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# Electron Transfer, Field Changes, Proton Translocation and Phosphorylation in Photosynthesis

## Coupling in the Thylakoid Membrane

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With 36 Figures

## 1. Poblems

Most of the contributions of this meeting are concerned with the consumption of $O_2$. The opposite way — the production of $O_2$ — is realized in photosynthesis through the cleavage of water.

On the mechanism of the cleavage of water which is one part of the very complex reaction system of photosynthesis only little is known. However, a number of informations are available on the primary processes of photosynthesis which "drive" the cleavage of water.

In these primary processes $NADP^+$ is reduced and ATP is generated through the energy of the visible light. In secondary processes $CO_2$ is reduced to sugar etc. through NADPH and ATP. This second path is well-known by Calvin [1].

In the following the primary processes will be discussed in more detail. Four characteristic events have been observed.

1. The reduction of $NADP^+$ is performed by an *electron transfer* from $H_2O$ to $NADP^+$. This leads to the cleavage of $H_2O$ with a simultaneous evolution of $O_2$ [2]. The electron transfer takes place through at least ten electron carriers [3].

2. The electron transfer is coupled in an unknown way with a *phosphorylation* which leads to the formation of ATP [4].

3. The electron transfer is accompanied by reversible *ion translocations* [5, 6]. Jagendorf et al. [5] observed reversible proton translocations during the electron transfer and Dilley et al. [6] reversible fluxes of other ions as potassium etc.

4. The electron transfer is furthermore coupled to changes of an *electrical field* [7]. Changes of an electrical field have been observed through special absorption changes [7].

Mitchell has postulated a special type of coupling between electron transfer and phosphorylation [8]. In this hypothesis the electron transport produces an electrical field and a pH-gradient across a membrane. The free energy, thus stored in a difference of the electrochemical potential of protons, should be used for the formation of ATP (Fig. 1).

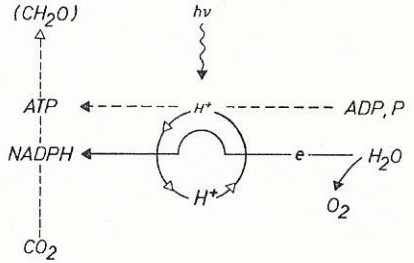


Fig. 1. Proposed relationship between electron transfer, $H^+$ translocation, phosphorylation and $CO_2$-reduction. Details § 1

Mainly one experiment supports one part of this concept. Jagendorf has shown that if an artificial pH-gradient is set up on chloroplasts, a formation of ATP can be observed in the dark [9]. On the other hand, in different hypotheses [10] it is assumed that phosphorylation is generated by an unidentified energy rich chemical intermediate which is produced by the electron flow. In this concept the observed movements of ions are regarded as a side path and the hypothesis of Mitchell has been rejected [10].

The arguments so far discussed in the literature with respect to the mechanism of phosphorylation are based on macroscopic experiments, that means on measurements of the products of the primary processes as NADPH, $O_2$, $H^+$ and ATP as function of different parameters. In the following the primary events in general and the phosphorylation in particular will be discussed on the basis of results which have been obtained on a molecular level. These

investigations have been combined with new macroscopic measurements on which will be reported additionally.

## 2. Methods

Informations on a molecular level have been obtained since intermediates which are in action during light excitation could directly be measured especially by transient absorption changes. In photosynthesis this kind of analysis requires the use of fast and sensitive techniques, since in photosynthesis only 0.1% of the pigments are photo-active and since the speed of formation of the intermediates and their disappearance are in the order of $10^{-1}$ sec to $10^{-8}$ sec.

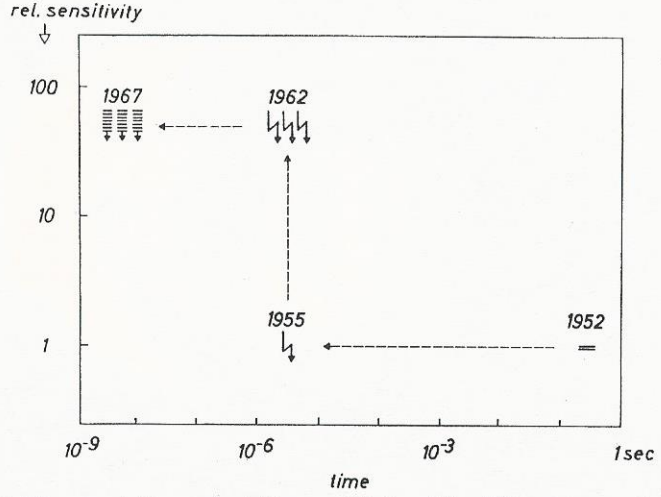


Fig. 2. Time resolution and relative sensitivity of the different types of spectroscopic pulse techniques [15, 18]. Details § 2

In *1952* sensitive methods for reactions in the range of seconds have been developed for photosynthesis by Duysens [11] (see Fig. 2).

In *1955* the flash techniques of Norrish and Porter [12] were refined for the field of biology and the time range in photosynthesis was extended down to $10^{-5}$ sec and the sensitivity increased up to 0.1% absorption changes [13].

In *1962* the repetitive pulse technique was introduced for the analysis of chemical and photochemical reactions [14]. By the repetitive technique the signal-noise ratio increases with the square root of the number of the events. In this way the sensitivity could be increased by a factor of about 100.



In *1967* this technique has been extended by using ultra short flashes and Q-switched giant laser pulses for excitation [15—17]. Equipments have been developed in which these ultra flashes and giant laser pulses can be run periodically. In combination with the repetitive technique it was possible to extend the time resolution down to the range of 10 nsec. The sensitivity of these techniques corresponds to 0.1% of absorption changes in the 10 nanosecond range as can be seen from Fig. 10. It is, of course, greater with longer times.

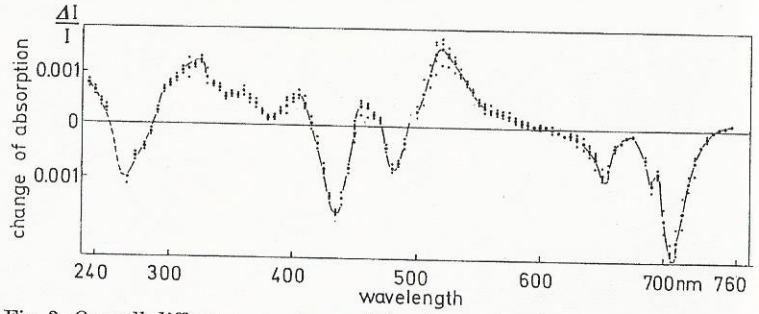


Fig. 3. Overall difference spectrum of the absorption changes in chloroplasts of spinach during photosynthesis [3]

With these pulse techniques intermediates of photochemical reactions in general and especially of photosynthesis can now be followed from the first beginning, that is from the excited *singlet* state condition in the time range of nanoseconds down to the end products of the primary processes. Details are given in [15, 18].

