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MEMBRANE POTENTIALS IN PHOTOSYNTHESIS[1]

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[1]Symbols and units used in this paper conform with the recommendations of the IUPAC council (SI units). The system used for electromagnetic quantities is the meter-kilogram-second one, with the permittivity of the vacuum ($\epsilon_0 = 8.854 \times 10^{-12}$ A $V^{-1}m^{-1}$) introduced to match the units of volt and ampere to the mechanical quantities. Deviating from this notation the length unit Å ($=10^{-10}$m) is used for atomic dimensions.

INTRODUCTION

Photosynthesis in green plants produces reducing equivalents in the form of $NADPH^+$ and free energy in the form of ATP, both used in the secondary processes to reduce carbon dioxide to sugar and other organic material. The primary processes occur at the inner membrane system of the chloroplast, which is formed from disk-shaped interconnected vesicles, the thylakoids.

The role of electrochemical events in the production of ATP has been perhaps the most embattled field in the study of photosynthesis during the last fifteen years. The experimental evidence accumulated during this period clearly favors Mitchell's hypothesis (103, 104) that the light-driven electron transport is vectorial in the thylakoid membrane and thereby generates an electrochemical potential difference of protons. The internal phase of thylakoids is more positively charged than the external one and becomes more acidic. The ATP synthase gains the free energy necessary for the synthesis of ATP from the translocation of protons downward the electrochemical potential gradient. The sequence of events in the thylakoid membrane is illustrated in Figure 1. Absorption of light quanta by two types of antenna systems promotes the transfer of electrons towards a more negative redox potential, ultimately from water to $NADP^+$. This is coupled to proton uptake from the external phase and to their release into the internal phase. The coupling factor for ATP synthesis, which is attached to the membrane from the outer side, reacts with protons from the internal phase via a proton well, probably formed by its binding-protein within the membrane.

The generation and the use of electrochemical free energy in photosynthesis of green plants is the topic of this review. Several reviews on this topic have appeared recently (63, 174, 175). In this article emphasis is laid on methods for the evaluation of the electrochemical phenomena and on the fields which deserve further study.

This article is limited to photosynthesis of green plants. The reader interested in electrochemical events in bacterial photosynthesis may refer to the recent review by Wraight, Cogdell & Chance (186).

DIGRESSION ON ELECTROCHEMISTRY

The term *membrane potential* is used to characterize the possibility to gain useful work from the translocation of ions across a membrane separating two aqueous

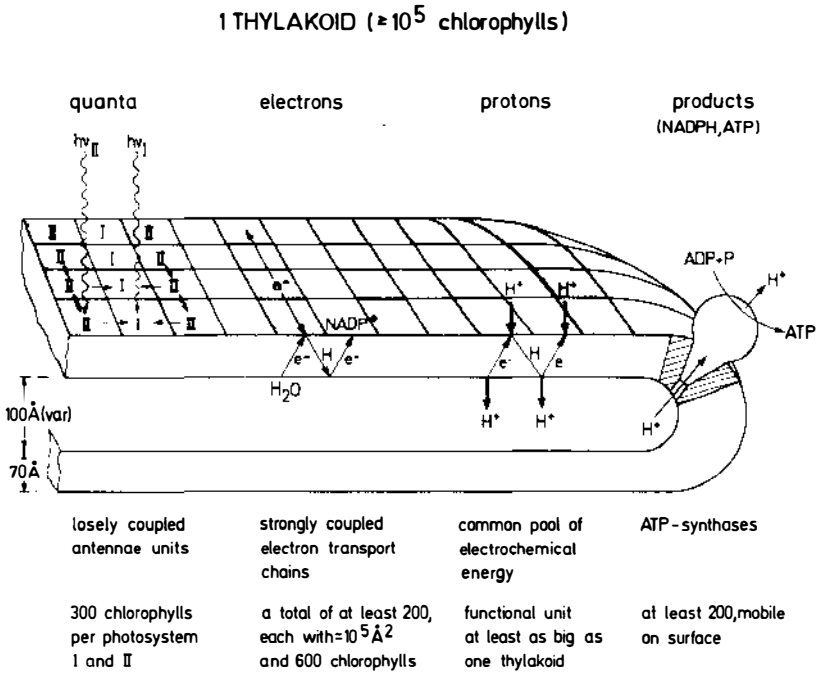

Figure 1 Schematic representation of the primary processes of photosynthesis in green plants (71b).

phases. The electrochemical potential of an ionic species k at a given point A is defined as the work to be performed to bring the ion from a point where its potential is arbitrarily defined as zero to point A. Due to the arbitrariness of the reference point only the difference between electrochemical potentials is observable. Useful work can be gained by translocating ions of the species k between points at different electric potential and/or at different concentrations of this ion. For an ion in aqueous solution the electrochemical potential is

$$\mu_k = \mu'_k + RT \ln a_k + z_k F \varphi, \qquad 1.$$

where μ'_k is the potential in the arbitrarily defined reference state, the activity a_k is related to the concentration c_k by a factor on the order of 1, z_k is the ionic valency of the species k, and φ is the electrical pontential. R, the gas constant, T, the absolute temperature, and F, the Faraday, are as usual.

Ions tend to move from points of high to those of lower electrochemical potential. The fact that ionic mobility is larger in the aqueous phases than in the membrane makes it reasonable to assume that the potential of any species k is constant

throughout each aqueous phase from the bulk to the boundary of the membrane $\mu_k^{bulk} = \mu_k^{boundary}$. The difference between the electrochemical potential across the membrane then reads

$$\Delta\mu_k = 2.3\ RT\ \lg(a_k^i/a_k^o) + z_k F\ (\varphi^i - \varphi^o), \qquad 2.$$

where the superscripts stand for the internal and the outer aqueous phase, respectively, and the natural logarithm was changed into the decadic one. For protons the negative decadic logarithm of the activity is defined as the pH value, hence the difference of the electrochemical potential becomes

$$\Delta\mu_H = -2.3\ RT\Delta\text{pH} + F\Delta\varphi = \text{pmf}, \qquad 3.$$

where pmf stands for proton motive force, introduced by Mitchell in analogy with the electromotive force.

The constancy of the electrochemical potential throughout the aqueous phases does not imply constancy of its chemical and electrical components. Considerable deviations may occur if the membrane carries fixed electric surface charges. As a satisfying quantitative description of surface potentials has not even been obtained for simple bimolecular lipid membranes [see Haydon & Hladky (52)] most authors have relied so far on a semiquantitative description based on the theories of Gouy, Chapman & Stern [Delahay (27)].

Figure 2 illustrates the thylakoid membrane. The density of negative fixed charges and dipoles is assumed to be higher at the inner surface. These give rise to a negative surface potential stretching out into the adjacent aqueous phase. Mobile ions adjust their concentration to this potential, with cations closer and anions more distant to the fixed charges. This finally screens the surface potential. The thickness of the diffuse double layer of ions depends on the ionic strength in the aqueous phases, at 10 mM it is about 30 Å. As the electrochemical potential is constant in each aqueous phase the variation of the electric potential is compensated by the variation of the chemical potential; for the proton it is the pH value. This is illustrated in the lower part of Figure 2.

The consequences of surface effects are most stringent if the thickness of the internal aqueous phase is comparable to the thickness of the diffuse double layer. Then, as illustrated in Figure 2, the electric potential in the internal phase will not decrease to zero and an electric potential difference will prevail between the internal and the external aqueous phase even if the membrane is leaky for any kind of ion. Maintenance of this potential difference does not require input of energy. Concomitant to this is a difference in the ion concentration and especially of the pH. Care must therefore be taken to assign a specific pH value or a specific electric potential to the internal phase. While some probes for the internal pH may detect the average value (some distribution methods), others may be sensitive to the pH at the boundary (certain dyes).

A semiquantitative estimate of the surface potential at the internal side of the thylakoid membrane yielded a value on the order of 100 mV [Rumberg (128)]; however, important parameters for a rigorous treatment are inaccessible as yet.

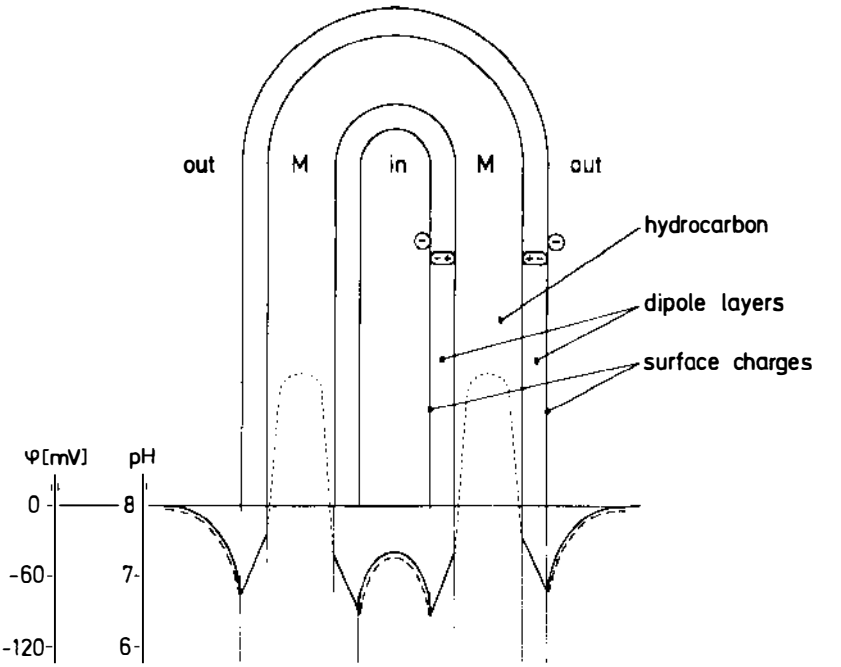

Figure 2 Section through one thylakoid illustrating the role of surface potentials. The solid line describes the electric potential, its continuation as a dotted line in the hydrophobic core of the membrane is determined by the electrostatic image forces created by a test charge located in this region. The broken line gives the pH value in the aqueous phases [for further details, see Rumberg (128)].

