thylakoids was obtained by studying electrochromic absorption changes of intrinsic membrane pigments, and it is therefore necessary to discuss this technique in some detail before passing on to the experimental results themselves.

B. Chloroplast Electrochromism as a Molecular Voltmeter

In recent years a variety of dye substances have been developed and tested for their ability to measure biological membrane potentials via absorption or fluorescence shifts (Waggoner, 1976). As extrinsic probes, these substances respond to voltage changes with half-times ranging from tens of seconds to microseconds and function by several different molecular mechanisms. However, the thylakoid membranes and other photosynthetics organelles (see, e.g., Dutton *et al.*, this volume) are intrinsically highly pigmented and offer the unique opportunity to monitor voltage-sensitive absorption changes in the resident native pigments. Observed changes occur with (yet instrument-limited) half-times of nanoseconds and appear to be genuine electrochromic shifts, as originally defined by Platt (1961). Fundamentally, a chromophore oriented in an intense electric field [commonly in biological membranes, 100 mV/nm ($= 10^7$ V/m)] can respond to a variation in field strength by a variation in the resonant wavelength for light absorption. [For a theoretical discussion see Liptay (1969) and Reich and Sewe (1977).] Such effects tend to be small in relation to the normal bandwidth for the chromophore and so must ordinarily be followed with a differential instrument which can be arranged to show either the shift in absorption peak itself or a change in absolute absorbance at a given wavelength. Initially, the obvious advantages of this kind of technique were partly offset by difficulties in discriminating genuine electrochromic changes from apparent absorption changes of spurious origin. Accumulated evidence defining and confirming the electrochromic effects in chloroplast pigments is summarized below.

1. Kinetics

Junge and Witt (1968) observed that, for certain absorption changes which appear when chloroplasts are excited by brief flashes, decay (following each flash) is accelerated by manipulations which increase the ionic conductivity of biological membranes: aging, osmotic shock, organic solvents, and ion-transporting antibiotics. This is illustrated in Fig. 3 for absorption changes (ΔA) measured at 520 nm and responding to a 10-μsec flash of light (delivered at time zero). In all three traces the rise of ΔA occurred exceedingly rapidly, essentially vertically on this millisecond time scale. For chloroplasts suspended in the normal buffer solution, containing sodium and potassium, the absorption signal decayed by 25% in 20 msec following the flash. Decay was hardly affected by addition of the potassium carrier valinomycin, provided no potassium was present (Fig. 3B), but was greatly speeded by valinomycin in the presence of potassium (Fig. 3C). In similar experiments with the channel-forming antibiotic gramicidin, a single molecule per 10^5 chlorophyll molecules (the average number for spinach thylakoids) yielded substantial acceleration of the decay, confirming that the field to which the absorption change responds must be delocalized over the entire thylakoid (in the time domain of a millisecond).

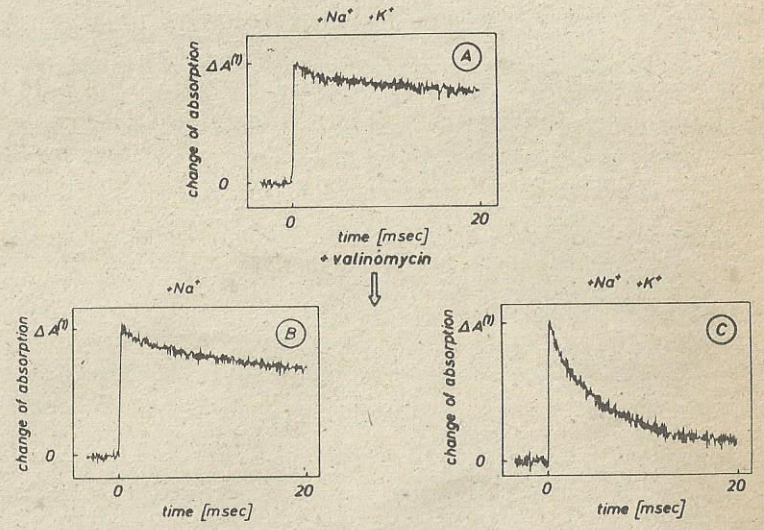

FIG. 3. Time course of the electrochromic absorption changes at 520 nm, observed after excitation of a chloroplast suspension with a short flash at $t = 0$. Increased ionic conductance, as by potassium in the presence of valinomycin, accelerates decay of the electrochemic absorption change (Junge and Schmid, 1971).

2. Spectrum

The difference spectrum of the absorption changes, which are sensitive to the ionic conductivity of the thylakoid membrane (Emrich *et al.*, 1969), can be synthesized from the separate electrochromic difference spectra of carotenoids and chlorophylls, measured in microcapacitors (Schmidt *et al.*, 1970). Two main peaks, at 428 and 520 nm, arise from a minor carotenoid fraction (< 20%: DeGrooth *et al.*, 1980; Schlodder and Witt, 1980), probably luteins complexed by chlorophyll *b* (Sewe and Reich, 1977a). Apparently it is formation of this complex which, by the prepolarization of lutein, produces a pseudolinear relation between the absorption shift and the membrane electric field. Pseudolinearity (at 520 nm) has been confirmed by several independent techniques (Reinwald *et al.*, 1968; Witt and Zickler, 1974; Amesz and DeGrooth, 1975; Schapendonk and Vredenberg, 1977).

3. Artificially Imposed Potentials

In bacterial chromatophores electrochromic absorption changes have been most convincingly demonstrated by exposing chromatophore vesicles to salt jumps in the presence of cation-specific ionophores (Jackson and Crofts, 1969). Despite the much greater light-scattering changes usually observed with thylakoids (Strichartz and Chance, 1972), recent experiments have demonstrated the same effect on thylakoids (Schapendonk and Vredenberg, 1977). In such experiments the absorption change is linearly proportional to the calculated (Nernst–Planck) diffusion potential, so that the technique yields a calibration of the flash-induced electrochromic change. Electric field transients across the thylakoid membrane generated via macroscopic electrodes, in thylakoid suspensions, can produce absorption shifts which duplicate those produced by light (Ellenson and Sauer, 1976; DeGrooth *et al.*, 1980; Schlodder and Witt, 1980). Although, for a variety of technical reasons, this technique cannot be used to calibrate the light-induced absorption shifts, it can be used to extend the range of voltages applicable to the thylakoid up to about 500 mV (see Gräber, this volume).

4. Limitations

It is evident that electrochromic absorption changes (mainly at 518 and 478 nm) are useful primarily as indicators of the electric field strength in the thylakoid membrane, with high time resolution. Limitations of this molecular voltmeter arise from four main sources. The most serious of these is *scattered light,* which can easily be mistaken for absorption transients. Even double-beam spectrophotometry cannot wholly correct this situation, which becomes especially severe in studies with long illumination times. Scattering changes do seem to be negligible, however, when the chloroplasts are illuminated with brief flashes at a low repetition rate. Under certain conditions, particularly at high flash energies, large *absorption changes not related to electrochromism* can occur in the wavelength domain around 520 nm. These can be distinguished from the electrochromic response by higher saturating light levels, more rapid decay (at least 1000-fold), and persistence in heat-inactivated chloroplasts. It has been attributed to metastable carotenoid triplets (Mathis, 1970; Wolff and Witt, 1969; Witt and Wolf, 1970).

Field nonuniformity, in either a lateral or a normal direction, may lead to the misinterpretation of electrochromic changes. Lateral nonuniformities can arise, for example, as a result of the membrane granularity (see above) and the fact that the detecting carotenoids are associated primarily with photosystem II (Sewe and Reich, 1977a; DeGrooth *et al.*, 1980; Schlodder and Witt, 1980), whereas two of the three electrogenic steps are associated with photosystem I. If the charge separation process is punctate or regional, and distant from the detecting carotenoids, then the transmembrane potential difference will be attenuated between the source and the detector. This kind of nonuniformity could become serious either when the electrical conductivity of the internal and external bulk phases is very low (as under cryogenic conditions; see Amesz and DeGrooth, 1975; Vermeglio and Mathis, 1974; Conjeaud *et al.*, 1976) or when the thylakoid is nonuniformly activated (e.g., by an extrinsic electric field.