## 3. Results

With the pulse techniques we have identified *ten* different types of absorption changes between 200 nm and 800 nm in *green plants* under physiological conditions [3]. These types were isolated from the overall absorption changes which are shown in Fig. 3. The ten types of absorption changes which cause these overall changes are depicted in Fig. 4. From the characteristic maxima and minima it can be concluded which particular reactions are responsible for these changes. These reactions are depicted on the right of

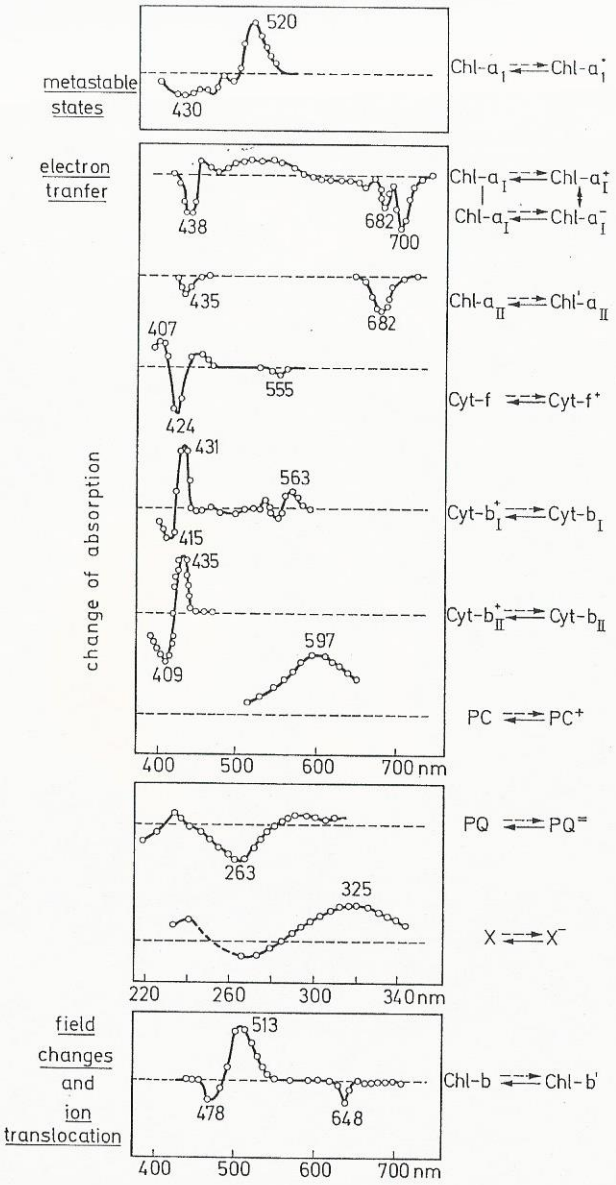


Fig. 4. (Legends see page 267)



Fig. 4. It can be distinguished between different sets of reactions which are depicted on the left of Fig. 4. One set is caused by the formation of *metastable states*, a second set by *electron transfers*, and a third one by changes of an *electrical field* and *ion translocations*.

A cytochrome-*f* reaction was first observed in red algae by Duysens [19]. He observed also unidentified changes in the range of seconds at 415, 475 and 520 nm [19]. The band around 700 nm in the second spectrum in Fig. 4 was discovered by Kok and attributed to the oxidation of a so-called pigment 700 [20, 33]. Changes of plastocyanine around 597 nm were reported by de Kouchkovski et al. [21].

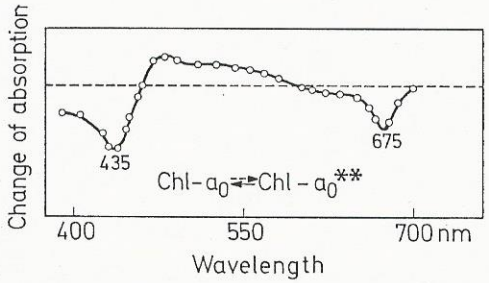


Fig. 5. Difference spectrum of the formation of the $\pi$-$\pi^*$-triplett state in the bulk of disaggregated chlorophyll-*a* in mutants of chlorella [22]

These *ten* types of absorption chanes are observable under physiological conditions. When, however, photosynthesis is stopped by disarrangement of the chlorophylls, all these changes disappear and a new *eleventh* type of changes occurs which is caused by the formation and deactivation of the $\pi$-$\pi^*$-triplet state of chlorophyll [22] (see Fig. 5). This indicates that under such conditions the excitation energy stays in the bulk of chlorophyll followed by physical deactivations without conversion into chemical energy.

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Fig. 4. Transient difference spectra during photosynthesis in green cells (chlorella or chloroplasts of spinach) analyzed by the pulse techniques. The spectra were isolated from the overall difference spectrum in Fig. 3. Chl-$a_I$ = chlorophyll-$a_I$ [45, 46], Chl-$a_I$ = chlorophyll-$a_I$ [44]. Chl-$a_{II}$ = chlorophyll-$a_{II}$ [34], Cyt-*f* = cytochrome-*f* [38], Cyt-$b_I$ = cytochrome-$b_I$ [26], Cyt-$b_{II}$ = cytochrome-$b_{II}$ [27], PC = plastocyanine [29], PQ = plastoquinone [25], substance X [25], Chl-*b* = chlorophyll-*b* [30]. The magnitude of the absorption changes is in the order of $^1/_{10}$%

The separation of the ten types of absorption changes from the overall spectrum has been achieved by different chemical and physical treatments of whole algae cells and chloroplasts under physiological conditions. Details are described in the literature of [3]. In principle, one can distinguish between the ten types of reactions already by one of their characteristic kinetics which are depicted in Table 1.

Table 1. *Some characteristic kinetics of the intermediates in photosynthesis at 20° C*

| Intermediates | | Range | Literature |
|---|---|---|---|
| | Relaxation Time | | |
| chlorophyll-$a_1$ | $3 \cdot 10^{-6}$ sec | visible | [**3**, 45, **46**] |
| chlorophyll-$a_I$ | $2 \cdot 10^{-2}$ sec[a] | visible | [**23**, 20, 33, 44] |
| chlorophyll-$a_{II}$ | $2 \cdot 10^{-4}$ sec | visible | [**24**, 34] |
| plastoquinone | $2 \cdot 10^{-2}$ sec | UV | [**25**, 36, 37] |
| substance X | $6 \cdot 10^{-4}$ sec | UV | [**25**, 3] |
| cytochrome-$b_I$ | reduced with $h\nu_I$ | visible | [**26**] |
| cytochrome-$b_{II}$ | reduced with $h\nu_{II}$ | visible | [**27**, 39] |
| | Rise Time | | |
| cytochrome-$f$ | $\sim 10^{-3}$ sec | visible | [**28**, 19, 38] |
| plastocyanine | $\sim 10^{-3}$ sec | visible | [**29**, 21] |
| chlorophyll-$b$ | $\leq 2 \cdot 10^{-8}$ sec | visible | [**3**, 30, 7, **17**] |

The literature especially on the kinetics is indicated by bold figures.
[a] When the donor Cyt-$f$ is in the oxidized state.

As can be seen from Table 1 the kinetics of the absorption changes in the visible region of chlorophyll-$a_1$, chlorophyll-$a_I$ and chlorophyll-$a_{II}$ and the changes in the ultra violet region of plastoquinone and X can clearly be distinguished by their very different relaxation times. Cytochrome-$b_I$ is reduced by excitation with $h\nu_I$-light, and cytochrome-$b_{II}$ is reduced by $h\nu_{II}$-light (on $h\nu_I$ and $h\nu_{II}$, see § 4). Cytochrome-$f$ and plastocyanine are characterized by finite rise times of $\sim 10^{-3}$ sec. The change of chlorophyll-$b$ has a rise time of $\leq 2 \cdot 10^{-8}$ sec and shows in any respect an outstanding kinetic [7] which is discussed below.

The analysis of these different types of absorption changes as a function of numerous parameters provides fairly detailed informations on the mechanism of photosynthesis.



In the following the results of the electron transport system are only briefly reported because details were presented in [3].