METHODS

The submicroscopic dimensions of the functional vesicles of photosynthesis allowed only for indirect measuring techniques for the voltage across their membrane and the pH value in the internal phase. Because there has been controversy, it seems worthwhile to review the virtues and problems of these methods first, before passing over to their application for the study of photosynthesis.

Electrochromism in Chloroplasts

The functional membrane of photosynthesis is highly pigmented intrinsically. This offers the unique advantage to detect the voltage across the thylakoid membrane via electrochromic absorption changes of the bulk pigments. Electrochromism is the effect of strong electric fields (of say 10^7 Vm^{-1}) on the absorption spectrum of dye molecules. The major effect is an almost homogeneous shift of an absorption band

by a few tenths of a nanometer. Field strength high enough to cause measurable bandshifts are common across thin biological membranes. If a voltage of 100 mV exists across a membrane 100 Å thick the electric field strength is 10^7 Vm^{-1}. The physical basis for electrochromism is outlined in the appendix.

Absorption changes which were later interpreted as electrochromic in origin were discovered first when Duysens (30) and Witt (173) started to excite algae and chloroplasts by flashing light for high resolution spectrophotometry [for recent reviews on instrumentation, see Rüppel & Witt (136) and Junge (71a)]. However, these absorption changes proved hard to understand for many years. Various concepts were proposed to associate the most prominent of these changes (around 520 nm) with events in the electron transport chain (e.g. 23, 35, 54, 55, 119, 127, 129, 131, 132). A discussion on whether these changes are predominantly due to carotenoids (23, 34, 35, 54, 161) or to chlorophyll *b* (35, 178) went on. An understanding of the absorption changes around 520 nm was furthermore complicated since, as we know now, at least three different phenomena give rise to apparent changes of absorption in this spectral region, namely electrochromism, the formation of carotenoid triplets, and transients in the light scattering properties of chloroplasts.

Finally, three lines of evidence led to the identification of some of these absorption changes with an electrochromic response of carotenoids and chlorophylls to a light-induced electric field across the thylakoid membrane.

KINETIC EVIDENCE Kinetic evidence was based on the time course of the absorption changes around 520 nm after flash excitation of chloroplasts. Fork, Amesz & Anderson (36) observed acceleration of the decay after treatment of chloroplasts with the detergent digitonin. Rumberg & Siggel (134) observed an acceleration under phosphorylating conditions. Stimulated by Mitchell's hypothesis (103, 104) they suggested that the absorption changes around 520 nm might indicate proton flux across the thylakoid membrane. Junge & Witt (75) finally observed that the decay of these absorption changes is accelerated by any treatment which might increase the ion conductivity of the thylakoid membrane (e.g. aging, osmotic shock, solvents, or ion-transporting antibiotics). There was no specificity for any special type of ion observed. Moreover kinetic effects were induced by as little as one molecule of the pore-forming antibiotic gramicidin D on 10^5 chlorophyll molecules, which demonstrated that the phenomenon indicated by the absorption changes is delocalized over a functional unit as large as one thylakoid disk, at least. From these observations Junge & Witt (75) concluded that the rise of absorption at 520 nm indicates the generation of an electric potential difference across the thylakoid membrane and its decrease the decay of the potential by ion flux. An example for the time course of the absorption change at 520 nm after flash excitation in dependence on the presence of the ion-carrying antibiotic valinomycin is given in Figure 3.

SPECTROSCOPIC EVIDENCE Spectroscopic evidence came from the similarity between the flash-induced "field-indicating" difference spectrum in chloroplasts [Emrich, Junge & Witt (31)] with the electrochromic difference spectrum of chlorophylls and carotenoids observed in vitro [Schmidt, Reich & Witt (147)]. These

Figure 3 Time course of the electrochromic absorption changes at 520 nm observed after exciting a suspension of isolated spinach chloroplasts with a short flash of light at time $t=0$. Addition of the potassium carrier valinomycin accelerates the decay of the electric potential generated by the light pulse (74).

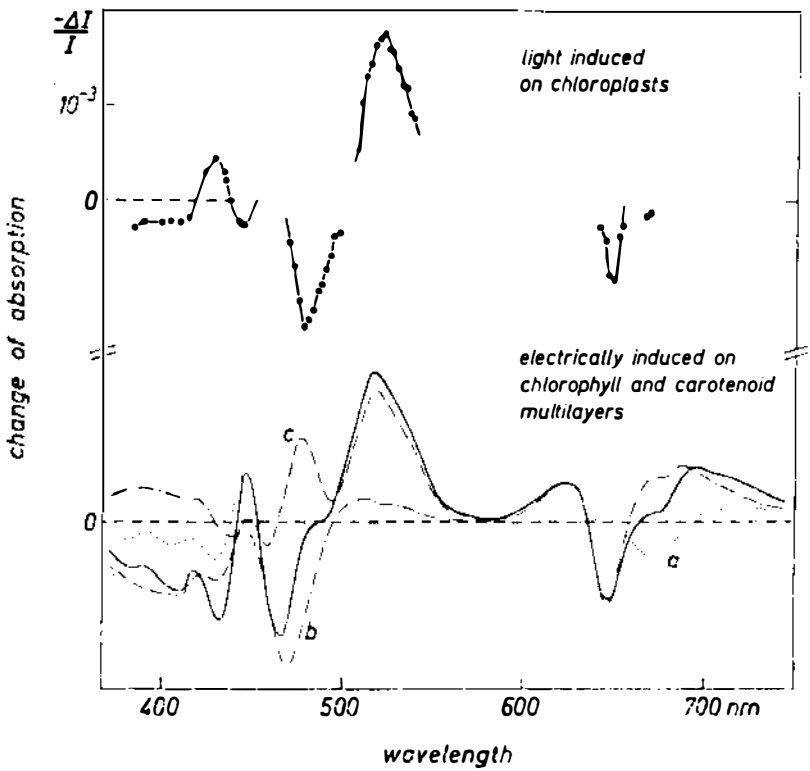

Figure 4 Difference spectrum in the light-induced electrochromic absorption changes observed in chloroplasts [*above,* after (31)] and of electrochromic absorption changes induced by high field strength applied to pigments embedded in microcapacitors [*below,* after (147)]. *a,* chlorophyll *a*; *b,* chlorophyll *b*; *c,* carotenoid. The solid line is a synthesis of the spectra *a, b,* and *c* to mimic the spectrum from chloroplasts.

spectra are illustrated in Figure 4. For separating the "field indicating" absorption changes in chloroplasts from those of other origins advantage was taken of the fact that the former are kinetically labeled by their sensitivity to ion transporting antibiotics, among other things (31). In vitro spectra were obtained with the pigments embedded into microcapacitors formed from lipoic monolayers (147). As evident from Figure 4, the agreement of the in vivo with the in vitro spectra is less satisfying in the region where the physically different types of chlorophyll *a* absorb in the red spectral region from 670 nm on. However, it was to be expected that their structure would not be mimicked in vitro. The major peak at 518 nm, however, could be attributed to electrochromism of carotenoids with a contribution from chlorophyll *b.* The large negative peak at 478 nm is largely attributable to chlorophyll *b.*

The spectroscopic argument was strongly backed by similar observations in bacterium chromatophores where the electrochromic difference spectrum is less complicated owing to the absence of chlorophyll *b* in the spectral region of the carotenoids [Jackson & Crofts (62), Schmidt & Reich (146), Borisevitch et al (15)].

EVIDENCE FROM ARTIFICIALLY INDUCED DIFFUSION POTENTIALS Evidence from artificially induced diffusion potentials was convincing in chromatophores from *Rhodospirillum rubrum*. Jackson & Crofts (62) imposed a diffusion potential by adding KCl to a suspension of chromatophores in a low-salt medium with the K^+-specific ionophore valinomycin present. They could induce absorption changes whose extent varied linearly with the diffusion potential and whose spectrum was almost identical to the light-induced one in the region of the carotenoids. Analogous studies with chloroplast fragments were less convincing [Strichartz & Chance (162)]. Dissimilarities between the light-induced and the salt-jump-induced difference spectra show that light-scattering artifacts may have been quite large.

LINEAR RELATIONSHIP A linear relationship between the extent of the electrochromic changes of absorption and the voltage across the thylakoid membrane is not trivial for many reasons (see Appendix). However, it is well established by experiments both in chromatophores (62) and in chloroplasts. In the latter, comparison of the flash-induced absorption changes at 520 nm with the response of macroscopic electrodes (electrostatic induction technique, see below) revealed a linear relationship (181). Moreover, comparison of the extent of the electrochromic absorption change with the number of protons taken up from the external phase after excitation of chloroplasts with one single or a group of short flashes has revealed a linear relation as well [(141), together with (122)]. This gave indirect evidence for the following linear relationship,

$$\Delta A \sim C\Delta\varphi = \Delta Q, \qquad 4.$$

where ΔA is the extent of the electrochromic absorption changes, e.g. at 520 nm, ΔQ is the charge displacement per unit area of the membrane, and C is the electric capacitance per unit area. This empirical relationship suggests that the membrane capacitance is independent of the voltage at least in experiments under excitation with flashing light.

The charge displacement per unit area in time defines the electric current density j. Hence the first derivative of the electrochromic absorption changes with respect to time is proportional to the electric current density. And the extent of these absorption changes measures the voltage. This is illustrated in Figure 5.

CALIBRATION Calibration of the changes of the electrochromic absorption into voltage and current density across the thylakoid membrane is difficult. A gross estimate on the voltage was based on the following considerations [Schliephake, Junge & Witt (141)].