Field nonuniformity, in a direction normal to the membrane, must always exist to a certain extent, because of surface potentials. Presumably, the electrochromic pigments monitor the field strength *within* the membrane, and this must be related to the *sum* of the difference of the bulk phase potentials *plus* the difference of surface potentials. Electrochromic shifts, therefore, could be distorted by asymmetric changes in membrane surface potentials. Most of our experiments have been designed to minimize this kind of error, by using brief light flashes at a low repetition frequency. [The change in surface potential occurring for displacement of two protons (a single-turnover flash with both photosystems active) should be less than 2 mV, as long as bulk phase monovalent electrolytes are in excess of 3 m*M*.] Under continuous illumination, the problem can become serious, however (Witt, 1979).

Finally, it should be mentioned that use of the electrochromic decay time course as a measure of the membrane's ionic conductivity is subject to the usual restrictions of *uniformity—in size and specific transport properties*—which must be applied to any ion flux measurement for vesicular populations (see Schmid and Junge, 1975).

5. Alternative Techniques

In recent years several other techniques have been developed for estimating the membrane potential in thylakoids under various conditions. Although

some quantitative discrepancies exist between results obtained by these other techniques and results obtained from electrochromic shifts, the qualitative pictures are generally in agreement. Fowler and Kok (1972, 1974) and Witt and Zickler (1973, 1974) used *macroscopic electrodes,* immersed in chloroplast suspensions, to detect bulk charge displacement resulting from nonuniform illumination. The latter authors were, in fact, able to use the technique to confirm that electrochromic signals in the thylakoids are linear indicators of the electric field strength. *Microelectrodes* have been used by Bulychev, Vredenberg, and their collaborators (Bulychev *et al.,* 1972; Vredenberg and Tonk, 1975; Bulychev and Vredenberg, 1976) to record light-generated voltage transients—probably partially shunted (both in resistance and capacitance)—in giant chloroplasts from *Peperomia metallica. Delayed fluorescence* from thylakoids, following a flash, has been shown to depend on the membrane potential, as determined either by artificially induced diffusion potentials (Barber and Kraan, 1970; Wraight and Crofts, 1971) or by externally imposed fields (Ortoidze *et al.,* 1979). Finally, Trissl (1980) and Trissl and Gräber (1980) have observed rapid photovoltages (via macroscopic electrodes) on *thylakoid membranes spread at a hexane–water interface.* Though this technique is restricted to artificial membrane structures, it yields a very high signal-to-noise ratio and a very high time resolution.

C. Electric Generators

1. Electrogenic Steps in the Linear Electron Transport Chain

In both photosystems (Schliephake *et al.,* 1968; Malkin, 1978) it appears to be the primary photochemical act—electron transfer from the donor molecule to the primary acceptor—which takes place across the dielectric core of the thylakoid membrane. At least this is the simplest conclusion which can account simultaneously for the speed and temperature insensitivity of the initial electrogenic process observed on flash excitation. Delayed fluorescence, which is believed to represent reversal of the photochemical reactions, has been shown by several different laboratories (Barber and Kraan, 1970; Wraight and Crofts, 1971; Ortoidze *et al.,* 1979) to be sensitive to the potential difference imposed across the thylakoid membrane. Under flash stimulation by a Q-switched ruby laser, intact thylakoids show an instrument-limited risetime of 20 nsec for the electrochromic absorption change; and this can be shortened to 2 nsec by spreading the thylakoid membranes at a hexane–water interface (Trissl and Gräber, 1980). Measurable shifts in electrochromic absorption survive at temperatures down at least to −50°C (Amesz and DeGrooth, 1975; Mathis and Vermeglio, 1975) and, although the signal following stimulation of photosynthesis I disappears at still lower

temperatures (−125°C; Conjeaud *et al.,* 1976), this should probably be attributed to field attenuation (hindered delocalization) between photosystem I and the detecting carotenoids located near photosystem II (Section III,B,4). The polarity of the light-generated electric potential difference is positive toward the internal aqueous phase, as demonstrated both by macroscopic electrodes (Section III,B,5) and by ionic redistributions (Deamer and Packer, 1969; Schröder *et al.,* 1972; Hind *et al.,* 1974). All evidence presently available indicates that none of the other electron transfer steps in the linear electron transport chain contributes appreciably to the *transmembrane* electrogenesis; in particular, there is no component of the electrochromic response which kinetically matches the restoration (re-reduction) of the primary electron donor at photosystem I, P700 (Junge, 1972).

2. Electrogenic Steps in Cyclic Electron Transport

Recently, several laboratories (Velthuys, 1978, 1979; Slovacek *et al.,* 1979; Crowther *et al.,* 1979; Horvath *et al.,* 1979; Bouges-Bocquet, 1980) have identified a slowly rising (milliseconds) change in absorption which appears to be genuinely electrochromic but is associated with an electrogenic step other than electron transfer. Apparently this change is observed only in freshly isolated chloroplasts which have been prepared with the outer membrane and stroma intact, retaining soluble ferredoxin. Figure 4 illustrates the slow absorption change superimposed on the fast changes due to photosystems I and II (control). Blockage with 3′-(3,4-dichlorophenyl)-1′,1′-dimethylurea (DCMU) abolishes all the light-induced absorption changes, but release of photosystem I with dithionite restores part of the fast component and all of the slow component. Thus, photosystem I must provide the driving force for the slow electrochromic change (Crowther *et al.,* 1979). Further experiments have shown that the redox state of the plastoquinone pool—and, thereby, photosystem II—has an important *regulatory* effect on the slow change.

Although there is not yet complete agreement on the origin of the slow electrogenesis, it is possible to synthesize a tentative scheme based on the combined work from the laboratories of Velthuys, Crowther, and Horvath. One such scheme is shown in Fig. 5 (lower diagram), complementary to a simplified scheme for the fast electrogenesis. The main point is that the slow electrogenic reaction is part of an electron–hydrogen loop linked to linear electron flow via the plastoquinone pool. A prerequisite for the slow reaction is reduction of cytochrome *f* (Velthuys, 1978), and it seems as if electron donation by the two-electron donor, plastohydroquinone (PQH_2), to the one-electron acceptor, cytochrome *f*, creates the driving force for an additional one-electron step, probably from plastosemiquinone (PQ^-) to a *b*-type cytochrome. It is this step which probably takes place across the dielectric core

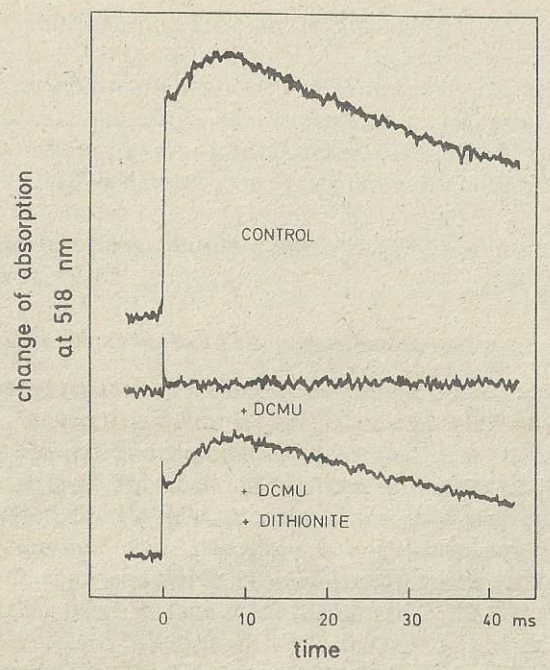

FIG. 4. Time course of the electrochromic absorption changes at 518 nm, during linear and pseudocyclic electron transport. (Top) Both photosystems and the pseudocyclic path active. (Middle) Both photosystems blocked by DCMU. (Bottom) Reactivation of photosystem I with the artificial reducing agent, dithionite (Crowther *et al.*, 1979).

of the membrane. Near the outer surface of the membrane, the electron would again be transferred to plastoquinone to yield the semiquinone. The regulatory effect of photosystem II could be accounted for by the fact that only fully reduced and protonated plastoquinone molecules can pass inward through the membrane, so that a source of additional electrons is required for the system to continue running. As suggested by Slovacek *et al.* (1979), the acceptor chain of photosystem I might also supply the needed electrons in a reaction involving soluble ferredoxin. [The situation may be analogous to that in blue-green algae (Knaff, 1977), where cytochrome b_6 is reduced by ferredoxin and oxidized via an ADP-sensitive step that probably involves proton pumping via plastoquinone.]