## 4. Electron transfer

In the following *only* those results on the electron transfer system are summarized which have been obtained by the described *spectroscopic* pulse techniques. On the *bottom* of Fig. 6 the characteristic absorption changes of the intermediates are depicted. In the *center* of Fig. 6 it can be seen from the direction of the *heavy arrows* that

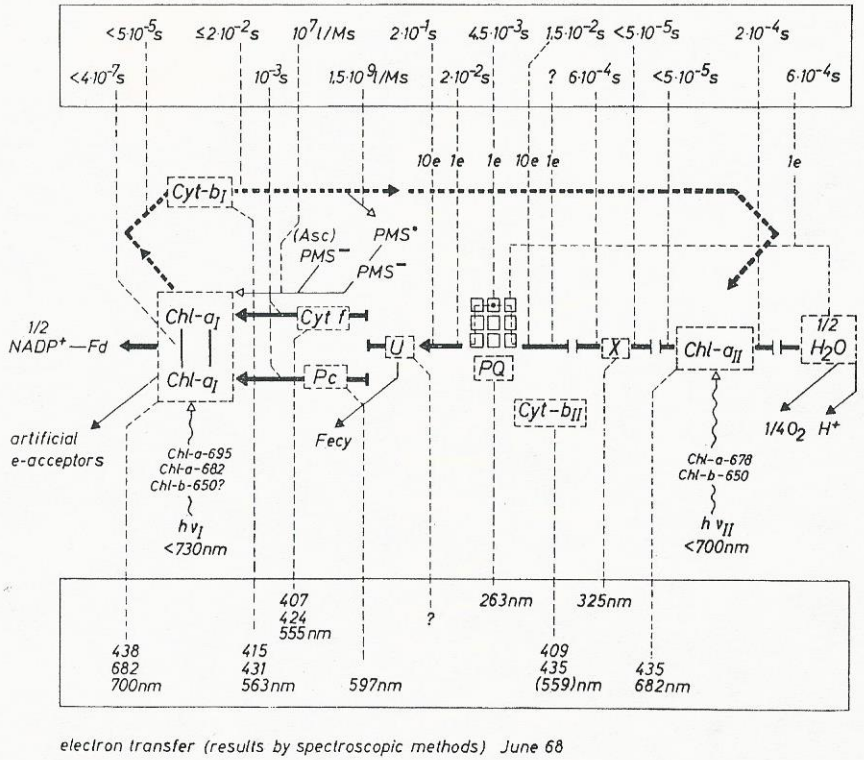


Fig. 6. Electron transfer system in photosynthesis according to the results by the pulse techniques. Details § 4. $NADP^+$ = nicotinamide adenine dinucleotide phosphate, Fd = ferredoxin, Chl-$a_I$ = chlorophyll-$a_I$, Cyt-$b$ = cytochrome-$b$, Cyt-$f$ = cytochrome-$f$, PC = plastocyanine, PQ = plastoquinone pool, X = chemically unknown, Chl-$a_{II}$ = chlorophyll-$a_{II}$. Fecy = Ferricyanide, PMS = N-methylphenazonium-methylsulfate

electrons are transferred from $H_2O$ to $NADP^+$ by the promoting forces of two active pigment systems [31]. As a precursor of $NADP^+$ the substance ferredoxin with a flavoprotein [32] was found by biochemists.

The two *wavy lines* represent the incident light quanta $h\nu_I$ and $h\nu_{II}$. These quanta are supplied to the two active chlorophyll molecules by energy transfer. The types of the chlorophyll out of the bulk which are engaged in the energy transfer are indicated by their absorption maxima, e.g. Chl-*a*-695 etc.

One of the two active pigments is chlorophyll-$a_I$ [33, 23], the other chlorophyll-$a_{II}$ [24, 34]. Chlorophyll-$a_I$ can be excited with wavelength $< 730$ nm, chlorophyll-$a_{II}$ with wavelength $< 700$ nm. The link between both chlorophylls is plastoquinone PQ [25, 36, 37]. PQ exists as a pool with a capacity for about ten electrons between chlorophyll-$a_I$ and chlorophyll-$a_{II}$. Intermediates between chlorophyll-$a_I$ and PQ are cytochrome-*f* [19, 38] and plastocyanine [21, 29] and U [37]. Intermediate between plastoquinone and chlorophyll-$a_{II}$ is X [3, 25]. An intermediate which is reduced by light reaction II is cytochrome-$b_{II}$ [27, 39], its location is, however, not accurately known.

A natural cyclic electron flow (*heavy dotted arrow*) including both light reactions and opposite to the linear flow of electrons from $H_2O$ to $NADP^+$ is mediated by cytochrome-$b_I$ [26].

Artificial interceptions of electrons *(thin arrows)* can occur at chlorophyll-$a_I$ by different types of electron acceptors [40] and at U by ferricyanide [41, 37]. Artificial injections of electrons can occur with $PMS^-$ at chlorophyll-$a_I$ [23].

On the *top* of Fig. 6 the time and rate constants of the electron transfers are depicted. Values between $10^{-7}$ sec and $10^{-1}$ sec have been measured [3]. The longest transfer time for *one* electron between two intermediates is $2 \cdot 10^{-2}$ sec. This time is observed between plastoquinone and U and caused by the oxidation of reduced PQ [25]. This time is identical with the bottle-neck time of the overall reaction of photosynthesis [3] which has been estimated by oxygen measurements as $2 \cdot 10^{-2}$ sec [42a]. Another characteristic transfer time for one electron, namely between $H_2O$ and PQ, takes according to Fig. 6 $6 \cdot 10^{-4}$ sec and is caused by the reaction of X [25]. This time has been checked by $O_2$-measurements in the following way [42b].



PQ can accept about ten electrons before the rate limiting oxidation time of reduced PQ ($2 \cdot 10^{-2}$ sec) becomes effective on the $O_2$-evolution. When, therefore, the $O_2$-yield is measured in flash groups with less than ten flashes, for instance two flashes, the $O_2$-yield as a function of the dark time between two flashes should indicate the transfer time from $H_2O$ to PQ [42b]. Results of such $O_2$-measurements are shown in Fig. 7. The half yield time is $6 \cdot 10^{-4}$ sec and corresponds exactly to the spectroscopic transfer time of X. A similar time was recently found by Joliot with a completely different technique [43].

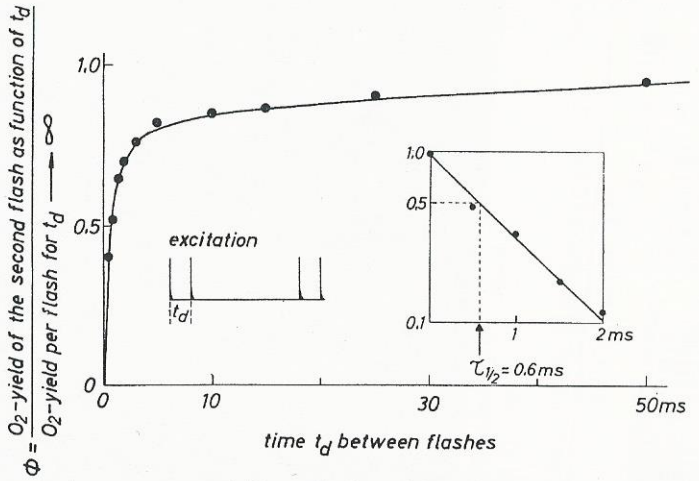


Fig. 7. Relative oxygen yield per flash in chloroplasts as a function of the dark time $t_d$ between flashes [42b]

A valuable consequence of this result is the following. In actinic flash light with a duration $\ll 6 \cdot 10^{-4}$ sec one and only one electron is transferred through the electron transport chain to the terminal acceptor.

Details of this electron transport system have been published in 1967 [3]. Since then, the following *new* data — already depicted in Fig. 6 — have been obtained.

1. With respect to the function of chlorophyll-$a_I$ it has been observed that absorption changes of chlorophyll-$a_I$ in the red are splitted into double-banded changes (see Fig. 4) which indicate that *two* chlorophyll-$a_I$ molecules are active in the reaction center I of photosynthesis [44]. The electron acceptor of chlorophyll-$a_I$ symbolized in [23] with Z is probably identical with the second chlorophyll-$a_I$ molecule. Therefore the absorption changes indicate not only the

oxidation of chlorophyll-$a_I$ as has been discussed in [33, 23], but probably also a simultaneous reduction of a second chlorophyll-$a_I$ [68].