The voltage induced by excitation of chloroplasts by a flash turning over each photosystem only once is due to the translocation of two elementary charges per

membrane area covered by one electron transport chain. Approximation of the membrane by a plate capacitor yields the following relation,

$$\Delta\varphi^{(1)} = 2ed/(\epsilon\epsilon_0 A) \qquad 5.$$

where $^{(1)}$ refers to excitation with a single turnover flash, e is the elementary charge, d is the thickness of the dielectric core of the membrane, ϵ is its dielectric constant, A is the membrane area covered by one average electron transport chain, and ϵ_0 is the known dimensional factor. Taking the thickness of the membrane as 50 Å [Kreutz (83)], the area per average chlorophyll as 220 Å^2 units [Thomas, Minnaert & Elbers (165), Wolken & Schwertz (185)], the average number of chlorophylls per electron transport chain as 700 and ϵ, the dielectric constant, as 2 in analogy to a homogeneous hydrocarbon then one obtains a voltage of

$$\Delta\varphi^{(1)} = 60 \text{ mV}.$$

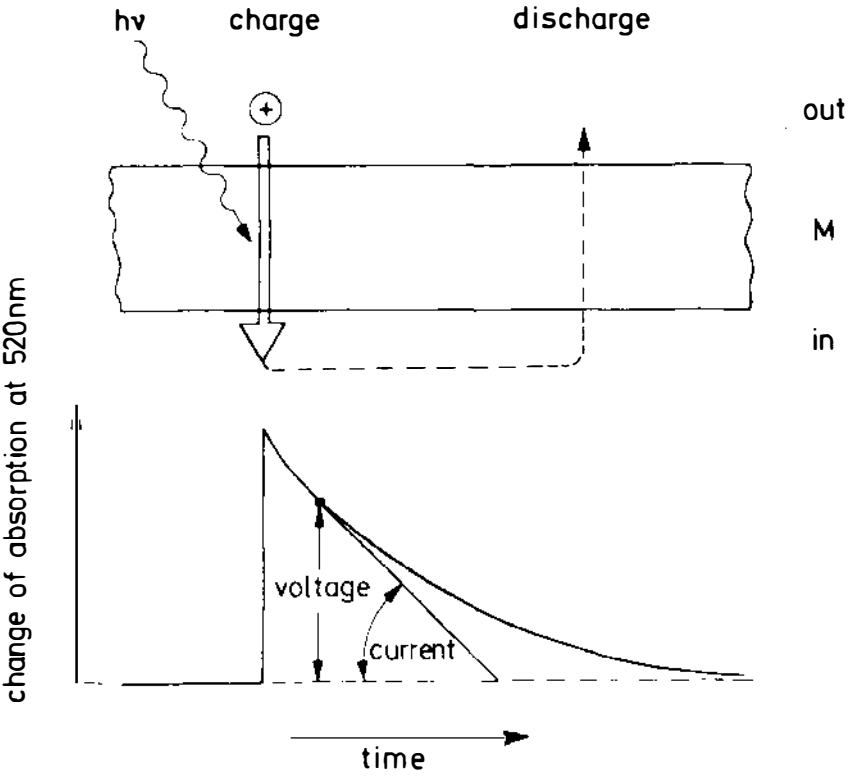

Figure 5 The interpretation of the flash-induced absorption changes at 520 nm in chloroplasts to be used as molecular voltmeters and amperemeters.

The data entering into this estimate for the voltage are not precisely known. For instance the dielectric constant of the membrane might be assumed to be 4, which would yield a figure of about 30 mV for the voltage induced by a single turnover flash rather than 60 mV.

Taking the same data for the average area per electron transport chain and the translocation of two elementary charges per flash as above, the electric current density can be estimated with somewhat greater accuracy [Junge & Schmid (74)]. If the decay of the absorption change is single-exponential after a flash, i.e.

$$d\Delta A/dt = -\Delta A/\tau,$$

where τ is the exponential decay time, according to equation 4, this implies

$$d\Delta Q/dt = -\Delta Q/\tau = j,$$

where j is the electric current density. With the above data for the translocated charges per area of one electron transport chain it follows that the current density just after the flash $j^{(1)}$ is

$$j^{(1)} = 1/\tau \times 10^{-4}\ Am^{-2}sec^{-1}.$$

If the decay time is 100 ms the peak current density is 1 $mAm^{-2}\ sec^{-1}$.

LIMITATIONS Limitations for the utility of electrochromic absorption changes for semiquantitative studies on the electric phenomena at thylakoid membranes arise from the following sources.

The light-scattering properties of thylakoids change under illumination. Transients in light scattering may be mistaken for absorption changes. Such transients are strongly dependent on ionic fluxes across the thylakoid membrane, hence they are kinetically related to electrochemical events. Even worse, the apparent difference spectrum of scattering transients is not too different from the spectrum of electrochromic absorption changes (Thorne et al 166). Therefore, even a double-beam spectrophotometer will not amend the confusion between electrochromic absorption changes and scattering artifacts. Fortunately, the light-scattering events occur at a time scale above 1 sec. Therefore they do not interfere with experiments at shorter time spans, especially with experiments at flash excitation, where the electric potential relaxes with a decay time of the order of 100 msec.

However, work on "electrochromic" absorption changes (e.g. around 520 nm) at a longer time range or involving large ion fluxes (as in salt jump experiments) does not necessarily bear on the electric potential across the thylakoid membrane. This holds for the following references (7, 85, 87, 162, 176). Thorne et al (166) have demonstrated techniques to identify scattering transients superimposed on absorption changes in chloroplasts.

Absorption changes observed in algae in the spectral region between 470 and 530 nm are not necessarily indicative of the electric potential across the photosynthetic membrane either. Unlike in chloroplasts there is no kinetic "labeling" of electrochromic absorption changes in algae, e.g. by ionophores. In fact, there are striking

differences between the observed difference spectra in algae [Witt, Müller & Rumberg (177), Joliot & Delosme (66)] and the kinetically "labeled" electrochromic one from spinach chloroplasts [Emrich, Junge & Witt (31)].

Two other types of absorption changes are superimposed to the electrochromic ones around 520 nm. One of these rises in less than 20 nsec and decays in some microseconds. It was attributed to the formation of carotenoid triplet, a reaction which protects the antennae chlorophylls from photooxidation (95, 96, 182, 184). The other component is spectrally very broad (36) and kinetically slow. It makes up for about 10% of the flash-induced electrochromic absorption changes at 520 nm (see 141, Figure 4). Its origin is not characterized.

Inhomogeneity of the sample with respect to its electric properties is another factor limiting the kinetic resolution of the electrochromic method. The electrochromic absorption changes result from an ensemble of more than 10^{10} vesicles in an optical absorption cell. A biphasic decay of these absorption changes after a flash may then be interpreted in two ways: either it reflects the inhomogeneity of the sample, some vesicles having higher others lower electric conductivity, or it reflects an intrinsic property of each vesicle in a homogeneous sample. Schmid & Junge (143) in studies on the action of ionophores on the thylakoid membrane could cure this ambiguity in the interpretation of kinetic data. Inhomogeneity of the chloroplast suspension might have caused the reported kinetically distinct phases of the absorption changes around 520 nm [e.g. the *I* and the *S* phases in (56) and (54)].

Electrostatic Induction

Fowler & Kok (37, 39) introduced a new technique for the observation of vectorial charge displacement in thylakoid membranes by macroscopic electrodes. The method is based on the fact that water at physiological ionic strength is a badly conducting dielectric. For events which are more rapid than the ionic relaxation it can be idealized as a nonconducting dielectric. The principle of the electrostatic induction technique is illustrated in Figure 6. One thylakoid is symbolized by a spherical vesicle. When excited with light from above, the upper part of the membrane shields the lower one by about 1%. Hence excitation with a flash of nonsaturating energy produces more charge displacement by reaction centers in the upper part of the vesicle than by those in the lower one. The dipoles created in the upper membrane are not fully compensated by the ones in the lower one. By electrostatic induction this causes a voltage between two macroscopic electrodes which are placed at different depth in the chloroplast suspension. The electric asymmetry will be finally compensated by ionic currents around each thylakoid. Fowler & Kok (37, 39) reported a relaxation of the voltage within some 10 μsec, but the relaxation time could be increased by orders of magnitude if the viscosity of the suspending medium was increased [e.g. by adding sucrose, see Witt & Zickler (180)]. Correlation of the voltage as measured by electrostatic induction with the electrochromic absorption changes at 520 nm under variation of the exciting energy yielded a linear correlation [Witt & Zickler (181)].

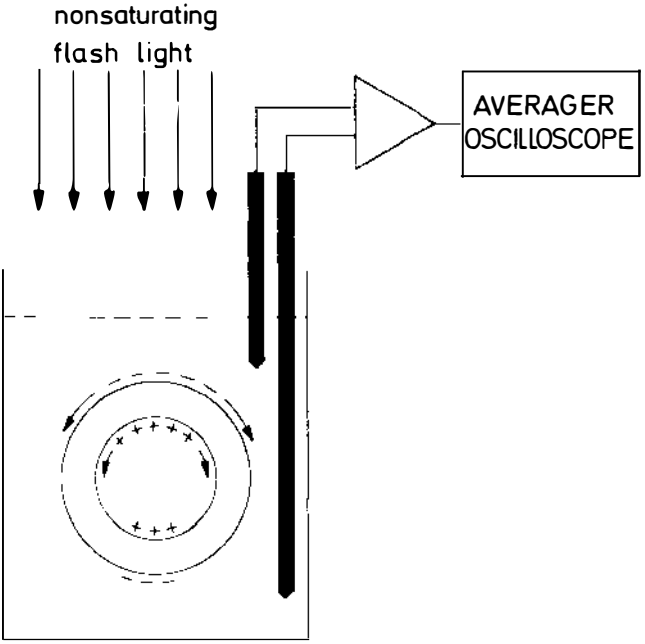

Figure 6 The principle of the electrostatic induction method (see text).