3. The Magnitude of the Electric Potential Difference

a. Excitation with Single-Turnover Flashes. Early estimates of the magnitude of the electric potential difference arising from a single turnover of

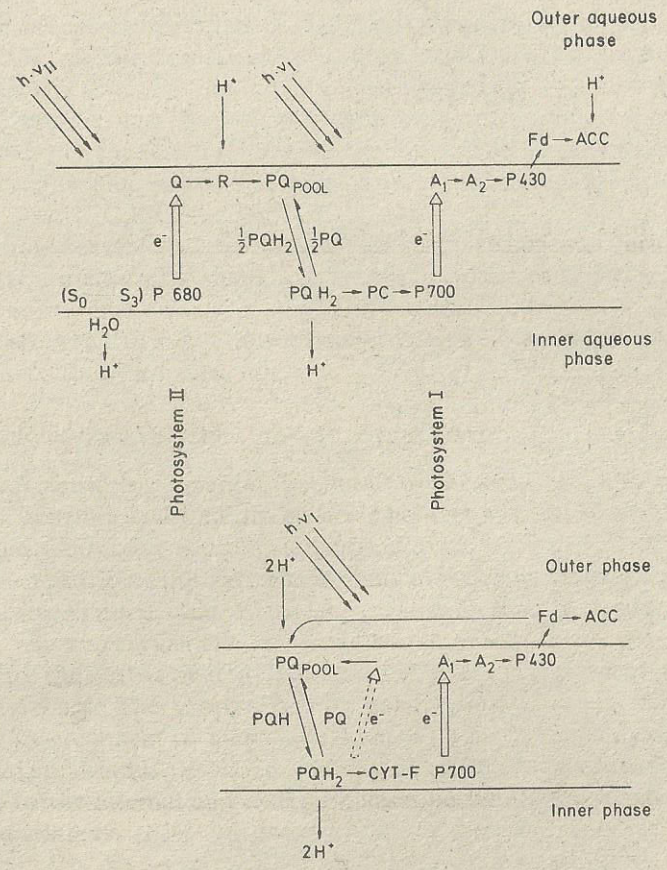

FIG. 5. Schematic presentation of proton pumping and generation of an electric potential difference under linear electron transport (top) and cyclic electron transport (bottom). Double arrows, electrogenic transfer, outwardly directed; dashed arrow, electron path responsible for the slowly rising component of the electric potential (see Fig. 4). P680, P700, reaction center chlorophylls, designated by the characteristic absorption peaks (in nanometers); PQ, PQH_2, pool plastoquinones in various oxidation states; Q→R, bound plastoquinone couple; PC, plastocyanin; Cyt-F, cytochrome *f*; $A_1 \rightarrow A_2 \rightarrow$ P430: acceptor chain; Fd, ferredoxin, iron–sulfur center; ACC, terminal electron acceptor; $S_0, \ldots, S_3$, redox states of the water-oxidizing enzyme.

the photosystems were based on assumptions about the average membrane area occupied by each reaction center and about the electric capacitance of the thylakoid membrane (Schliephake *et al.*, 1968). Allowing 220 Å^2 per chlorophyll, 600 chlorophylls per pair of reaction centers, and 0.5 μF/cm^2 (the generally accepted value for ordinary planar lipid bilayer membranes) gives

48 mV for the membrane potential from a single short flash. Microelectrode measurements on intact giant chloroplasts from *P. metallica* have recently given values near 40 mV (Bulychev and Vredenberg, 1976). Zickler *et al.* (1976) have discussed a possible underestimation due to impalement shunting by this technique. Although this is certainly no valid objection for kinetic arguments, it is still to be tested whether capacitive mismatching of the detector amplifier causes voltage readings that are too low (argument by Dr. Trissl). Attempts have also been made to calibrate the light-induced electrochromic changes by means of salt jumps in the presence of valinomycin (with bacterial chromatophores, Jackson and Crofts (1969); with chloroplasts, Schapendonk and Vredenberg (1977), taking care to minimize light-scattering changes. By this means an apparent maximal single-turnover voltage of 58.5 mV was obtained for chloroplasts, which reduced to 35 mV under certain assumptions about the permeability ratio for K^+ and Cl^-. Much higher estimates, 105–135 mV, have been reported by Zickler *et al.* (1976) based on the action of a voltage-gated, channel-forming antibiotic, alamethicin.

In view of the indirect nature of all these estimates, it is difficult to favor one over another, but a *figure near 50 mV seems probable.* As already mentioned (Section III,B,4), changes in membrane surface potential accompanying a single-turnover flash should not seriously compromise this value.

b. Excitation with Continuous Light. Under conditions of steady, saturating illumination, the electric potential difference measured across the thylakoid membrane becomes a function of the method of measurement. Methods which indicate the bulk phase potential difference—such as redistribution of permeant ions (Rottenberg *et al.*, 1972; Schröder *et al.*, 1972) or microelectrodes (Bulychev and Vredenberg, 1976)—give values near 10 mV (thylakoid interior positive); but methods which monitor the intramembranal field—such as delayed fluorescence (Barber, 1972) or the electrochromic absorption changes (Gräber and Witt, 1974)—indicate values 10-fold greater, ~100 mV. This discrepancy, which is highly reproducible, can be taken as evidence of a strong asymmetry of membrane surface charge, produced as a result of sustained electron transport and proton pumping (Rumberg and Muhle, 1976; Rumberg, 1977; Witt, 1979).

The following time course of events can be constructed following the onset of a step illumination. Initially, the bulk phase potential difference rises to a high value, two- to fourfold that observed with short-flash illumination. Under the influence of this membrane potential, a massive ion exchange begins, with cations driven outward and anions inward. As the internal phase becomes increasingly acidic, protons themselves take over an increasing proportion of the outward current driven by the field (see Gräber and Witt, 1976), and the steady state is reached when proton efflux equals the active inward

proton pumping. The high effective conductivity for protons, conjoined with a negative feedback effect upon the velocity of electron transport, caused by internal acidification (up to 3 pH units), leads to a progressive decrease in the electric portions of the bulk-phase potential difference. At the onset of illumination the rapid rise in the electric potential and the much slower rise in the chemical potential of the proton reflect the difference between the electrical and the chemical buffering capacities of the membranes, as pointed out in Section II,B.