2. Chlorophyll-$a_{II}$ has until now only been indicated by absorption changes in the red [24]. In the meantime the *whole* difference spectrum in the visible region with peaks at 436 nm and 682 nm has been measured by the repetitive pulse technique [34] (see Fig. 4).

3. An intermediate which is obviously a physical precursor of the reaction of chlorophyll-$a_I$ or chlorophyll-$a_{II}$ is indicated by the absorption changes of chlorophyll-$a_1$ [45, 3]. The spectrum has been *refined* by the repetitive technique (see Fig. 4 top) [46]. The reaction is, however, as yet not included in the scheme of Fig. 6.

4. A precise difference spectrum of plastocyanine has been obtained with a time resolution down to $10^{-5}$ sec. From the kinetics it follows spectroscopically that cytochrome-*f* and plastocyanine are operating *parallel* to each other in the electron chain.

5. Analysis between the reaction of cytochrome-$b_I$, the PMS radical, the fully reduced $PMS^-$ and chlorophyll-$a_I^+$ leads to a *PMS-reaction cycle* as depicted in Fig. 6 [47]. Cytochrome-$b_I$ reacts only with $PMS^\cdot$ and reduces $PMS^\cdot$ into $PMS^-$. $PMS^-$ reacts directly with chlorophyll-$a_I^+$ [23]. The rates of this artificial cycle correspond to the rate of the artificial PMS mediated cyclic phosphorylation. Therefore the PMS-cycle in Fig. 6 is probably responsible for this artificial cyclic phosphorylation.

6. Plastoquinone acts not only as a link in form of a pool between the two light reactions. The pools of the different electron chains are furthermore combined with each other through a *strand* of plastoquinone. In this way at least ten chains are electronically connected with each other in a parallel arrangement [48] (see Fig. 35).

## 5. Electrical field changes

### 5.1 The field indicator

In photosynthesis one type of absorption changes indicates the changes of an electrical field across a molecular membrane [7]. Since changes of this field are due to ion fluxes, these fluxes can be studied by the spectroscopic technique, too. Because of the prompt indication by the absorption changes, the field changes can be



measured through the pulse technique down to the range of 10 nanoseconds. So the possibility is given to develop also a molecular scheme of the ion translocation system in addition to the molecular scheme of the electron transport system.

The type of absorption changes which is attributed to the change of an electrical field is shown in Fig. 8. The decrease at 478 nm and 648 nm indicates that absorption changes are caused by a reaction of a chlorophyll-b [30].

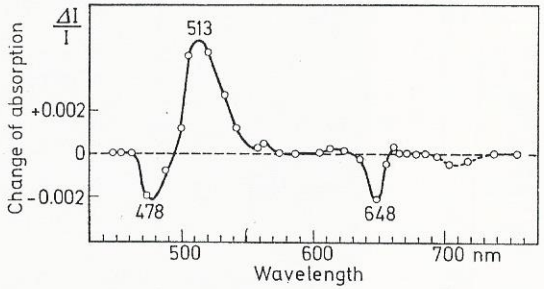


Fig. 8. Difference spectrum of the reaction of chlorophyll-*b* in chlorella cells [30]

### 5.2 Rise and decay of the field

The speed of the absorption change at 515 nm is shown in Fig. 9. In the light a fast increase takes place and in the dark a decay with a half-life time in the range of $10^{-2}$ to $10^{-1}$ sec [13]. This value depends on temperature, pH and the degree of "uncoupling" (see § 8.4). In spinach chloroplasts, which are so carefully prepared that uncoupling is minimized, the rate constant $k_o^e$ of the intrinsic decay at 20 °C and pH = 8.4 is

$$k_o^e = 8 \text{ sec}^{-1}$$

(e = electrical field driven, o = intrinsic).

The rise is extremely fast [3, 17]. By the repetitive giant laser pulse technique on an average a half rise time less than

$$2 \cdot 10^{-8} \text{ sec.}$$

has been measured (s. Fig. 10). We assume that the fast rise indicates the formation of an electrical field across a membrane in the light and the decay with $k_o^e$ the intrinsic breakdown

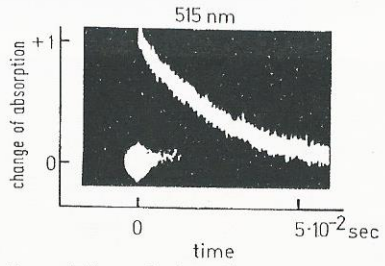


Fig. 9. Time variation of the relative absorption change of chlorophyll-*b* at 515 nm in chlorella cells [13]

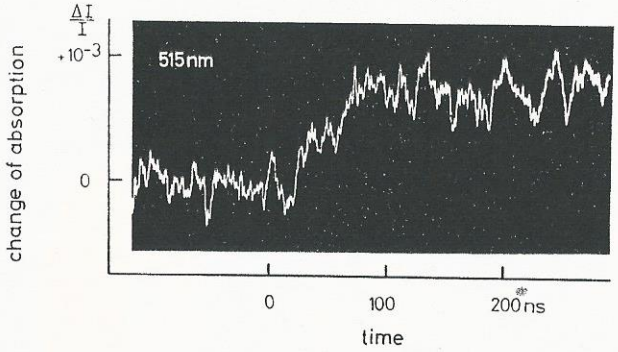


Fig. 10. Time variation of the rise of the absorption changes of chlorophyll-*b* at 515 nm in chlorella cells [3, 17]

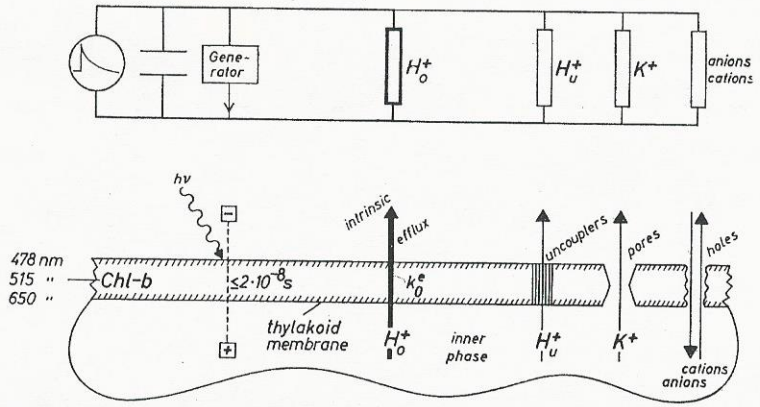


Fig. 11. *Bottom:* Scheme of the field formation (intermitted thin line) and the ion translocation in chloroplasts across the thylakoid membrane. *Top:* Electrical analogue. Details § 5.2



of the field in the dark (Fig. 11). The membrane is considered as a wall which separates an inner phase from an outer one. An electrical model is given in Fig. 11 top. We will first give proof that the absorption changes of chlorophyll-*b* are caused by a field. Secondly, it will be shown that the wall is the membrane of the thylakoids. Thirdly, evidence is given that the intrinsic break-down $k_e^o$ of the field is caused by intrinsic proton fluxes $H_o^+$.