Delayed Light Emission

In addition to prompt fluorescence, delayed light emission from Photosystem (PS) II was observed with decay times up to seconds [for a review, see Lavorel (88)]. Delayed light emission in the time range of some milliseconds was attributed to the reversal of the photochemical charge separation in PS II. For the recombination of an electron-hole pair to yield the excited singlet state of antennae chlorophyll *a* the energy deficiency of the pair with respect to the excited state has to be compensated. The rate of delayed light emission is then proportional to the Boltzmann factor of this energy gap,

$$L \approx \exp(-E_a/RT), \qquad 6.$$

where E_a is the activation energy for the recombination (per mole).

Mayne (98) was the first to relate delayed fluorescence to the "high energy state" of the thylakoid membrane. Later Barber & Kraan (10) reported on the influence of an artificially induced diffusion potential on the intensity of delayed light emission. This was interpreted by Wraight & Crofts (187) as follows: The activation energy for delayed light emission is the difference between the energy of the excited singlet of chlorophyll *a* (E^*) and the span of the redox potential between the

primary electron donor and the primary acceptor (E_D–E'_A). If the donor and the acceptor are in contact with different aqueous phases, i.e. if the primary charge separation is directed across the membrane, then the electric potential difference across the membrane enters into the activation energy. A similar argument introduces the pH difference if the redox reactions involve shifts of acid-base equilibria. Hence the activation energy E_a becomes

$$E_a = E^* - (E'_D - E'_A + F\Delta\varphi - 2.3RT\Delta\text{pH}). \quad 7.$$

Merging equations 6 and 7, Wraight & Crofts (187) could quantitatively describe the then available data on the dependence of the delayed light emission on the electrochemical potential of the proton across the thylakoid membrane. Barber (9) used this relationship to calibrate the electric potential difference across the thylakoid membrane under continuous illumination. However, it is questionable whether the figure of about 100 mV that he obtained represents the "bulk phase potential difference" or the difference between the surface potentials at both sides of the membrane (see Digression on Electrochemistry).

Microelectrodes

Because of the submicroscopic internal phase of thylakoids one would not expect that microelectrodes could prove useful for studying electric phenomena at the thylakoid membrane. However, two groups have detected light-induced voltage transients by inserting microelectrodes (tip radii 1000–4000 Å) into grana stacks of chloroplasts from *Peperonia metallica* in situ [Bulychev et at (21), Vredenberg (171), Vredenberg & Tonk (172)]. Vredenberg (171) claimed that the recorded voltage transients reflect the electric potential difference between the internal phase and the stroma phase of thylakoids. The observed time course of these transients seems to support this hypothesis. However, quantitative conclusions drawn from such observations (172) are not too convincing as long as there is no independent control for impalement damage.

Measuring Protons

On illumination of broken chloroplasts Jagendorf & Hind (64) observed an alkalization of the external phase by means of a glass electrode. The major disadvantages of using glass electrodes in studies on proton transport is their slow response [for a fast electrode, see Schwartz (152)] and their limitation to events in the external phase.

pH transients in the internal phase of thylakoids were resolved by indirect techniques. Hager (51) attributed activity changes of the internally sequestered violaxanthin de-epoxidase to the influence of the light-induced acidification of the internal phase. Rumberg & Siggel (135) assumed that the slowing down of the reduction of chlorophyll a_I in the initial phase after the onset of illumination is due to the internal acidification. They calibrated this effect for a quantitative measurement of the internal pH value. Several authors used the redistribution of weak acids between the two aqueous phases in response to the light-induced pH difference across the thylakoid membrane. The distribution of amines was determined either by cation-sensi-

tive electrodes [Crofts (25), McCarty (99)], by radioactive assay [Rottenberg, Grunwald & Avron (125,126)], or by fluorescence changes of the internally located amine [Shuldiner, Rottenberg & Avron (156)]. Whether the distribution methods are quantitative for the evaluation of the internal pH is subject to discussion. Amines, which were used as pH indicators, were shown to act as proton carriers across the thylakoid membrane. This implies that they alter the pH difference they ought to indicate [Portis & McCarty (117)]. Moreover, some amines seem to bind specifically to the internal side of the thylakoid membrane. This makes it difficult to obtain a rigorous understanding of the relation between their distribution and the pH value in the internal phase [Fiolet, Bakker & VanDam (32)].

Despite these complications all the above techniques for the determination of the internal pH yielded an acidification of the internal phase by about three pH units when starting from a pH of eight units in the dark. The time resolution of distribution methods is slow. To resolve rapid pH transients following flash excitation of chloroplasts, pH-indicating dyes were used in rapid spectrophotometry. From the first application of such dyes to biological membranes [Chance & Mela (22)] there was discussion of possible artifacts attributable to binding changes, solvatochromic effects, and even redox reactions of these dyes, making ambiguous the interpretation of absorption changes attributed to pH changes (24, 32, 33, 107).

Controls to eliminate the influence of such artifacts on the results obtained with pH-indicating dyes were given by Junge & Ausländer (72). Another problem, the difficulty in discriminating whether observed absorption changes of a pH-indicating dye result from pH transients in the external or in the internal phase of thylakoids, was overcome by using appropriate permeating and nonpermeating buffers [Ausländer & Junge (4)]. pH-indicating dyes, together with rapid flash spectrophotometry, proved useful for resolving the rapid pH changes following flash excitation of chloroplasts. If, as under flashing light, the pH changes are small (e.g. 0.1 units), the absorption changes of pH-indicating dyes are linearly related to pH changes. However, if larger pH changes are induced as under longer illumination, problems similar to those of the amine distribution methods complicate a quantitative evaluation. Pick & Avron (114) studied this complication for the pH_{in} indicator neutral red introduced by Ausländer & Junge (4).

GENERATION OF AN ELECTROCHEMICAL POTENTIAL DIFFERENCE OF THE PROTON

The Electric Generator

Our understanding of the mechanism of electric potential generation in thylakoids has arisen mainly from studies on the electrochromic absorption changes (see Methods).

ELECTROGENIC REACTION STEPS Two electrogenic reaction steps were identified. It was shown that both photosystems contribute about equally to the electric potential difference after excitation of chloroplasts with a short flash of light [Schliephake, Junge & Witt (141)]. Three lines of evidence suggested that the primary

photochemical charge separation crosses the thylakoid membrane: 1. at room temperature the electric potential rises within less than 20 nsec (unresolved) after excitation of chloroplasts with a short flash of light [Wolff et al (183]; 2. the electric potential is generated even at rather low temperatures [Mathis & Vermeglio (97), Amesz & DeGrooth (1)]; 3. the reverse reaction of the primary photochemical act, which produces the emission of delayed light, is sensitive to an artificially induced diffusion potential across the thylakoid membrane [Barber & Kraan (10), Wraight & Crofts (187)].

The polarity of the electric potential is positive inside, as concluded from studies on the redistribution of permeant ions between the two aqueous phases (see below). This implies that the respective primary electron donors of both photosystems, chlorophyll a_I and a_{II}, are located at the inner side of the thylakoid membrane while the primary electron acceptors are at the outer one. From kinetic correlation of the electron transport reactions with the time course of the electric potential it was inferred that the donor and the acceptor in PS I, at least, are not embedded within the dielectric core of the membrane, but are located close to its surface [Junge (70)]. There is no evidence for a third electrogenic step in *isolated chloroplasts.* However, a slowly rising component of absorption changes at 520 nm observed in *algae* was interpreted to indicate that there might exist a thermally activated third electrogenic site [Joliot, Delosme & Joliot (67)]. As the argument was entirely based on the absorption changes around 520 nm, which in algae are not to be identified as electrochromic by their kinetic behavior, this interpretation is still questionable.

DELOCALIZATION OF THE ELECTRIC FIELD The very rapid charge separation in both photosystems generates localized dipole fields within the membrane, which, however, will delocalize over the thylakoid membrane by ion redistribution in both aqueous phases. Clear evidence for this delocalization of the electric field came from the following observation: the decay of the electric potential after flashing light is accelerated down to zero level if only one molecule of the pore-forming antibiotic gramicidin D is given to every 10^5 chlorophyll molecules in a chloroplast suspension [Junge & Witt (75)]. Hence, at the millisecond time scale the electric field is a collective property of a functional unit at least as big as one thylakoid disk. The time scale of the field delocalization by ionic redistribution can be inferred from experiments by the electrostatic induction technique (see Methods). These studies revealed that any imbalance of the electric field around one whole thylakoid relaxes in about 10 μsec (39, 180).

The major open question as to the electric potential generation is directed at the unknown structure that conducts electrons, which are photochemically "ejected" from chlorophyll *a* across the thylakoid membrane into the primary acceptor. It has been speculated that the protein which probably holds the photochemical reaction center including its electron acceptor might act as an injection semiconductor [Tributsch (168)]. On the other hand, model studies with bimolecular lipid membranes doped with chlorophyll and carotenoids indicate that carotenoids might act as conductors across lipid membranes for electrons which are photochemically released from chlorophyll *a* [Mangel, Berns & Ilani (94)].

The generation of the electric potential difference by the two photochemical reaction centers is illustrated in Figure 7.