The specific proton uptake under steady illumination of thylakoids (in the absence of uncoupling agents) amounts to 0.2–0.5 H^+ per chlorophyll. Since protons are the only actively pumped species, and since there is no evidence for coupled proton–cation exchange or proton–anion cotransport across the thylakoid membrane, all other ionic species can be assumed to adjust their respective concentrations in the bulk phases according to the Nernst–Planck relationship: $ZF\,\Delta\psi = RT \ln (C_{in}/C_{out})$. The quasi-steady state value of the electric potential difference between bulk phases, however, will depend on the concerted action of all permeant species. The most limiting ionic species in this process is likely to be that present at the largest internal concentration. As an example, in a typical experiment with isolated swollen chloroplasts (internal aqueous volume ≅ 50 liters/mole chlorophyll) the KCl concentration of the suspending medium is 30 m*M*. Under dark conditions the internal concentration of KCl will also be 30 m*M*, and under steady, saturating illumination, a maximum of 0.5 K^+ ions per chlorophyll will be driven out, which—with 0.02 mole chlorophyll/liter—will decrease the internal K^+ concentration at most by 10 m*M*. The Nernst–Planck potential for a K^+ concentration ratio (inside/outside) of 20:30 is 10 mV, interior positive, in close agreement with the steady state values actually observed by methods which measure the bulk phase potential difference.

4. Electrical Conductivity of the Thylakoid Membrane

A reasonable estimate for the electrical conductivity of the thylakoid membrane can be obtained from the decay of electrochromic absorption changes following a light flash (Fig. 3). Typically, the time constant for decay (τ) ranges between 10 and 100 msec, depending on the quality of the chloroplast preparation. Assuming a specific capacitance of 0.5 $\mu F/cm^2$, then, gives a range of 2×10^{-4} to 2×10^{-5} S/cm^2 for the membrane conductivity.

Under flashing light or moderate illumination, the conductivity is dominated by K^+, Cl^-, and Mg^{2+}, not by protons, as is evident from the slow relaxation of pH differences across the thylakoid membrane (= 5–10 seconds; Junge and Ausländer, 1974). Isolated chloroplasts display only poor ion selectivity (Barber, 1972), so that ionic control of conductance depends

strongly on the composition of the suspending medium (Dilley and Vernon, 1965; Hind *et al.*, 1974; Schröder *et al.*, 1972). In intact chloroplasts, Mg^{2+}—which is the most abundant ion (2.33 μmoles/mg chlorophyll; Barber, 1977)—dominates conductivity of the thylakoid membrane and counterbalances most of the pumped protons (Barber *et al.*, 1974; Krause, 1973; Portis and Heldt, 1976). With steady, saturating illumination and the consequent acidification, an outward proton current (probably flowing through the phosphorylation coupling factor) dominates the membrane conductivity (Gräber and Witt, 1976).

5. Structural Aspects of the Electrogenic Reactions

Primary charge separation is directed across the thylakoid membrane. In photosystem I, the electron donor is a special pair of chlorophyll *a* molecules (absorbing at 700 nm and named P700) located near the internal membrane–water interface. This location has been inferred from the polarity of the light-generated electric field, plus the observation that the reduction of P700 by plastoquinone via plastocyanin is not electrogenic (Junge, 1972). (Plastocyanin is a water-soluble protein known to be located inside the thylakoid, near the inner surface of the membrane.) The fact that the potential difference indicated by electrochromic absorption shifts can occur in at least 2 nsec (Section III,C,1; Trissl and Gräber, 1980a) suggests strongly that the primary electron acceptor must reside close to the outer surface of the membrane. The primary acceptor is possibly another pair of chlorophyll *a* molecules which, according to Shuvalov *et al.* (1979) can be reduced in less than 60 psec. At present, however, the time resolution of the electrochromic measurements is insufficient to permit spatial localization of the individual members of the acceptor chain (see Sauer *et al.*, 1978). For photosystem II, studies of the emission of delayed fluorescence have suggested that one of the later acceptors is located 25 Å from the primary electron donor (Ortoidze *et al.*, 1979), within a single protein complex (Fig. 10).

[The probable subunit composition of the protein which contains the primary electron donor in photosystem I, as described by Bengis and Nelson (1975), is also shown in Fig. 10, along with one possible spatial arrangement. The *y* axes of the two chlorophyll *a* rings (Breton, 1976; Junge and Eckhof, 1974), and probably also the *x* axes (Junge and Schaffernicht, 1979), are tilted only slightly out of the plane of the thylakoid membrane.]

It is not yet clear in molecular terms how electrons can be rapidly channeled, or "tunneled," across the thylakoid membrane. Since neither the chlorophyll rings themselves nor the β-carotenes associated with the photosystem I complex are significantly inclined to the plane of the thylakoid membrane (Junge *et al.*, 1977; Junge and Schaffernicht, 1979), there is no identifiable "molecular wire" which spans the thickness of the membrane. A

proposal has been made (Tributsch, 1972) that the interior of the protein complex acts like an injection semiconductor, so that the force driving photoinjected electrons across the membrane is exerted by asymmetric surface potentials (Duniec and Thorne, 1979). Unfortunately, both the direction of charge asymmetry required for this mechanism and the predicted decrease in driving force with increased ionic strength of the medium are incorrect (Table I and Section III,C,3,b, and Fig. 7 of Trissl and Gräber, 1980b). It is more reasonable at present to suppose that electron conduction occurs *across* a series of large π-electron systems, as is known from studies on photoprocesses in model membranes (Mangel *et al.*, 1975) and monolayer assemblies (Kuhn, 1979; Moebius, 1979), but it must be admitted that experimental support for this picture is thus far inadequate.

6. Localized versus Delocalized Electric Field

Physical considerations argue that the act of photochemical charge separation must generate localized dipole fields between electron–hole pairs on a picosecond time scale, and that ionic conduction in the adjacent aqueous phases must distribute this field more evenly over the membrane capacitance but on a longer time scale. By means of macroscopic electrodes (Section III,C,5), Witt and Zickler (1973) were able to show that only about 10 μsec is required for the electric field to "homogenize" over the entire membrane of a single thylakoid. The process can be greatly slowed, however, by low-temperature treatment of intact thylakoids (−125°C, see Section III,C,1; Vermeglio and Mathis, 1974; Conjeaud *et al.*, 1976), because the ionic conductivity of the internal and external phases becomes exceedingly small at such temperatures.

IV. PROTOLYTIC REACTION STEPS

A. Survey

Figure 5 diagrams how protolytic reactions can be linked to electron transfer. With pure linear electron transport (upper diagram), two protons per electron are transferred from the external to the internal aqueous phase of the thylakoids. Of the two sites for proton uptake from the outer phase, one is associated with the terminal electron acceptors (ACC) and the other is associated with the reduction of a special quinone which is probably also active during pseudocyclic electron transfer (lower diagram). Of the two sites for proton release into the internal phase, one is associated with the oxidation of water (i.e., the primary electron donor to photosystem II), and the other is

associated with oxidation of the PQH_2, which again may be active during pseudocyclic electron transfer. Present evidence indicates that the linkage between proton uptake or release and the redox reactions is trivial, so that for chloroplasts—unlike mitochondria and halobacteria—there has not been a need to invoke special proton pumping action by the proteins.

Single-turnover flashes at low frequency produce only very small pH changes inside the thylakoid (~0.05 pH units), and approximately 99.99% of the pumped protons are buffered away. With continuous illumination, however, the influx of protons and concomitant efflux of K^+ and Mg^{2+} (driven by the potential difference) cause a pH drop of more than 3 units, which becomes the major driving force for photophosphorylation (see Gräber, this volume). Still larger pH differences between the internal and external bulk phases are prevented by a negative feedback effect of the internal pH on the rate of electron transfer.

At present, a major controversy concerns the pathway that protons take, from the "sources" on the internal side of the thylakoid membrane into the ATP synthetase. In order to resolve this controversy, it has been necessary to devise molecular probe strategies for proton subcompartmentation. Our most accurate information has come from spectrophotometry with pH-indicating dyes, which have proved to be highly resolving both in kinetic and spatial studies. Once again, it is necessary to discuss some aspects of the technique before presenting the experimental results.