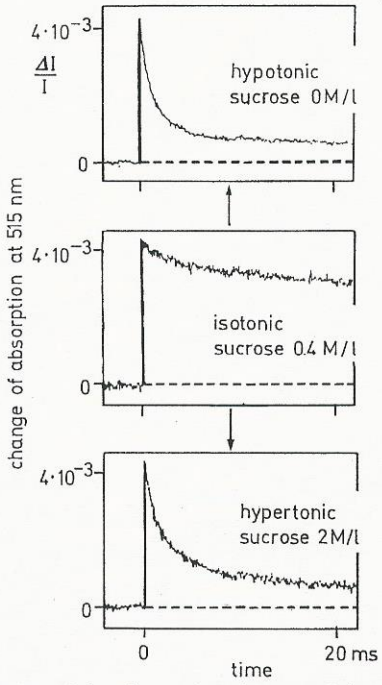


Fig. 12. Time variation of the absorption changes of chorophyll-b at 515 nm in chloroplasts suspended in three different solutions of sucrose [7]

### 5.3 Proof for the field

It should be possible according to the proposed scheme (Fig. 11) to change the intrinsic break-down of the field into a fast one by artificially increased permeability of the membrane for ions. Artificial by-passes should be possible:

1. by production of *holes* permeable for arbitrary cations and anions,

2. by production of *pores* permeable for specific ions only, e. g. $K^+$,
3. by increasing the permeability of the membrane, for protons $H_u^+$ through so called *uncouplers*.

These three by-passes are depicted in Fig. 11 on the right side.

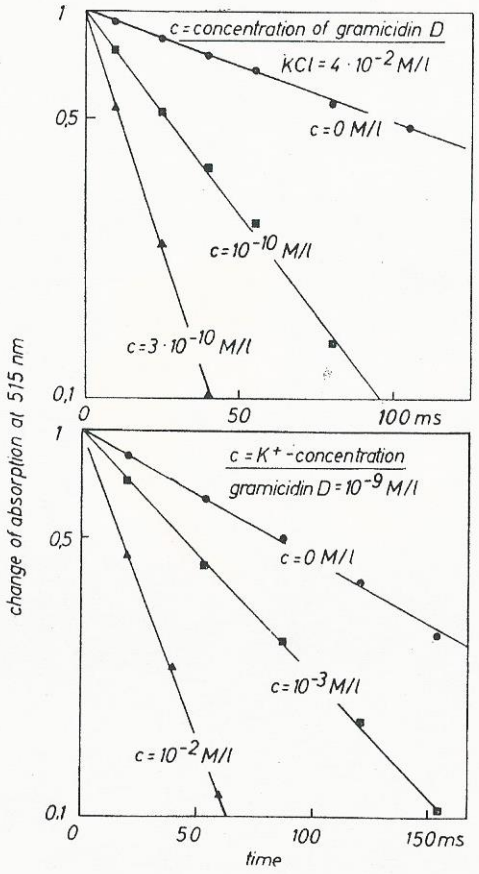


Fig. 13. Time variation of the absorption changes of chlorophyll-*b* at 515 nm in chloroplasts. *Top:* as function of the gramicidin concentration. *Bottom:* as function of the $K^+$-concentration [7]

1. *Holes* have been produced by osmotic procedures as by suspension of chloroplasts in hypo- or hypertonic solution. If additionally the concentration of arbitrary ions, e.g. $Cl^-$ or $Mg^{++}$, was increased, the decay of the changes has been accelerated. Fig. 12 shows that the decay is speeded up by such



procedures [7]. One finds for the rate constant $k_h^e$ of the decay with $Mg^{++}$ at 20 °C and pH = 7,4

$$k_h^e = a_h \cdot c_{Mg^{++}} \text{ with } a_h = 7 \cdot 10^5 \text{ l/M} \cdot \text{sec}$$

(e = electrical field driven, h ≙ holes).

2. A more differential intervention can be achieved when, on addition of gramicidin, *pores* are introduced in the membranes. According to Chappell and Crofts such pores if introduced in erythrocytes or mitochondria are permeable for alkali ions only [50]. Indeed, with increasing concentrations of gramicidin at constant concentration of potassium (Fig. 13 top) as well as with increasing concentration of potassium at constant concentrations of gramicidin (Fig. 13 bottom), the intrinsic decay time of the absorption changes is accelerated more than ten times [7]. The rate constant $k_p^e$ of the decay is for $K^+$ at 20 °C and pH = 7.4

$$k_p^e = a_p \cdot c_{K^+} \text{ with } a_p = 2 \cdot 10^{+4} \text{ l/M} \cdot \text{sec}$$

(at a gramicidin/chlorophyll-ratio of $10^{-4}$)

(e = electrical field driven, p = pores).

Furthermore, it can be demonstrated that the acceleration of the decay at 515 nm by gramicidin pores does not depend on the concentration of other ions ($Mg^{++}$, $Ca^{++}$, $Cl^-$) than alkali ions (Table 2, top). Within the different species of alkali ions, the half-life follows the order of the cation mobilities which are proportional to decreasing hydration energies (Table 2, bottom).

Table 2. *Acceleration of the decay of absorption changes of chlorophyll-b at 515 nm by various ions in gramicidin D treated chloroplasts* [7]

| Added Salt ($c = 10^{-3}$ M) | Acceleration ($t_{1/2}^0/t_{1/2}^1$) | Cation Mobility [$V^{-1}$ $sec^{-1}$ $cm^2$] |
|---|---|---|
| None | 1 | |
| $MgCl_2$ | 1.04 | |
| $CaCl_2$ | 1.06 | |
| LiCl | 1.2 | 30.8 |
| NaCl | 2.8 | 40.5 |
| KCl | 4.1 | 60.7 |
| CsCl | 6.1 | 63.7 |

3. Crofts [51] has indirectly shown that so-called *uncouplers* as methylamine hydrochloride increase the permeability of membranes for protons. This has been shown directly also for other uncouplers (§ 8.3). From Fig. 14 it can be seen that with increasing concentrations of such uncouplers the intrinsic decay is enhanced [35, 58]. For different kinds of uncouplers as desaspidin (Des), di-chlorophenolindophenol (DPIP) and methylamine hydrochloride Meth) the rate constants $k_u^e$ of the decay are at 20 °C and p H = 7.4

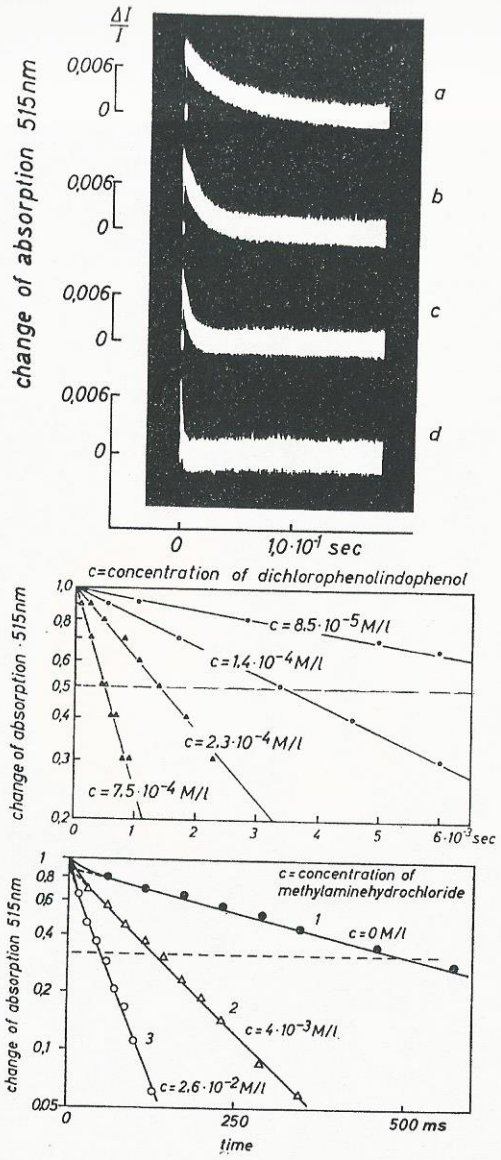


Fig. 14. Time variation of the absorption changes of chlorophyll-*b* at 515 nm in chloroplasts as function of the concentrations of uncouplers. *Top* and *center:* dichlorophenolindophenol [35]. *Bottom:* methylamine hydrochloride (pH = 7.4) [58]



$$k_u^e = a_u \cdot c_u \text{ with } a_{Des} = 1.3 \cdot 10^8 \text{ l/M} \cdot \text{sec},$$
$$a_{DPIP} = 2.7 \cdot 10^6 \text{ l/M} \cdot \text{sec},$$
$$\text{and } a_{Meth} = 1.7 \cdot 10^3 \text{ l/M} \cdot \text{sec}.$$

(e = electrical field driven, u = uncouplers).