The Proton Pump

Under continuous illumination of broken chloroplasts, Jangendorf & Hind (64) observed the uptake of protons from the external phase. By several indirect techniques (for references, see Methods) it was demonstrated that the protons disappearing outside are transferred into the interior of the thylakoid. The sites of proton binding from the outer phase and of proton release into the inner phase were identified by flash spectrophotometric studies with pH-indicating dyes. Schliephake, Junge & Witt (141) observed two sites of proton uptake at the outer side of the membrane, each associated with one of the two photosystems. These sites were attributed to the reduction of plastoquinone by PS II and to the reduction of the terminal electron acceptor by PS I [Junge & Ausländer (72)]. Under excitation, with repetitive short flashes turning over each photosystem only once, the proton/electron stoichiometry of these sites is $1H^+/e^-$ for the site at PS II (2, 72, 141) and variable depending on the nature of the terminal electron acceptor for the site attributable to PS I. The latter is $1H^+/e^-$ if O_2 via benzyl viologen is used as terminal acceptor, it is $0.0H^+/e^-$ for ferricyanide which does not bind a proton on reduction at physiological pH, and it is $0.5H^+/e^-$ for the natural acceptor $NADP^+$ (72).

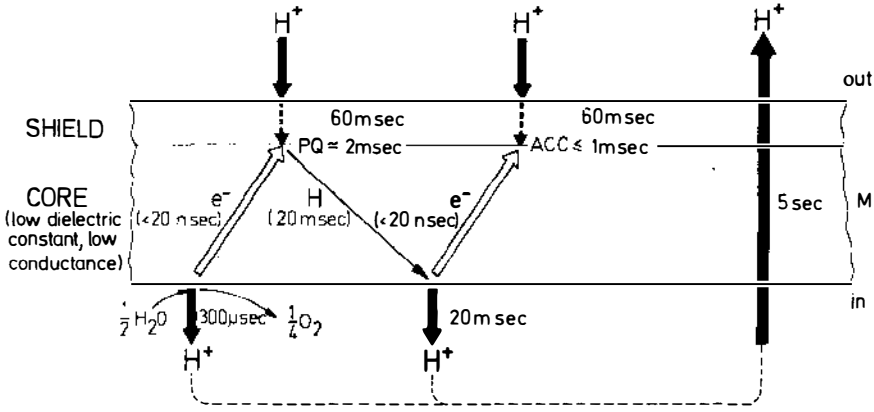

Figure 7 The proton pump in the thylakoid membrane. Open arrows symbolize rapid electron transfer across the membrane in both photochemical reaction centers. This produces the electric potential difference. Some redox reactions are followed by proton uptake from the external and proton release into the internal phase (heavy inward arrows). A removable diffusion barrier for protons shields the proton uptake sites at the outer side of the dielectric core of the membrane from the outer aqueous phase. The time constants for the electron transport across the membrane and the reactions with protons are indicated. The proton permeability of the dielectric core of the membrane (in the absence of ATP synthesis) is low as evident from the time constant of 5 sec for the relaxation of a pH gradient across it.

These stoichiometries, however, were recently challenged by Fowler & Kok (40), who claimed a stoichiometry of $2H^+/e^-$ for each of these sites (methyl viologen as terminal acceptor). These authors, however, did not determine the absolute number of protons taken up and the absolute number of electrons turned over in the same experiment, in contrast to the above cited authors who used pH-indicating dyes. There is an indication that Fowler & Kok (40) might have overestimated the proton/electron stoichiometry by their glass electrode technique. Two sites of proton release into the internal phase were identified with neutral red, a dye which indicates pH changes in the internal phase if used together with a nonpermeating buffer [Ausländer & Junge (4)]. The proton/electron stoichiometry is equal for both sites (4, 46). It was determined by means of nonpermeating pH-indicating dyes after reappearance of the internally liberated protons in the external phase to be $1H^+/e^-$ for each site [Junge & Ausländer (72)]. The two sites of proton release into the internal phase were attributed to the oxidation of water (2, 38, 72) and to the oxidation of plastohydroquinone (2, 72). The time constants for proton release at these two sites matched those for the respective redox reactions. They were resolved by the pH_{in} indicating dye neutral red: $\tau_{1/2} \simeq 300$ μsec for the water proton and $\tau_{1/2} \cong 20$ msec for the plastoquinone proton, respectively [Ausländer & Junge (4)].

In contrast to the kinetic fit between the proton release into the internal phase and the redox reactions causing it, proton uptake from the external phase is considerably delayed against the reduction of plastoquinone and the terminal acceptor, respectively (60 msec against about 1 msec). Grünhagen & Witt (49) speculated that this might be due to the mutual fixation of the internally liberated protons and the proton hole at the outer side of the membrane by Coulomb interaction. They published circumstantial evidence for a coupling of the external alkalization to the decay of the electric potential across the membrane. This was disproved when Ausländer & Junge (3) showed that the delay could be eliminated if a "permeability barrier" for protons was removed from the outer side of the membrane by mechanical (e.g. sand grinding) or chemical (detergents) mistreatment of chloroplasts or by addition of proton-carrying agents. At the extremes, proton uptake from the outer phase could be accelerated to match almost perfectly the velocity of the respective redox reactions (3). The chemical nature of this permeability barrier for protons which shields the reducing sites of both photosystems against the external aqueous phase is still unknown. As the proton taken up by plastoquinone is released into the internal phase after 20 msec, but the proton hole caused by the reduction of plastoquinone refills after 60 msec only, it has been postulated that there exists a proton reservoir beneath the shielding structure which protonates the reduced acceptor first and is then refilled from the external phase (3). The relevance for photophosphorylation of the proton deficiency pool generated between the core of the membrane and the shielding layer is unknown.

The above results on the stoichiometry and kinetics of the proton pump were obtained under flashing light. The question arises whether they can be confirmed under continuous illumination. Studies on the proton/electron stoichiometry under continuous light yielded nonuniform results. Some authors reported a stoichiometry

of $2H^+/e^-$ (61, 130, 134, 152, 153, 164) in agreement with reports cited above. Other authors reported lower (44, 58) and higher stoichiometries (28, 78, 91). Schröder, Muhle & Rumberg (150) pointed out that stoichiometries lower than 2, if recorded with a glass electrode, might be due to the fact that this slow device necessarily ignores the first rapid transient of proton efflux after cessation of continuous illumination. They demonstrated that apparent stoichiometries of e.g. $1H^+/e^-$ are measurable if the membrane permeability for protons is higher than the permeability for any other ion. Jagendorf (63) criticized some authors who obtained higher stoichiometries for comparing the (higher) initial rate of proton uptake with the steady-state rate of the electron transport. Although the controversy over proton/electron stoichiometry under continuous light is not fully settled, it seems most probable that it is $2H^+/e^-$, as under flashing light.

This strongly supports the scheme illustrated in Figure 7. The proton pump is directly linked to the electron transport chain which zigzags across the membrane as originally proposed by Mitchell (103, 104). This scheme is supported by biochemical studies on the sidedness of the electron transport chain as well [for review, see Trebst (167)].

Besides the above ambiguities in the proton/electron stoichiometry there are two other open questions which deserve further study. Experiments on the quantum yield for proton uptake under illumination of chloroplasts with red light yielded figures higher than $5H^+/h\nu$ [Dilley & Vernon (29), Heath (53)] which is incompatible with the above H^+/e^- stoichiometries in the light of the generally accepted quantum yield of $1e^-/h\nu$ for each photosystem. The apparent discrepancy is unsettled. In analogy to his recent concepts for the proton pump in mitochondria Mitchell (106) postulated the existence of another electron-hydrogen loop operating between the two photosystems. This loop should involve *b*-type cytochromes. The above experiments on isolated chloroplasts were not specifically aimed at this possibility, as the *b*-type cytochromes were inefficient electron carriers under their conditions. Specific experiments are necessary to evaluate this postulate.

Passive Ion Permeability of the Thylakoid Membrane

The active inwardly directed translocation of protons is accompanied by the outflux of cations and the influx of anions. Several authors have measured the extrusion of potassium (29, 112, 150), the extrusion of magnesium (29, 57) and the uptake of chloride (26, 150). They agreed that these fluxes practically compensate the proton uptake for electroneutrality under continuous illumination. Estimates of the permeability coefficients of these ions were based on the influence of a diffusion potential on the intensity of delayed light emission (see Methods). A poor selectivity was obtained [Barber (9)]. It seems that the nature of the dominating counterion in isolated chloroplasts is determined by the ionic composition of the suspending medium rather than by an intrinsic preference of the thylakoid membrane for any ion. There is circumstantial evidence, however, that magnesium is the dominating cation in intact chloroplasts (11, 81, 82).

Agents which change the ionic conductivity of artificial bimolecular lipid membranes act on the thylakoid membrane as well. Some of the observed peculiarities

are perhaps worth mentioning. The pore-forming antibiotic gramicidin D already accelerates the decay of the electric potential difference at relative concentrations as low as one molecule per 10^5 chlorophyll molecules [Junge & Witt (75)]. At relative concentrations of $1 : 10^3$ it starts to act as an uncoupler, increasing the proton permeability of the membrane, while at a proportion of 1 : 10 it starts to interrupt electron transport chains in the same way as detergents [Junge (68)]. Alamethicin acts as a voltage-dependent pore-forming agent in a manner very similar to its action in model membranes [Zickler & Witt (188)]. The potassium carriers valinomycin and nonactin act with similar turnover times as in model membranes; however, only one molecule out of 100, which are reversibly bound to the thylakoid membrane, is active in ion transport [Schmid & Junge (143)]. This was attributed to the relatively high protein and pigment contents of the membrane, which probably leaves only small fractions of the membrane open for the action of mobile carriers. Valinomycin at higher concentrations seems to increase the proton permeability and finally interrupts electron transport chains [Telfer & Barber (163)]. Some proton carriers like carbonyl-cyanide-*m*-chlorophenylhydrazone, in addition to this specific action, interfere with the water-oxidizing system and enhance the relaxation between its different oxidation states [Renger (123)].