B. Spectrophotometric Detection of pH Changes with pH-Indicating Dyes

Dyes used to measure pH changes in suspensions of cell organelles, over the physiological pH range and with minimal disturbance of biological activity, include bromcresol purple ($pK = 6$; Chance and Mela, 1966), neutral red ($pK = 6.6$; Lynn, 1968; Ausländer and Junge, 1975), bromthymol blue ($pK = 6.8$; Schliephake *et al.*, 1968), phenol red ($pK = 7.3$; Schröder *et al.*, 1972), and cresol red ($pK = 7.9$; Junge and Ausländer, 1974). These dyes distribute over the aqueous phases and membrane spaces according to their solubility and respond to pH changes by easily measured absorption changes.

However, straightforward interpretation of the absorption changes can be hampered by several complications, including overlapping absorption shifts in intrinsic pigments and unknown distribution among different internal compartments. The compartmentation problem has many possible ramifications: Individual compartments may show different pH changes in response to experimental manipulation; steady state dye concentrations in separate compartments may be unequal (they will be unequal if compartment pH values are dissimilar); apparent dye pK values may differ between compartments, as observed, for example, with dyes bound to membranes or proteins (Fernandez and Fromherz, 1977); and large pH changes will cause massive redistribution of the dye. A fortuitous combination of circumstances has allowed pH-indicating dyes to be used quite satisfactorily for short-flash studies, where the above complications can be minimized. The technique to be described, however, does not carry over well either to studies with continuous illumination (Pick and Avron, 1976; Siefermann-Harms, 1978) or to experiments on organelles other than thylakoids (Schnetkamp *et al.*, 1980).

A dye such as cresol red, which is of low lipid solubility at the ambient pH, reports mainly the pH in the outer aqueous phase. The reason for this is probably not the impermeability of the thylakoid membrane to such indicators but the small size of the total internal aqueous volume of thylakoids relative to the outer volume (the ratio is smaller than 10^{-3} in a typical experiment). On the other hand, a dye such as neutral red resides in the membrane to a large extent and reports pH changes from both phases. By combining such indicator dyes with the use of appropriate penetrating or nonpenetrating buffers, it is possible to define unequivocally the phase of predominant pH shift following flash excitation, as demonstrated in Fig. 6. In this case bovine serum albumin (BSA) has been used as a nonpenetrating buffer and imidazole as a penetrating buffer. In the absence of dye (Fig. 6A), the light flash produces a slight change in absorbance due to the intrinsic chloroplast pigments (Junge *et al.*, 1979); this intrinsic response must be substracted from that observed in the presence of dye. When dye is added (Fig. 6B), the flash produces absorption changes both with neutral red and with cresol red. External buffering (with BSA, Fig. 6C) eliminates the response of cresol red, but full buffering (BSA plus imidazole, Fig. 6D) is required to abolish the response of neutral red. Thus, cresol red, without buffer, can be used to estimate pH changes in the external aqueous phase; and neutral red, with strong BSA buffering, can be used to estimate flash-induced pH changes in the internal aqueous phase of thylakoids (Ausländer and Junge, 1975).

The possibility that the dyes and buffers might have spurious biological effects (because of direct interaction with proteins or lipids) which could be misinterpreted has been eliminated by showing that 10 different buffers diminish the flash-induced change in internal pH in *direct proportion to their calculated buffering capacity* (Junge *et al.*, 1979). The same experiments have also indicated that the "internal" pH measured by neutral red is in fact that of the internal aqueous phase, not some restricted compartment within the membrane itself, since even very hydrophilic buffers—once allowed to penetrate the thylakoids—diminished the flash-induced absorption change in neutral red.

An absolute calibration curve for the flash-induced pH changes inside

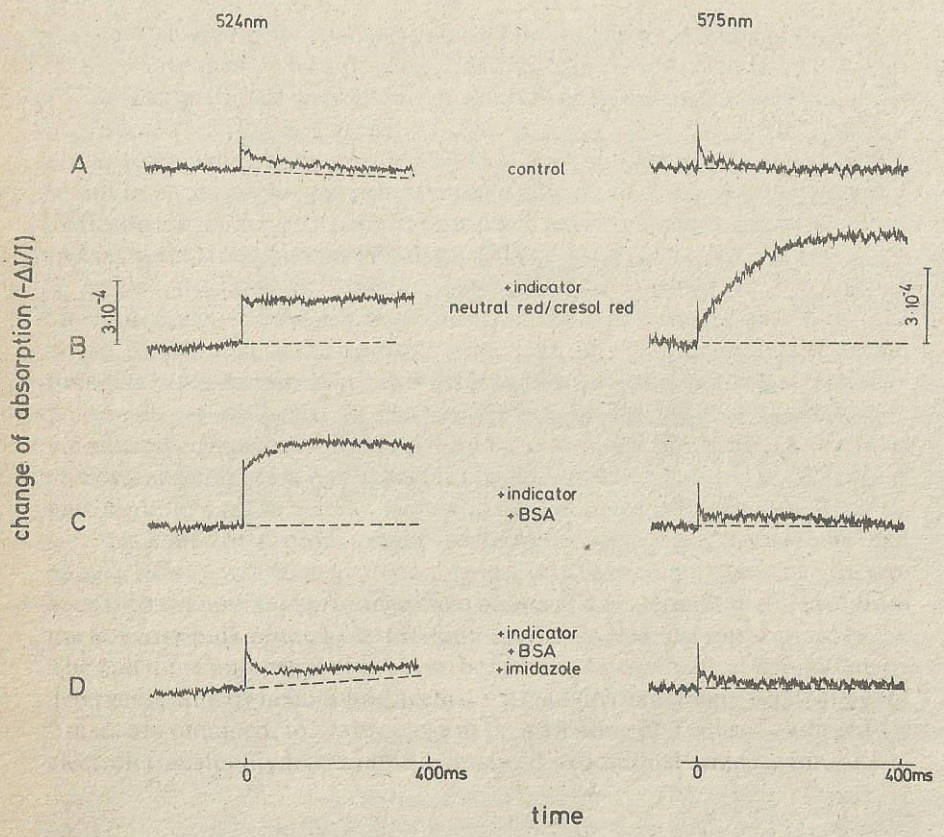

FIG. 6. pH-indicating absorption shifts of dyes in a chloroplast suspension. (Left) Neutral red, which distributes to both internal and external phases. (Right) Cresol red, which is restricted to the external bulk phase. Bovine serum albumin (BSA) is used as a nonpermeating buffer, and imidizole as a permeating buffer. Valinomycin was added to virtually eliminate electrochromic absorption changes at 524 nm (Junge *et al.*, 1978a).

thylakoids can be obtained in (Fig. 7) by plotting the magnitude of the absorption change in neutral red against the pH of the medium, under the condition that flashes are spaced far apart (here, 33 seconds) to avoid accumulation of protons. The resultant plot resembles the theoretical sensitivity of a pH indicator having an apparent pK of 7.25 (solid curve in Fig. 7). The alkaline shift in pK, from the 6.6 expected in aqueous solution, is consistent with the model studies of Fernandez and Fromherz (1977) and can be taken as evidence that the indicating molecules are actually bound to the negatively charged thylakoid membrane. The inset in Fig. 7 demonstrates incremental absorption changes in neutral red obtained when flashes are fired at short intervals