The results allow the following conclusions. The physical property which can reflect accelerations of the decay by so different types of interventions as 1) by holes for arbitrary ions, 2) by pores for alkali ions only and 3) by increase of the membrane permeability for protons, is very probably an electrical field [7]. In the meantime further results have been obtained which support this interpretation [68]. It is as yet however not known by which mechanism the absorption changes of chlorophyll-*b* indicate the field (Stark-effect ?).

### 5.4 The field bearing membrane and the function unit

The acceleration of the break-down of the field by gramicidin (Fig. 13) can be achieved with extremely low concentrations. Table 3 shows that already one gramicidin molecule on $2 \cdot 10^5$ chlorophyll molecules transfers 50% of the intrinsic slow break-down into

Table 3. *Conversion of the slow phase of the absorption changes of chlorophyll-b at 515 nm into a fast phase in chloroplasts in dependence of the ratio gramicidin D:Chlorophyll* [7] [60]

| GRAM-D | Chl | Slow phase of the chlorophyll-b absorption change |
|---|---|---|
| 1 | : ∞ | 100% |
| 1 | : $10^6$ | 86% |
| 1 | : $5 \cdot 10^5$ | 75% |
| 1 | : $2 \cdot 10^5$ | 52% |

a fast one. This means that about one gramicidin molecule operates on a function unit containing at least $10^5$ chlorophyll molecules [7]. $10^5$ chlorophyll molecules are spread over an area of 5000 Å × 5000 Å [52]. Therefore the function unit for the electrical field must be a membrane with an area of at least such a size. A comparison of this function unit with structure elements in chloroplasts shows that the measured area has the same order of magnitude as the so-called thylakoids [7]. Thylakoids are disc-shaped closed vesicles surrounded by a double layer membrane of proteins and lipids including the pigments [53] (see Fig. 15). According to these results the field formation occurs across such a thylakoid membrane between the inner and outer phase. This is already indicated in Fig. 11.

It follows from these results also a valuable practicable criterion for the quality of isolated chloroplasts: the ratio of the slow and fast phase of the absorption changes of chlorophyll-*b* must correspond to the ratio of undamaged and damaged thylakoids [7].


Fig. 15. Electron micrographs of thylakoid piles in chloroplasts. *Top:* view from the side [66]. *Center:* view from the top [67]. *Bottom:* scheme of one cross section through the thylakoid



It is long known that the function unit of one electron transport chain including the energy conducting bulk of chlorophyll corresponds to a size of about $10^3$ chlorophyll molecules [42a] which covers an area of 300 Å × 300 Å [52]. Therefore one thylakoid consists of several hundred units of electron transport chains.

With ratios of gramicidin:chlorophyll = 1:10 field changes are not measurable while the water cleavage works at high rates (see Fig. 16). By gramicidin of such high concentrations the thylakoid is

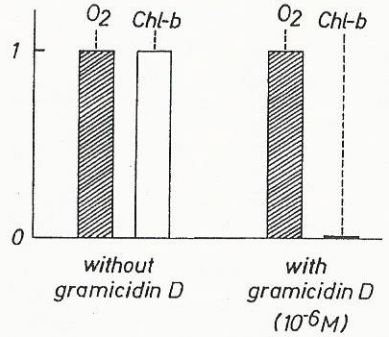


Fig. 16. Relative $O_2$-production and absorption changes of chlorophyll-*b* in chloroplasts without and with $10^{-6}$ M/l gramicidin D (Gramicidin:chlorophyll = 1:10)

damaged so that no field formation can occur. The undamaged districts of the thylakoid membrane, however, are obviously great enough to include the smaller electron transport units (300 Å × 300 Å), so that $O_2$-evolution is not diminished.

### 5.5 The field strength

The none-aqueous insulating layer in the thylakoid membrane, the lipid layer, has a thickness of about l = 30 Å [53]. In the next chapter it is experimentally shown that with one electron transfer in one chain *two* elementary charges are translocated across the membrane [54]. One chain covers an area of A = 300 Å × 300 Å (see above). With an effective dielectric constant of $\varepsilon = 2$ (phospholipid membrane) it follows for the voltage produced in a short flash of $\ll 6 \cdot 10^{-4}$ sec [54]

$$V_0 = \frac{2e \cdot l}{A \cdot \varepsilon_0 \cdot \varepsilon} \approx 50 \text{ mV}.$$

Because the absorption changes of the chlorophyll-b increase from short flashes to longer, maximal by a factor 4 [13] the maximal voltage across a thylakoid membrane amounts up to

$$V_{max} \simeq 200 \text{ mV}.$$

This can be concluded because there exists a linear relationship (s. § 6.3) between the magnitude of the absorption changes and the voltage.

### 5.6 Formation of one half of the field at each of the two light reactions

In the elementary act of photosynthesis only one electron is transferred through the chain. This is realized by excitation with a short flash ( $\ll 6 \cdot 10^{-4}$ sec). The magnitude of the changes under these conditions is shown in Fig. 17, left. When, however, light reaction I

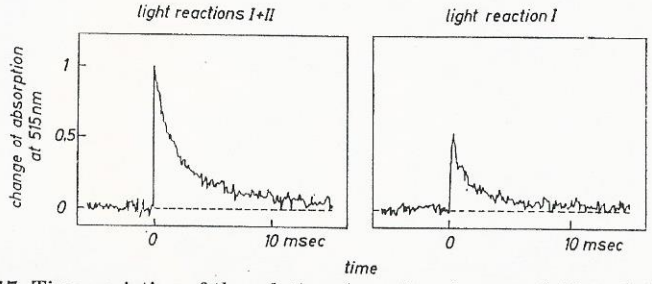


Fig. 17. Time variation of the relative absorption changes of chlorophyll-*b* at 515 nm in chloroplasts. *Left:* excitation of light reaction I and II. *Right:* excitation of light reaction I only [54]

is excluded, for instance by saturating light reaction I with 700 to 730 nm background light (this excites only light reaction I), one half of the absorption changes disappear [30]. When, on the other hand, the contribution of light reaction II is prevented, for instance by poisoning the light reaction II by DCMU, one half of the absorption changes disappear also (see Fig. 17, right). This means that one half of the formation of the field is caused by light reaction I and the other by light reaction II [54]. These two field formations are depicted in Fig. 18.

This result has been additionally proved by measuring the action spectra of the absorption changes of chlorophyll-*b*. Two spectra have been obtained. Both correspond to the action spectra of light reaction I and II respectively [54].