Electrochemical Potential of the Proton under Continuous Illumination

Illumination of chloroplasts with a square pulse of light causes the rise and decay of the electrochemical potential of the proton which is qualitatively illustrated in Figure 8.

After the onset of illumination the electric potential difference rises up to a relatively high level. Judged for the electrochromic absorption changes at 520 nm it reaches between two and four times higher values than under excitation with a single short flash. Under the influence of this electric potential difference, cations move outwardly and anions inwardly. While the internal phase becomes more and more acidic, protons take over an increasing proportion of the electric current in response to the electric field. In the steady state the proton efflux fully compensates the active proton pumping directed inwardly. The internal acidification feeds negatively back on the velocity of the electron transport (see below). This, together with the increasing proton conductivity (because of higher proton concentration), leads to a decrease of the electric potential towards the steady state.

The steady level of the electric potential is controversial. Studies on the steady-state extent of the "electrochromic" absorption changes in *Chlorella vulgaris* seemed to indicate that it is even larger than that induced by a single flash of light [Gräber & Witt (45)]. For isolated chloroplasts figures ranging between 75 and 105 mV were reported based on delayed light emission [Barber (9)]. Both these reports are contrasted by studies on the redistribution of ions in response to the electric potential difference in the steady state. Schröder, Muhle & Rumberg (150) concluded that there was a steady-state potential of 10 mV only. To account for this discrepancy Rumberg (128) pointed out that membrane-bound probes for the electric potential (as electrochromism and delayed light emission) are sensitive to the difference between the surface potentials on both sides of the membrane, while

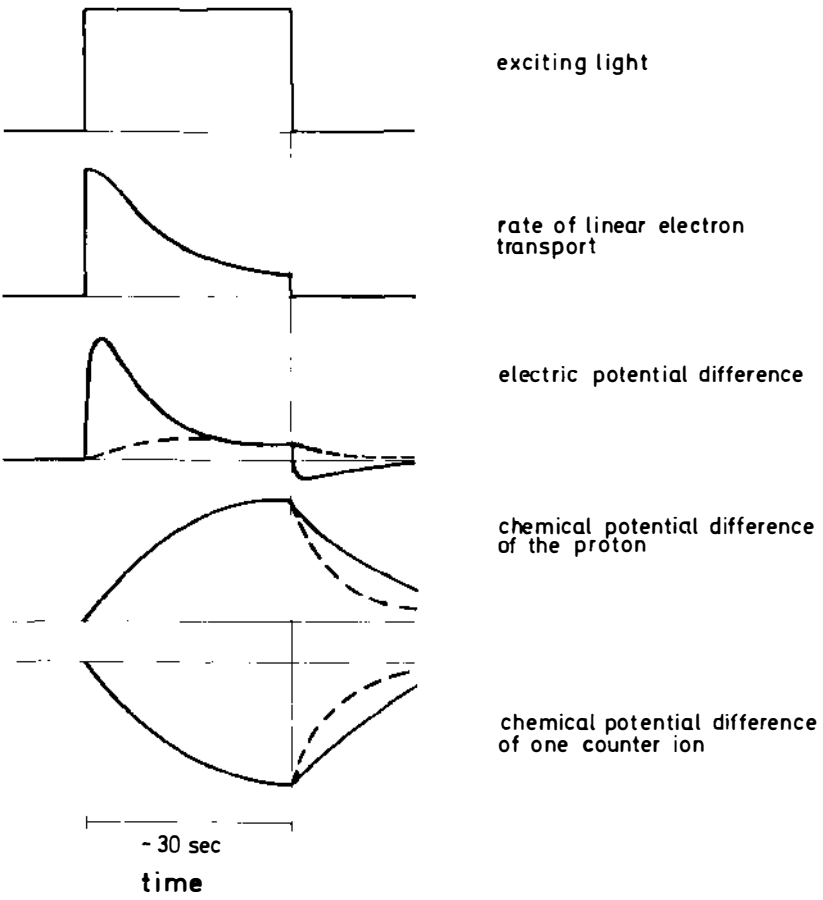

Figure 8 Qualitative drawing of the time course of the electrochemical events in chloroplasts after excitation with a square pulse of light (see text). Broken lines illustrate the events if the permeability of the membrane for one counterion dominates the one for the proton.

distribution methods evaluate the average potential difference between the bulk phases (see Digression on Electrochemistry).

If the external phase of chloroplasts is kept at a pH of 8 the internal phase reaches pH 5 under continuous illumination. This is the uniform result produced by several indirect techniques to determine the internal pH (for references, see Methods). Let us take a reasonable estimate for the internal volume of thylakoids, e.g. 50 liter per mole chlorophyll [Reinwald (121), Bamberger, Rottenberg & Avron (8)]. At pH 5 there will be about 5×10^{-4} free protons per chlorophyll in the internal phase. It is known, however, that about 0.5 protons per chlorophyll have disappeared from

the external phase [Neumann & Jagendorf (108)]. This result implies that the internal phase buffers away 99.9% of the inwardly translocated protons.

The electric potential difference in the steady state is probably less than the one under excitation with a short flash of light. The latter corresponds to the displacement of two elementary charges per electron transport chain (about 700 chlorophylls) (141). This implies that about 99% of the inwardly translocated protons are electrically compensated by cations extruded and anions taken up. If the light is shut off after establishment of the steady state, the electric potential generator stops as well. The actively supported electric potential is substituted by a diffusion potential. The polarity of the latter depends on whether the permeability of the proton dominates over the permeabilities of the other ions. If it is larger, the polarity of the electric potential difference is inverted after the end of illumination. If it is smaller, the polarity of the diffusion potential is the same as that of the light-induced one. This is illustrated in Figure 8. Although it is probable that Figure 8 anticipates the events after cessation of illumination these events are not well documented up to the present time.

Feedback of the Electrochemical Potential on the Electron Transport

As evident from the vectorial electron transport scheme in Figure 7 there are two sites where the electron transport might be influenced by the electric potential difference across the thylakoid membrane (the two photochemical reactions) and four sites of interaction with protons from either aqueous phase. It is known that the rate of the electron transport decreases when the electrochemical potential difference increases.

Rumberg & Siggel (135) reported on the influence of the internal pH on the velocity of the reduction of oxidized chlorophyll a_I (P_{700}) by plastohydroquinone. The half-rise time under flash excitation varied from 16 msec at pH 8 to 90 msec at pH 5. Siggel (157) presented a mechanistic model for the influence of the pH value on this reaction. He postulated that two protons have to be taken over by some weak acid before plastohydroquinone plus plastoquinone reaches the semiquinone status, which reacts further to reduce chlorophyll a_I via plastocyanin. Bamberger, Rottenberg & Avron (8) gave evidence that the rate of the electron transport depends on the external pH as well. Studies by Haehnel (50) on the influence of the external pH on the rate-limiting step between plastoquinone and chlorophyll a_I, however, revealed that this influence is almost negligible under flashing light. However, the external pH may indirectly affect the membrane permeability for protons or regulate the activity of the water-oxidizing system and thereby affect the rate of the electron transport under continuous light. This question deserves further study. Proton carriers, by decreasing the steady-state level of the pH difference, accelerate the rate of the electron transport (uncouplers).

PHOTOPHOSPHORYLATION

Hypotheses

The discussion on the mechanism of photophosphorylation centered around one major subject, whether or not the electrochemical potential difference of the proton

across the thylakoid membrane is a necessary intermediate between the electron transport and the synthesis of ATP. While the emphasis was first on chemical intermediates [Slater (158, 159)] or conformational intermediates [Boyer (16), Boyer, Cross & Momsen (17), Boyer et al (18), Green et al (48), Slater (160)] it later became almost generally accepted that the electrochemical potential difference in fact links the electron transport with phosphorylation. This was first proposed by Mitchell (103, 104). His hypothesis has three salient features:

1. Redox reactions are vectorial in the inner membranes of chloroplasts and mitochondria. Alternating electron-hydrogen transfer across the membrane generates an electrochemical potential difference of the proton.
2. The coupling membrane has a low leak conductivity for protons (or hydroxyl anions) so that the electrochemical potential is dissipated only slowly.
3. The ATP synthase is located anisotropically in the coupling membrane. By translocating protons downhill along their electrochemical potential, the enzyme gains the free energy necessary for the synthesis of ATP from ADP and inorganic phosphate.

While these three essentials of Mitchell's hypothesis are well substantiated by experimental facts (see foregoing sections and subsequent section) further details (e.g. proton/ATP stoichiometry, proton/electron stoichiometry) are still controversial. The detailed mechanism of how the ATP synthase couples proton translocation to the formation of one phosphoester bond is unknown. Mitchell (105) discussed a mechanistic model that depends only on the right steric configuration of the reactants ADP and phosphate with respect to the electrochemical gradient of the proton across the membrane. The enzyme in this model had only to install this configuration without serving as an energy transducing unit by itself. An alternative concept proposed by Boyer and collaborators (17, 18) visualizes conformational states of the enzyme as energy transducers between protons and the nucleotides. The interaction of the protein with protons from one side is thought to alter the binding constants of nucleotides at another site. A whole wealth of experiments on electrochemically triggered conformational changes of the ATP synthase and on energy-dependent nucleotide binding (see next section) seems to suggest an energy-transducing role of the enzyme.

Energetic Role of the Electrochemical Potential Difference

Two extensive reviews on the role of the electrochemical potential of the proton for photophosphorylation have appeared recently [Jagendorf (63), Witt (175)]. Hence the following sums up the crucial experiments rather briefly.