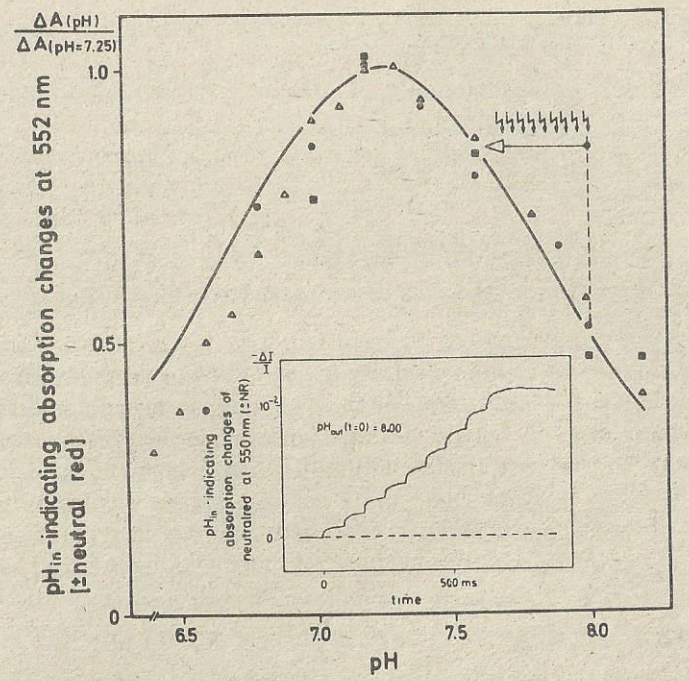

FIG. 7. Calibration of pH-indicating absorption changes in neutral red by means of flash-induced changes over a range of external pH values. The solid curve is a calculated fit assuming the apparent pK of internal (bound) dye to be 7.25. Inset: time course of absorption changes in neutral red during nine closely spaced (75-msec) cumulated pulses, from a starting pH of 8 (see superset diagram). The acidification per flash in this experiment is 0.05 units (Junge *et al.*, 1978b, 1979).

(here, 75 msec) so that the pH changes accumulate. Comparison with the calibration curve yields 0.05 pH units of acidification for each flash (Junge *et al.*, 1979).

C. Proton Pumps

1. Proton Release during Water Oxidation

Technically, the water-oxidizing enzyme system is perhaps the most interesting part of the photosynthetic apparatus. With the end of the petroleum era at hand, hydrogen gas produced at the expense of sunlight is an attractive alternative. The green plant has developed a technique for extracting electrons from water (with the possibility of producing hydrogen gas; see Hall, 1977) by using light quanta in the visible spectral region. The high efficiency

of this naturally occurring process has not yet been technically simulated. To produce one molecule of oxygen from water requires the removal of four electrons and four protons from two molecules of water:

$$2H_2O(liq.) \rightarrow O_2(gas) + 4H^+(aq.) + 4e^-$$

If this process occurred stepwise in water the abstraction of the first electron would be far more endothermic than to be driven by a quantum of red light. Apparently nature has coupled highly endothermic reactions with less endothermic ones to adapt the energetic requirements of each step to the free energy of red light. Experimentally this is apparent from the fact that oxygen is produced only after the accumulation of four oxidizing equivalents (after the input of four quanta of light). Actually, upon excitation of dark-adapted chloroplasts, a release of dioxygen occurs immediately after excitation with the third in a group of short flashes (for a review, see Joliot and Kok, 1975). This has been interpreted to indicate that the singly oxidized state of the enzyme system is the most stable state in the dark. Little is known about the chemical details of the enzyme system which stores the oxidizing equivalents. At least four manganese atoms are required for its function, which seem to change their oxidation states (Wydrzynski *et al.*, 1976). However, it is premature to decide whether the four oxidizing equivalents are stored upon manganese proper, before reacting with two water molecules, or whether "bound" water complexed by manganese is successively oxidized and only finally released as O_2 (Renger, 1977).

In order to discriminate between these two alternatives, a number of different laboratories have examined the release of protons with successive flash-driven transitions between oxidation states. The results are summarized in Table II, where the oxidation states are designated S0, S1, . . . , S4, and the

TABLE II

SURVEY OF STOICHIOMETRIES AND STEPS OF PROTON RELEASE BY THE WATER-SPLITTING ENZYME

Protons released per electron[a]				
S0-S1	S1-S2	S2-S3	S3-S4, S0	Reference
0	0	0	4	Fowler and Kok (1974)
0.75	0	1.25	2	Fowler (1977)
1	0	1	2	Saphon and Crofts (1977)
0	1	1	2	Junge *et al.* (1977)
1	1	1	1	Hope and Morland (1979)
1	0	1	2	Velthuys (1980)
1	0	1	2	Förster *et al.* (1981)

[a] S0, S1, . . . , S4 designate the sequential discrete oxidation states of the water-splitting enzyme. S0-S1, . . . , S4-S4 designate the corresponding transitions between oxidation states.

transitions are designated S0-S1, . . . , S3-S4. While the disagreement among different laboratories and even within one laboratory is obvious, one major point of agreement is also clear: Except in the early experiments of Fowler and Kok (1974), at least three of the four transitions are accompanied by proton release. Superficially, this is in agreement with the suggestion of Renger (1977) that bound water is successively oxidized, but Sauer (1980) has pointed out that hydroxyl ions could be the specific counterion for oxidized maganese in the enzyme complex. Since OH^- binding is equivalent to H^+ release, the stoichiometric experiments would not then discriminate between the two alternative mechanisms. Further attempts to distinguish the two alternatives have been based on detailed analysis. Measurements (via neutral red) of successive flash-induced acidifications on a microsecond time scale have shown each proton ejection step to be multiphasic, with the initial rise having a time constant of ~ 100 μsec (Junge and Ausländer, 1978). The multiphasic rise has been analyzed, and components attributed to certain transitions between subsequent oxidation steps (Förster *et al.*, 1981). It became clear that one portion of proton release precedes the turnover of a supposed precursor of water oxidation, which became apparent in electron paramagnetic resonance spectroscopy ("signal II vf"; Babcock *et al.*, 1976).

2. Protolytic Reactions Involving Plastoquinone

The known electron transfer sequence beginning in photosystem II is as follows: the red-absorbing chlorophyll unit designated P680 (Döring *et al.*, 1968); pheophytin *a* (Klimov *et al.*, 1980); a tightly bound plastoquinone molecule (Q), originally defined as the quencher of chlorophyll fluorescence; a second, closely associated quinone molecule designated R; (Stiehl and Witt, 1968; Bouges-Bocquet, 1973; Velthuys and Amesz, 1974); and then the "pool" plastoquinone molecules, which amount to at least six per pair of reaction centers and serve as a kinetic buffer. Upon transfer of one electron into the oxidized Q/R couple, the state Q/R^- is formed rapidly and remains stable for several seconds (Pulles *et al.*, 1976). After a second electron enters, to make Q^-/R^-, the couple dismutates to Q/R^{2-}, which is the first form that can transfer electrons onward to the quinone pool (Bouges-Bocquet, 1973; Velthuys and Amesz, 1974).

From a comparison of the equilibrium constants for electron transfer within the Q/R system with the equilibrium constants for dismutation of duroquinone in aqueous ethanol solution, Diner (1977) proposed that protonation of plastoquinone occurred at the state of R^{2-}, which would predict binding of zero protons on each odd-numbered flash and two protons on each even-numbered flash, beginning with fully oxidized Q/R. Experiments on proton uptake from the outer phase, associated with plastoquinone reduction, have shown that one proton is taken up per electron transferred

(i.e., per single-turnover flash) during low-frequency, repetitive excitation (Schliephake *et al.*, 1968; Junge and Ausländer, 1974; Ausländer and Junge, 1975). The predicted periodicity in proton uptake by plastoquinone under excitation of dark-adapted chloroplasts with a series of flashes has not been observed experimentally (Fowler, 1977a). The situation is very similar to that found in bacterial chromatophores (see Dutton *et al.*, this volume), where a diffusion barrier for protons was postulated to cover the reduction sites of the special bound ubiquinones (Wraight, 1978).