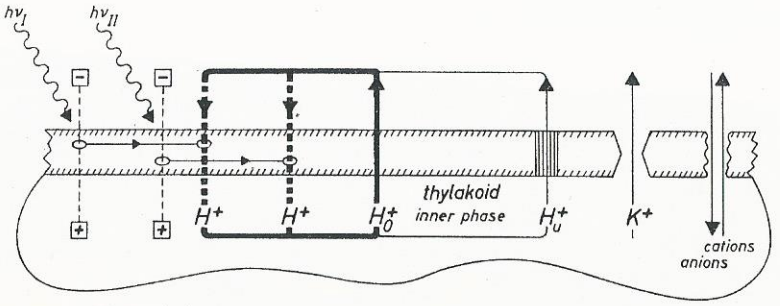


Fig. 18. Extended scheme of the field formation (intermitted thin lines) and ion translocations across the thylakoid membrane of chloroplasts. Detail § 5.6 and § 6.1

## 6. Field driven proton translocation

### 6.1 Influx of one proton at each of the two light reactions

With the formation of the field in a short flash of $\ll 6 \cdot 10^{-4}$ sec a proton influx can be observed. This has been measured directly by pH-increases in the outer phase of the thylakoid. This was possible by measuring the colour changes of pH-indicators which have been added to the chloroplast suspension. The very small colour changes have been detected with the repetitive pulse technique [54].

In such short flashes with one electron transfer two protons are translocated (see Fig. 19, left). This corresponds to the result in steady state light in which on an average the uptake of two protons per one electron transfer has been observed [55]. When, however, the

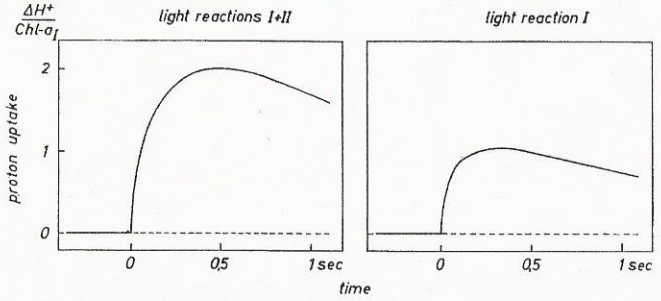


Fig. 19. Proton uptake per one electron chain measured by added pH-indicators in chloroplasts. *Left:* excitation of light reaction I and II. *Right:* excitation of light reaction I only [54]. Duration of excitation $10^{-5}$sec

contribution of one light reaction, e.g. light reactions II, is prevented (as in § 5.6), only one proton is translocated (see Fig. 19 right). This means that by each of the two light reactions one proton is translocated [54]. Because each of the two light reactions produces one half of the field (see Fig. 17), one half of the field is coupled with the translocation of one proton. (This is depicted in Fig. 18).

The half time of the pH-increase in a short flash which has been measured by pH-indicators added to the suspension, takes $\leq 8 \cdot 10^{-3}$ sec. This value represents the time of the uptake of one proton at each light reaction [54].

### 6.2 Proton influx and field stabilization

It is unlikely that the proton uptake in $\leq 8 \cdot 10^{-3}$ sec is the reason for the rapid formation of the electrical field in $\leq 2 \cdot 10^{-8}$ sec.

Therefore it must be assumed that first the field is set on by an electron transfer and that this field causes the $H^+$ uptake in a secondary process. This can in principle be realized by one of the different possibilities discussed by Mitchell in his hypothesis (8).

According to the results reported above and regarding the facts which are known on the electron transfer system in photosynthesis — see for instance [3] — the coupling between the two light reactions, electron transfer, field formation, and proton translocation can be formulated in the following way. The principle is depicted in Fig. 20, details and data in Fig. 36.

The events during the transfer of one electron, realized in a short flash ($\ll 6 \cdot 10^{-4}$ sec) will be called stage I (Fig. 20, left), those which occur in a long flash ($\sim 10^{-2}$ sec) will be called stage II (Fig. 20, right).

In *stage I* each of the two photoactive chlorophylls $a_I$ and $a_{II}$ absorbs one quantum $h\nu_I$ and $h\nu_{II}$ respectively. This absorption is followed by an electron transfer. This transfer is assumed to occur at each light reaction diagonal through the membrane by anisotropically arranged molecules. The transfer should occur from the electron donor located at the inner surface to the acceptors located at the outer surface of the membrane (Fig. 20a). This is possible in $\leq 2 \cdot 10^{-8}$ sec. The component of this transfer which is perpendicular to the membrane would cause within this time the formation of an electrical potential $V_0$ across the membrane. At the end of this process at *each* light reaction one negative charge ⊟ is located at the outer surface



of the membrane and one positive charge ⊞ at the inner surface. (In the following the chemical names of the charged molecules known from Fig. 6 are put in brackets).

The negative charge ⊟ ($X^-$) of light reaction II at the outer surface of the membrane reduces plastoquinone PQ within

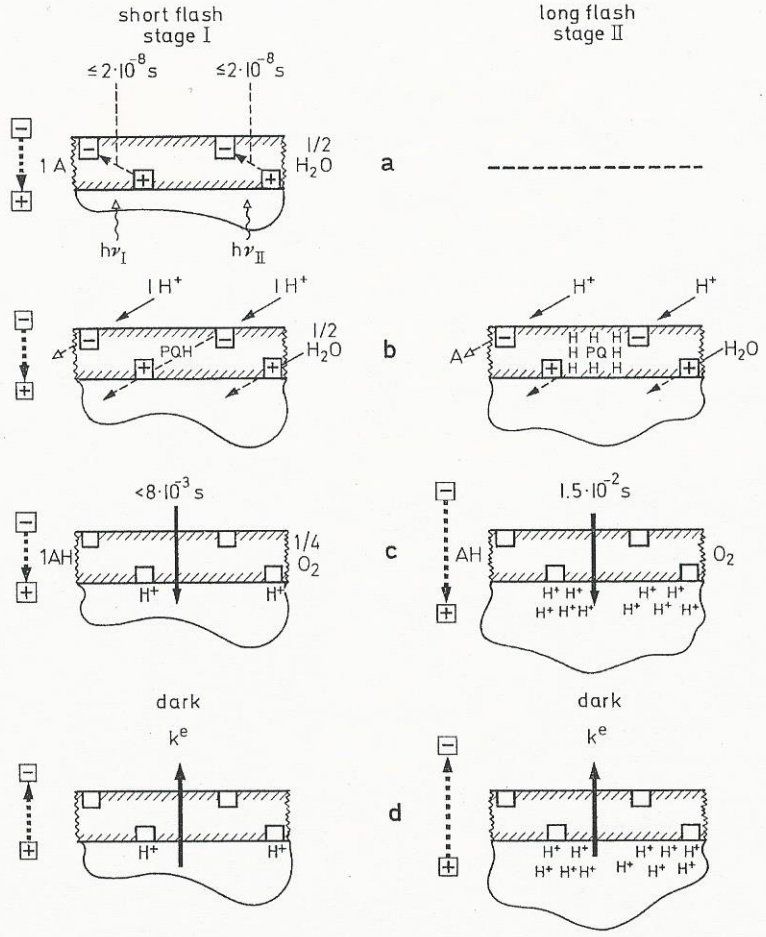


Fig. 20. Scheme of the coupling between electron transfer, field formation and proton translocation in the thylakoid membrane of chloroplast in short flashes (stage I) and long flashes (stage II). Details § 6.2 and § 6.3

$\leq 6 \cdot 10^{-4}$ sec (Fig. 7). This is accompanied by an uptake of one $H^+$ from the outer phase in $\leq 8 \cdot 10^{-3}$ sec [54] (see Fig. 20b, left).

$$^1/_2\,PQ + \boxed{-} \rightarrow {}^1/_2\,PQH_2 + \square - H^+_{extern}$$

The neutral molecules $^1/_2\,PQH_2$ (symbolized in Fig. 20 with PQH) translocate a hydrogen from the outer surface of the membrane to the positive charge $\boxed{+}$ of the light reaction I (Chl-$a_I^+$) at the inner surface which oxidizes (via cytochrome-$f$) $^1/_2\,PQH_2$.