Jagendorf & Uribe (65) showed that an artificially induced pH gradient across the thylakoid membrane can induce ATP synthesis without the need for a functional electron transport chain (101). Uribe (169) showed that an artificially induced diffusion potential across the thylakoid membrane without a concomitant pH gradient produces ATP as well. Witt, Schlodder & Gräber (179) even more drastically demonstrated electrically driven ATP synthesis by exposing chloroplasts to an electric field pulse in aqueous suspension. That the electric component of the electro-

chemical potential and the chemical component can compensate for each other was suggested by experiments in which a diffusion potential was superimposed onto a pH difference, which by itself was insufficient to drive ATP synthesis (155, 156, 170).

These qualitative results on the conversion of electrochemical energy into the free energy of the ATP/ADP couple are backed by kinetic studies. Rumberg & Siggel (135) observed the lowering of the steady-state level of the pH difference under phosphorylating conditions, a result later confirmed by Pick, Rottenberg & Avron (115). An acceleration of the decay of the electrochromic absorption changes was reported by Rumberg & Siggel (134) and Junge, Rumberg & Schröder (73). Figure 9 illustrates this acceleration, which was observed after excitation of freshly isolated chloroplasts with a group of short flashes. This was interpreted to indicate an extra flux of charges across the membrane under phosphorylating conditions (73). That the additional electric conductivity of the thylakoid membrane under phosphorylating conditions is necessary for, and not just a side effect of, phosphorylation, as suggested by Girault & Galmiche (42), was confirmed by the following observations:

1. The acceleration is sensitive to the specific antibody against the coupling factor of photophosphorylation [Schmid, Shavit & Junge (144)].
2. If an additional electric conductivity is induced, say for potassium ions (by valinomycin) which is as effective as the channel linked to the coupling factor, the ATP yield is halved [Junge, Rumberg & Schröder (73)].
3. An electrically conducting channel which competes with the coupling factor for electric energy can be induced by as little as one molecule of gramicidin D on 10^5 chlorophyll molecules which excludes an unspecific action of the antibiotic on the coupling factor [Boeck & Witt (14)].

These experiments have demonstrated very clearly that the ATP synthase uses electrochemical energy and that it translocates protons across the thylakoid membrane to gain the necessary free energy.

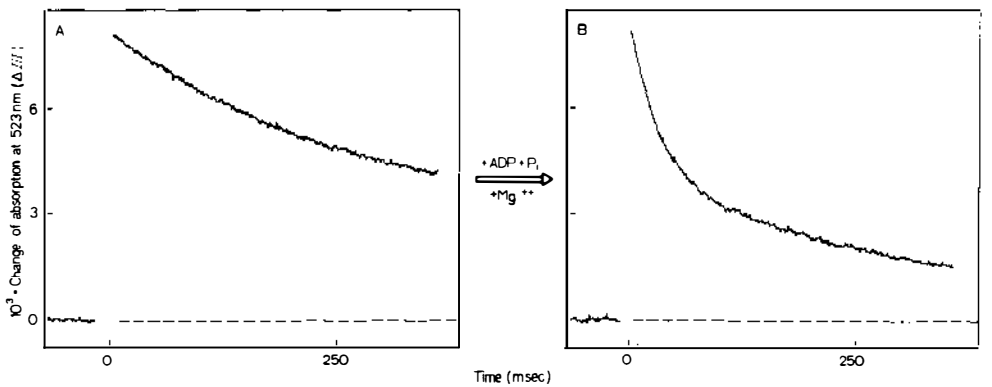

Figure 9 The decay of the electric potential difference (measured via the electrochromic absorption changes around 520 nm) in the absence (*left*) and in the presence (*right*) of substrates for photophosphorylation (73).

There is evidence that this enzyme in fact has access to protons from both aqueous phases, as required for the above type of action. The major part of the coupling factor for photophosphorylation (CF1) according to electron microscopic studies (12, 102, 111) protrudes into the external phase of thylakoids. That it is available for protons from the internal phase is suggested by the following experimental results: McCarty (99) observed that thylakoids lose the ability to take up protons if the coupling factor is extracted. They accumulate protons again if the coupling factor is reincorporated. By flash spectrophotometry it was demonstrated that the electric conductivity of the thylakoid membrane and the proton conductivity are increased by more than two orders of magnitude after extraction of the coupling factor (43, 142, 144). This suggests that the enzyme is connected to the internal phase by a proton well, which might be formed by its binding protein within the membrane.

The stoichiometry of protons translocated over ATP molecules formed is still a matter of controversy. The published figures ranged from two (60, 134) to four (151). Recent work carried out with different techniques seems to focus on a stoichiometry of $3H^+/ATP$[Junge, Rumberg & Schröder (73), Schröder, Muhle & Rumberg (150), Portis & McCarty (118), Gräber & Witt (47), Rumberg, Schröder & Schnecke (133)]. This deviates from two as postulated by Mitchell (104). It is still in apparent discrepancy to the highest ATP/e^- yield so far reported [Horton & Hall (59)]. This ATP/e^- ratio of 1 under the assumption of a H^+/e^- stoichiometry of 2 for the linear electron transport implies a H^+/ATP ratio of 2 only. However, there is only circumstantial evidence for the absence of cyclic electron transport in the experiment by Horton & Hall (59) and ratios as high have not been confirmed by other laboratories so far.

Taking a H^+/ATP stoichiometry of 3 as the most probable one, it may be asked whether the electrochemical potential difference under continuous illumination can account for the observed ATP/ADP ratio under these conditions. According to Mitchell's hypothesis (66) the following relation will hold,

$$\nu(F\Delta\varphi - 2.3\ RT\ \Delta pH) \geq \Delta G'_0 + RT \ln [(ATP)/(ADP)\cdot(P)], \qquad 8.$$

where G'_0 is the standard free energy for the formation of ATP under consideration of the magnesium concentration and the pH in the medium. Taking into account the figures for G'_0 as determined by Rosing & Slater (124), the ATP/ADP ratio sustained by chloroplasts under continuous illumination as observed by Kraayenhof (80) implies a figure of +13.5 kcal/M for the right side of equation 8. Assuming a pH difference of three units between the two aqueous phases without any additional electric potential difference, a stoichiometry of $\nu = 3H^+/ATP$ implies a figure of +12.4 kcal/M for the free energy to be gained by the translocation of three protons across the membrane. This shows that a stoichiometry of 3 was just sufficient to account for the ATP/ADP ratio observed in chloroplasts under continuous illumination.

Mechanistic Details of Photophosphorylation

While the energetic question of photophosphorylation seems to be solved in favor of Mitchell's chemiosmotic concept, the detailed mechanism of the ATP synthase

has remained open. The phenomena described below might once prove useful for our understanding of this mechanism.

LAG AND THRESHOLD PHENOMENA The rate of ATP synthesis depends non-linearly on the electrochemical potential across the thylakoid membrane. Indirect evidence for this came from the observation of an intensity lag for photophosphorylation (13, 84, 139, 154) as well as a time lag (76, 140). Later studies suggested that a certain level of the electrochemical potential has to be exceeded before ATP synthesis becomes efficient. With flashing light of low repetition frequency where the electric potential difference energetically exceeds the pH difference, Junge, Rumberg & Schröder (73) observed that ATP synthesis required a certain critical electric potential difference. This critical level is obvious from Figure 10 (*right*) where the acceleration of the electric potential decay stops at a certain level of this potential. The existence of such a critical potential was challenged by Witt and co-workers (14, 174, 175) until reconfirmed by Gräber & Witt (47). These authors found that the critical level for efficient phosphorylation did not depend only on the *electric* potential, but also on the *chemical* potential of the proton. A critical level of the electric potential for phosphorylation in algae under excitation with flashing light was reported by Joliot & Delosme (66). As pointed out by Junge (69a) this critical potential could not be interpreted by energetic arguments based on equilibrium

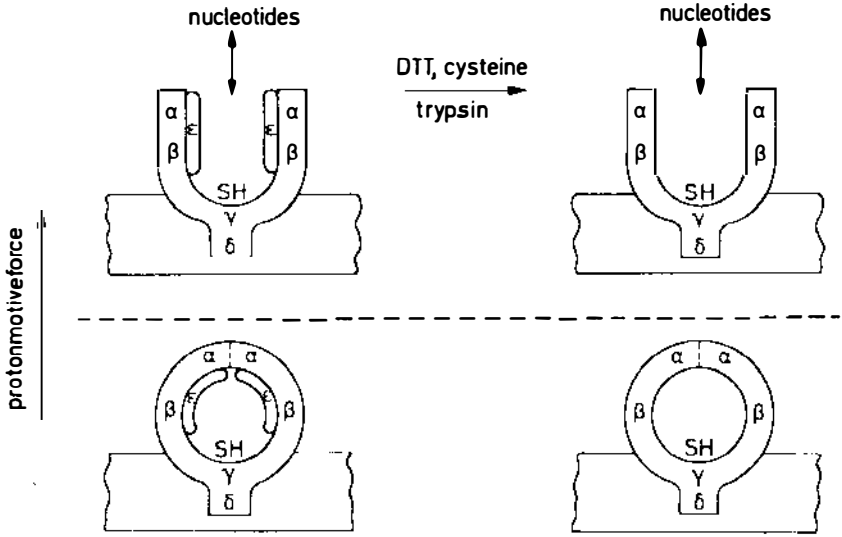

Figure 10 Conformational changes of the coupling factor for photophosphorylation (CF1) under the influence of the electrochemical potential difference of the proton across the thylakoid membrane; (*below*) no electrochemical potential difference, (*above*) with the electrochemical potential difference, (*left*) the unmodified coupling factor, (*right*) the coupling factor modified (removal of the inhibitory subunit by trypsin).

thermodynamics; it represents a kinetic phenomenon. The electrochemical potential may modulate the activity of the ATP synthase in either of two ways: either by allosterically switching the enzyme from an inactive into an active conformation or by the special current-voltage behavior of the proton channel through the enzyme, which is always active (69a). A quantitative model for an electrically triggerable enzyme [Junge (69b)] as well as a Monod-type cooperative proton carrier model [Schröder (149)] have been fitted to the kinetic data on the dependence of the phosphorylation rate on the electrochemical potential.