A demonstration of the time course for acidification of the internal aqueous phase, following a single-turnover flash, is given in Fig. 8. The rapid phase, represented by the upstroke (which is itself complex on a microsecond time scale; see Section IV,C,1) is associated with water oxidation. The slow phase can be attributed to plastoquinones, on the basis of the fact that 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), a quinone analog and antagonist, abolishes the phase (Ausländer and Junge, 1975). The half-time for proton release via the plastoquinones is thus about 20 msec, whereas that for proton uptake—determined previously (Ausländer and Junge, 1974)—was 60 msec. The simplest way to accommodate this discrepancy is to suppose that proton uptake occurs at an external site shielded from the aqueous bulk phase by a diffusion barrier (Ausländer and Junge, 1975). The half-time for proton uptake can be reduced by mechanical disruption, by detergents, and by uncoupling agents, so that—in the extreme—half-times of 2 msec are obtained, tolerably close to the half-time of 0.6 msec observed for electron transfer (hydroquinone formation). Additional evidence for a diffu-

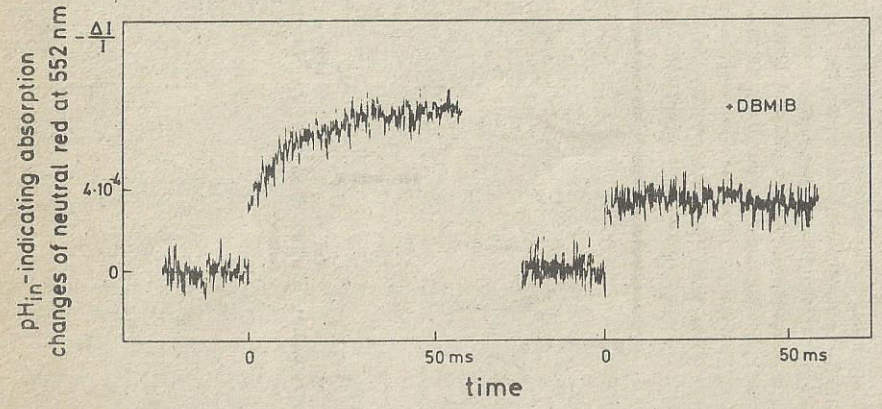

FIG. 8. Time course of flash-induced, pH_{in}-indicating absorption changes in neutral red for discriminating protolysis associated with water oxidation and with plastoquinone oxidation. (Left) Both photosystems—and therefore both proton steps—functioning. (Right) Plastoquinone blocked by DBMIB, so that only the water oxidation step releases a proton.

sion barrier, impeding the access of artificial electron acceptors to photosystem II, has been presented by Renger (1979). The gross topological arrangement of proton systems, with their kinetic constants, in the thylakoid membrane is summarized in Fig. 9. [The right-hand portions (leakages and the ATP synthetase, which are not otherwise discussed here) are included for the sake of providing an overall picture. Details of the ATP synthetase are given by Gräber (this volume).]

3. Protolytic Reactions Involving the Terminal Electron Acceptor

Proton uptake at the terminal acceptor is determined entirely by the chemical properties of the acceptor. With the non-proton-binding acceptor ferricyanide, no proton uptake is observed upon reduction of photosystem I; in the case of oxygen, mediated by benzyl or methyl viologen, one proton per electron is taken up, and we can infer that reduction of $NADP^+$ results in the uptake of 0.5 proton per electron (Junge and Ausländer, 1974). Like the uptake site at plastoquinone, that at the terminal acceptor appears also to be shielded by a diffusion barrier (Ausländer and Junge, 1974).

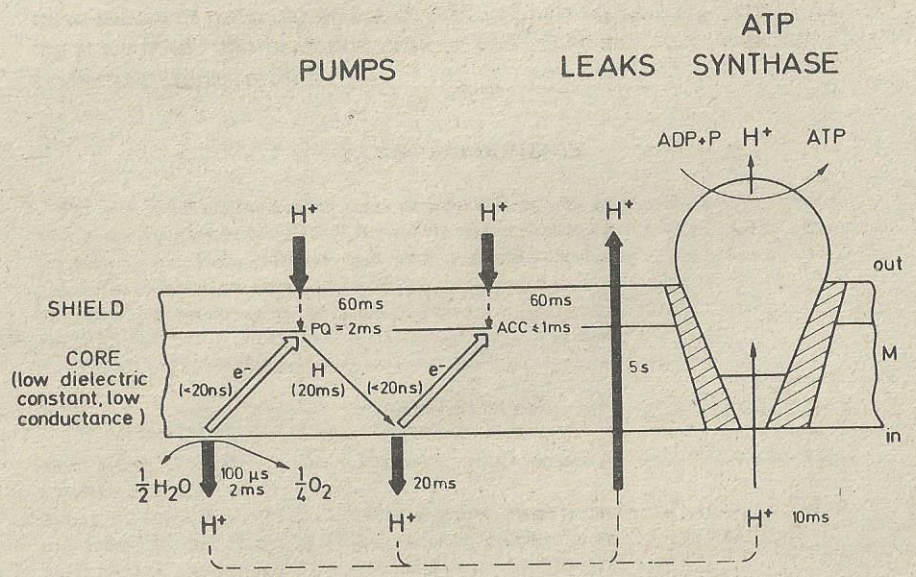

FIG. 9. Kinetic parameters for electric potential generation (double arrows), proton uptake from the external phase and release into the internal phase (heavy downward arrows), proton leakage from thylakoids (heavy upward arrow), and useful proton flow through the ATP synthetase. On the outer side of the membrane, protons are taken up from buffering groups under a diffusion barrier or shield. These postulated buffering groups can be refilled from the outer aqueous phase only after a finite delay.

4. Overall Stoichiometry and Magnitude of the pH Difference

Under conditions in which the linear electron transport chain (but not the cyclic system) is operative, the net stoichiometry of proton pumping is two H^+ transported from the external aqueous phase to the internal aqueous phase for each electron transferred from water to the terminal acceptor (assuming benzyl or methyl viologen). This conclusion has also been reached by many other investigators (see, e.g., Karlish and Avron, 1971; Saphon and Crofts, 1977b; Chow and Hope, 1977; and the reviews of Trebst, 1974; or Dilley and Giacinta, 1975), using both flashing light excitation and continuous illumination.

Under continuous illumination, very substantial bulk phase pH changes can result from proton-pumping activity. With a starting pH of 7–8, and ADP absent, the internal phase can become acidic by as much as 3 units (Rumberg and Siggel, 1968; Rottenberg *et al.*, 1972) during linear electron transport, and by as much as 3.8 units during cyclic electron transport (with the artificial redox mediator pyocyanin; Pick and Avron, 1976). Some restriction of acidification may occur as the external pH is experimentally lowered, but Portis and McCarty (1973), using low pK amines, demonstrated a pH difference of 2.8 units with $pH_o = 6.5$. Thus, the proton pumps and redox systems can still run at least down to ΔpH 3.7.

V. COMMENTS ON THE PATHWAY OF PROTONS TO THE ATP SYNTHETASE

Although it is not the purpose of this article to cover the mechanism of proton reaction during actual ATP synthesis (see the discussions by Gräber, Kagawa, and Kozlov and Skulachev, this volume), some brief comments on the routing of protons from the redox pump to the ATP sites do seem in order. After the strenuous discussions on the nature of energy flow in photophosphorylation had faded away (for an afterglow, see the multiauthored review by Boyer *et al.*, 1977), and the general tenets of proton coupling—as formulated by Mitchell (1961, 1966)—had been accepted, the question arose of whether the relevant protons arrive at the ATP synthetase via the internal bulk (osmotic) volume (Mitchell, 1961, 1966, 1977, 1978) or via special localized conducting subspaces either within the membrane (Williams, 1959, 1961, 1976, 1978) or at the membrane–water interface (Kell, 1979).