ELECTROCHEMICAL ACTIVATION OF ATPASE ACTIVITY For the ATP synthase to operate in reverse as an ATPase which hydrolyzes ATP it would be energetically favorable if there were no electrochemical potential (positive inside) across the thylakoid membrane. However, it was shown that the membrane-bound enzyme in class II chloroplasts requires two conditions for ATPase activity at higher rates: incubation with thiol reagents or trypsin plus electrochemical energization of the thylakoid membrane either by light [Petrack & Lipmann (113)] or by an artificially induced pH difference [Kaplan, Uribe & Jagendorf (77)]. Later it was inferred that thiol reagents or trypsin interfere with an inhibitory subunit (ϵ unit) of the coupling factor [Nelson, Nelson & Racker (110)]. Figure 10, which is adapted from the work of Bakker-Grunwald & VanDam (6) on ATP hydrolysis and of Nelson (109) on the coupling factor, summarizes the results on the electrochemical activation of ATP hydrolysis. The generation of an electrochemical potential of the proton across the membrane, once it exceeds a critical level, exposes the coupling factor to the attack by thiol agents and trypsin which modify (or remove) the inhibitor. If the inhibitor is removed the electrochemical potential alters the enzyme conformation so that it is right for the rapid hydrolysis of ATP. Once stimulated it sustains the electrochemical potential by pumping protons inwardly. The critical level of the pH difference for hydrolysis [Bakker-Grunwald (5)] is similar to that for ATP synthesis [Shuldiner, Rottenberg & Avron (156)] about three units at an external pH of 8.5 units.

It is unknown whether the activation of ATP hydrolysis depends on the extent of the electrochemical potential diffence, which includes the electric component, or on the internal pH only. In the above cited work (5) there is some indication for the latter. The matter deserves further studies.

ELECTROCHEMICAL ACTIVATION OF CONFORMATIONAL CHANGES Ryrie & Jagendorf (137) showed that the membrane-bound coupling factor for phosphorylation exchanges about 50 protons for tritium if the membrane is energized electrochemically. If the membrane was de-energized, the exchanged tritium ions were then sequestered in the enzyme and inaccessible to further exchange. Tritium incorporation was prevented in the presence of uncouplers which decrease the pH difference across the membrane under continuous illumination. Later a whole wealth of markers for energy-dependent conformational changes of the coupling factor were discovered. It was reported that energization exposes thiol groups on the γ-subunit of the coupling factor to the attack by N-methylmaleimide [McCarty

& Fagan (100)]. Irreversible effects of sulfate on the coupling factor [Ryrie & Jagendorf (138)] and the energy-dependent incorporation of nucleotides into it [Magnusson & McCarty (92)] were observed.

This leaves no doubt that electrochemical energization of the thylakoid membrane induces dramatic conformational changes of the coupling factor of photophosphorylation. However, it is unknown whether these conformational changes are triggered by the internal pH only, or whether they depend on the electrochemical potential difference of the proton. The latter would imply that they are "energy consuming." It is unknown whether the conformational changes detected by different markers reflect one and the same event. It is furthermore unknown whether they are on an alternative side path to phosphorylation, abusing electrochemical energy, whether they indicate a conformational high energy intermediate on the path to ATP, or whether they represent the triggering of the enzyme into an active conformation.

Appendix: Electrochromism

If a molecule is exposed to a strong electric field it may be reoriented and its absorption spectrum will change. Considering the ample evidence for a rather fixed orientation of chloroplast bulk pigments in the thylakoid membrane (19, 20, 41), the orienting effect may be neglected in the following. The term *electrochromism* was introduced by Platt (116) for the influence of an electric field on the absorption spectrum of a dye. The effects are theoretically defined [e.g. Liptay (89, 90)] and experimentally well established for numerous organic [e.g. Labhard (86), Schmidt & Reich (145)] and biologically relevant dyes [e.g. Malley, Feher & Mauzerall (93), Kleuser & Bücher (79), Schmidt, Reich & Witt (147)]. It has been found that the strongest influence acts on the center position of an absorption band, while the band shape and the absorption strength remain more or less unaffected (e.g. 145). The result is a homogeneous band shift. The first two terms in a power series expansion of the shift in the center frequency are

$$\Delta\nu = -h^{-1}(m_e - m_g)\cdot E - h^{-1}E:(\alpha_e - \alpha_g)\cdot E. \qquad 9.$$

The shift depends on the interaction of the excited state (subscript e) and the ground state (subscript g) with the electric field vector E. The interaction occurs via the permanent dipole moment m of the respective state and via the dielectric polarizability α. The vector m as well as the tensor α have definite orientations within the molecular coordinate system. Hence the magnitude of the frequency shift depends on the orientation of the dye molecule relative to the direction of the electric field. Owing to a higher polarizability of the excited state the second term always contributes a shift towards lower frequencies, i.e. longer wavelength.

For most dyes the order of magnitude of these shifts is small (for exceptions, see below) in comparison with the bandwidth. For example, if the difference between the dipole moments is 1 D (3.33×10^{-30} A sec m), which gives the order of

magnitude for many pigments, exposure to a field strength of 2×10^7 V/m^{-1} produces a shift of the center frequency of 10^{11} sec^{-1}. This corresponds to a wavelength shift of 0.08 nm around a center wavelength of 500 nm. In most cases the contribution from the polarizability, second order in the field strength, is even smaller. For lutein, an almost symmetrical carotenoid with 10 conjugated double bonds, Schmidt & Reich (145) determined a difference between the polarizabilities along the long axis of 1.01×10^{-39} A sec V^{-1}m^2 (or 910 Å^3 in el.stat.cgs). At the same field strength of 2×10^7 Vm^{-1} this produces a frequency shift of only 3×10^{10} sec^{-1}.

Molecules with a center of symmetry (the carotenoid lutein is a good approximation) have no permanent dipole moment either in their ground or in their excited state, hence the electrochromic band shift should depend by second order in the electric field strength (see equation 9). In chloroplasts, however, linear electrochromic effects of carotenoids were observed (see Methods). To account for this apparent discrepancy Schmidt, Reich & Witt (148) proposed the following: When embedded into a membrane a molecule is exposed to directed electric fields resulting from polar or dipolar groups of adjacent molecules. This polarizes even a symmetrical molecule permanently. If the biasing electric field is much stronger than the variable observed one, a second-order response of the dye may be transformed into a pseudolinear one. This is illustrated in Figure 11*B*.

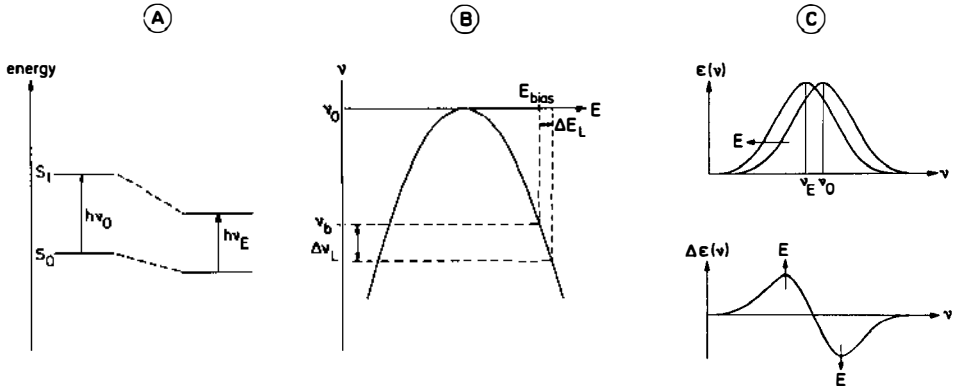

Figure 11 Schematic representation of the influence of high electric field strength on the absorption spectrum of a dye. *A.* Shifts of the energy of the excited (subscript *e*) and the ground state (*g*) of the dye and in consequence modification of the resonant frequency for light absorption (υ_0 changed into υ_E). *B.* Dependency of the resonant frequency on the electric field strength. It is assumed that second-order effects prevail. If the dye is exposed to a strong biasing field the second-order dependence may become pseudo–first order with respect to the smaller variable component (E_L). *C.* The electrochromic band shift (*above*) and its difference spectrum (*below*). The extent of the difference in extinction ($\Delta\epsilon$) is proportional to the electric field strength.

The small frequency shift of an absorption band causes changes in the molar decadic extinction coefficient ϵ of the dye. For an asymmetric molecule the first three terms in a power series expansion of these changes are (120)

$$\Delta\epsilon = h^{-1}(m_e - m_g)\cdot E\ \partial\epsilon/\partial\nu + (2h)^{-1}\ E:(\alpha_e - \alpha_g)\cdot E\ \partial\epsilon/\partial\nu + (2h^2)^{-1}[(m_e - m_g)\cdot E]^2\partial^2\epsilon/\partial\nu^2 \quad 10.$$

In a first approximation the difference spectrum plus-minus electric field is proportional to the first derivative of the absorption band. This is illustrated in Figure 11 *C*. An increase of the field strength causes an increase of the extent of the difference of the molar extinction, but no change of the spectral profile. Hence the negative and the positive lobes in the difference spectrum should not be misinterpreted as being due to a band shift from the center position of the negative lobe to that of the positive one. Misconception of this kind has caused some confusion in the interpretation of electrochromic absorption changes in chloroplasts (55).

Starting from the pioneering work by Platt (116) on electrochromism of merocyanine dyes an increasing number of reports have appeared on larger shifts of absorption bands in response to an electric field. Band shifts in the order of 10 nm, however, cannot be explained by the above mechanism. They are due to a field-induced shift of a chemical equilibrium, e.g. between two mesomeric states of a dye with different peaks of absorption.

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