Several observations suggest the latter alternative: (1) Ort *et al.* (1976), studying ATP synthesis under illumination by short light pulses (10–100 msec), found that permeating buffers had much less effect on synthesis than expected from the calculated buffer suppression of the bulk phase pH change. (For more recent aspects of these experiments, see Dilley *et al.*, this volume.)

(2) The existence of some kind of subspace seems to be confirmed by the time relationships of electron transport-driven proton uptake and release, as described in Section IV,C,2. (3) Especially in mitochondria, proton/ATP stoichiometries obtained from kinetic experiments are largely at variance with those obtained by static head experiments (reviewed by Kell, 1979), suggesting that the ATP synthetase does not see the average bulk proton motive force (Van Dam *et al.*, 1978). But rejoiners to these observations have already been offered: (1) At least two other laboratories (Davenport and McCarty, 1980; Vinkler *et al.*, 1980) have reached opposite conclusions in repeating the experiments of Ort *et al.* (1976). (2) The space or shield defined by the experiments of Ausländer and Junge (1974) is on the outside of the thylakoid membrane, not the inside. (3) The stoichiometric problems are especially obvious in mitochondria and perhaps may not be extrapolated to chloroplasts.

The issue has been further attacked experimentally by determining how fast released protons equilibrate with the internal aqueous volume of the thylakoids and how fast they disappear into the ATP synthetase, using flash spectrophotometry with neutral red. As already mentioned in Section IV,B, almost all distributed buffers—even the most hydrophilic ones—suppress flash-induced absorption changes in neutral red, so there seems little doubt that the dye estimates pH in the internal bulk phase. Furthermore, even the most rapid component ($\tau_{1/2} \cong 100$ μsec) of pH change after flash excitation of photosystem II is sensitive to water-soluble buffers in a manner dependent on the internal osmotic volume (Fig. 4 in Junge *et al.*, 1978a). Therefore, exchange of protons among the water-oxidizing sites, the neutral red-measuring space, and the osmotic space is at least as rapid as 100 μsec. However, *relaxation* of the flash-induced pH change, during ATP synthesis, occurs with a half time of 10–12 msec (Fig. 5 in Junge *et al.*, 1978a). Thus, at least with the relatively low-velocity ATP formation that occurs during flashing light, there is ample time for protons to equilibrate with the internal aqueous space before they enter the ATP synthetase.

VI. SUMMARY

Figure 10 presents, in an architectural form, our present view of molecular events involving proton transport in the thylakoid membrane. Photon excitation in either (or both) photosystems produces extremely rapid electron transfer from special chlorophyll *a* molecules on the inner side of the thylakoid membrane to acceptors on the outer side. The resultant charge shift is expressed both as a bulk phase potential difference and as an asymmetry of membrane surface potentials, the two together contributing to the electric field assayed by intrinsic membrane probes. In addition, intact chloroplasts

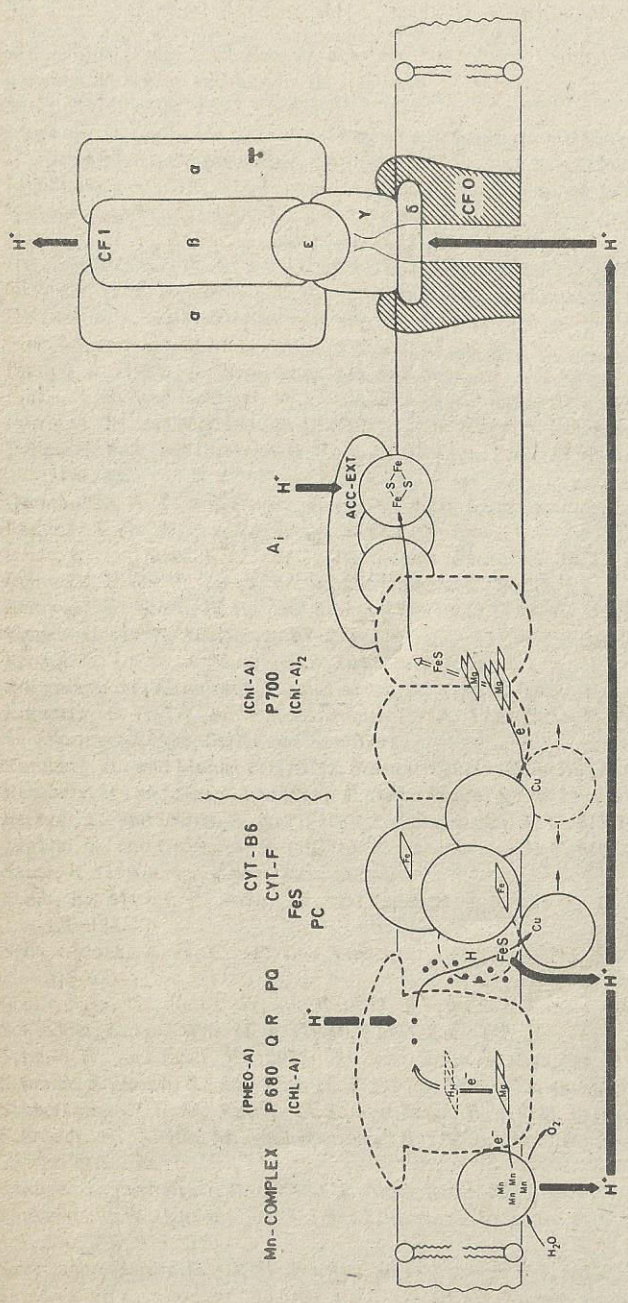

FIG. 10. Artist's view of the arrangement of electron transfer proteins and the ATP synthetase in the thylakoid membrane. The membrane is shown as a lipid bilayer with the known proteins sized according to their molecular weights. Double arrows, electrogenic electron transfer; thin arrows, nonelectrogenic electron transfer; heavy arrows, proton transfer steps. Certain groups active in electron transfer are specified: managnese in the water-oxidizing complex, chlorophyll *a* in both photosystems, pheophytin *a* in photosystem II, plastoquinones (the dots near photosystem II), copper in plastocyanin, the cytochrome hemes, and several iron-sulfur groups. The vertical serration locates a likely lateral separation between the serial components. (Photosystem II is preferentially located in the stacked inner portions of thylakoid membranes, whereas photosystem I and the ATP synthetase are preferentially in the nonstacked portions. Physiological connection between the photosystems is provided by lateral diffusion of phastocyanin and probably also plastoquinone.) Except for the shape of the ATP synthetase (CF_1–CF_0), the drawn shape of the proteins is arbitrary.

carry out a third, slower electrogenic reaction (under conditions of pseudocyclic electron transport) which involves photosystem I and can be modulated by photosystem II. Observable proton transfer seems directly linked to the redox reactions (of water, plastoquinone, and the terminal acceptor), and there is not yet convincing evidence for a special proton pump or proton-pumping protein.

On the outer side of the membrane, proteins can act as shields and/or intermediate proton donors to the reduced quinones, so that relative alkalinization of an intramembranal (or surface) subcompartment is possible. On the inner side of the thylakoid membrane, protons are released rapidly by the water-oxidizing enzyme system, but comparatively slowly by plastohydroquinone. Both the electric potential difference between the bulk phases and the pH difference appear to be a collective property of a large functional unit containing at least 10^5 chlorophyll molecules and corresponding broadly to the size of one thylakoid. It is well established that the electric potential difference and the pH difference are exchangeable as driving forces for photophosphorylation (see Gräber, this volume), but under steady illumination the electric component between the bulk phases appears negligible.

Our current research in this field is being directed toward the molecular mechanisms of water oxidation, of the extremely rapid charge separation taking place in the photoreaction centers, and of the manner in which the ATP synthetase utilizes proton flow.

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