This reaction is accompanied by a release of one $H^+$ into the inner phase (see Fig. 20c). This oxidation takes place in $2 \cdot 10^{-2}$ sec [25].

$$^1/_2\,PQH_2 + \boxed{+} \rightarrow {}^1/_2\,PQ + \square + H^+_{intern}$$

The positive charge $\boxed{+}$ (Chl-$a_{II}^+$) of light reaction II at the inner surface of the membrane oxidizes (via intermediates) $H_2O$ which is accompanied by a release of one $H^+$ into the inner phase. This takes place in $2 \cdot 10^{-4}$ sec (reaction time of Chl-$a_{II}$) [24].

$$^1/_2\,H_2O + \boxed{+} \rightarrow {}^1/_4\,O_2 + \square + H^+_{intern}$$

The negative charge $\boxed{-}$ (Chl-$a_I^-$) of light reaction I at the outer surface of the membrane reduces (via intermediates) the acceptor A ($\equiv$ NADP$^+$) which is accompanied with a $H^+$ uptake from the outer phase.

$$A(^1/_2\,NADP^+) + \boxed{-} \rightarrow AH(^1/_2\,NADPH \cdot H^+) + \square\,H^+_{extern}$$

In this way

a) an electrical field is set on by an electron transfer across the thylakoid membrane,

b) this field is stabilized by the translocation of two protons (without counter-ions) from the outer into the inner phase of the thylakoid,

c) one electron is transferred from $H_2O$ to A(NADP$^+$).

If in a second *stage II* a flash of such a duration is used that the plastoquinone pool of 3 to 5 PQ molecules is just completely reduced (in $1.5 \cdot 10^{-2}$ sec with n = 6 to 10 electrons) by n turn-overs of light reaction II, the same happens as demonstrated in stage I (see Fig. 20, right). The difference is only the quantity in the number of electron transfers, $H^+$ translocations, and the magnitude of the field strength. This is discussed in the next chapter. A proton uptake which corresponds to the size of the PQ pool has already been measured in [55].



### 6.3 Proton influx, field formation, and plastoquinone reduction

A consequence of the assumptions in stage I (Fig. 20, left) is — regarding for instance only the events at light reaction II — that with a tranfer of one electron and the set on of *one half* of the electrical field (§ 5.6) and the influx of *one* $H^+$ (§ 6.1) the formation of *one half* $PQH_2$ should be observable. This expected relationship is documented in Fig. 21 (see flash-sign).

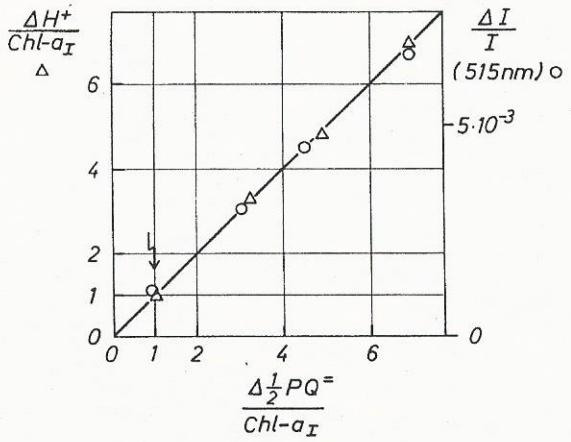


Fig. 21. Proton uptake ($\Delta H^+$) and field changes (absorption changes at 515 nm) at light reaction II in chloroplasts as function of plastoquinone reduction ($^1/_2\,PQH_2$). The values are measured in respect to one electron transport chain (measured by Chl-$a_I$) [57]

A consequence of the assumptions in stage II (Fig. 20, right) is — regarding for instance again only the events at light reactions II — that the 1:1:1 correspondence between the on set of $^1/_2$ field, $H^+$ influx and $^1/_2\,PQH_2$ formation, which has been proved for one electron in stage I, should hold in stage II also for n electrons. In stage II simultaneously with the reduction of the PQ-pool through n electrons by n turn-overs of light reaction II, the uptake of n $H^+$ and a n-fold increase of the field (absorption change at e.g. 515 nm) should occur. A corresponding strict coincidence of all these three components has been shown [57] and is demonstrated up to n = 6 in Fig. 21.

The linear relationship between proton uptake, plastoquinone reduction and absorption changes at 515 nm in Fig. 21 indicates additionally that the relationship between the membrane potential and its indication by the absorption changes at 515 nm is a linear one.

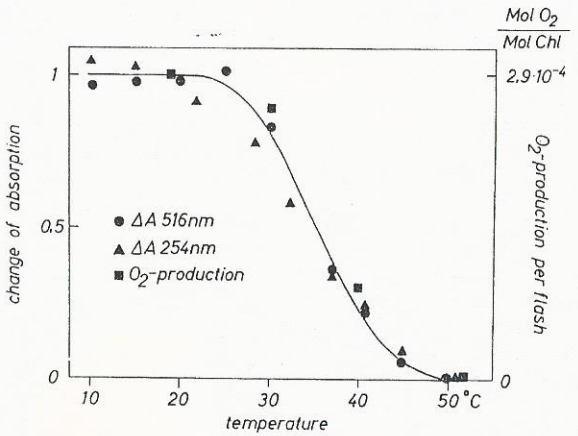


Fig. 22. Relative absorption changes of chlorophyll-*b* at 516 nm, plastoquinone at 254 nm and $O_2$-production as function of temperature in chloroplasts [56]

A further consequence of the assumptions in stage I and II is — regarding again only light reaction II — that with the set on of the field and plastoquinone reduction also the $O_2$-evolution should be strictly correlated under different conditions. This has been demonstrated in [56]. One example is given in Fig. 22.

### 6.4 Proton efflux and field decay

In the dark the relaxation of stage I and II takes place by an efflux of the protons back into the outer phase of the thylakoid accompanied by a break-down of the field (Fig. 20d).

The intrinsic break-down of the field in the dark can be followed by the decay of absorption changes of chlorophyll-*b* (s. Fig. 9), the proton efflux by the pH-decrease in the dark (see Fig. 19).

It is assumed that the intrinsic break-down of the field is caused by the *proton* efflux $H_0^+$ (see Fig. 11 and 18). It is also possible that ions other than protons (potassium, chloride etc.) cause the break-down.



A direct proof that *proton* efflux causes the intrinsic breakdown is given when the kinetics of the field decay and pH-decrease are identical under different conditions. Such measurements have as yet not been possible because the expected fast pH-decrease ($10^{-2}$ to $10^{-1}$ sec) has as yet not been resolved by glass electrode or pH-indicator techniques. The assumption can, however, be proved indirectly.

It has been shown by glass electrode measurements in permanent light that under different conditions the rate of proton efflux is always proportional to the rate of electron flow (see § 8.4). There-

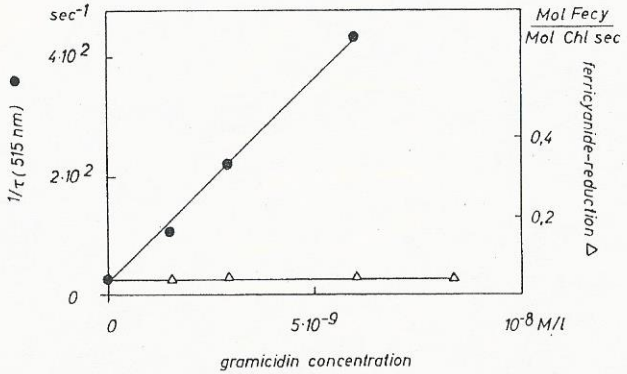


Fig. 23. Reciprocal half-life of absorption changes of chlorophyll-*b* at 515 nm and the rate of electron transfer measured by the ferricyanide reduction in chloroplasts as function of the concentration of gramicidin [7]

fore, the field decay—characterized by the rate constant $1/\tau_{515}$ — is caused by a proton efflux when it can be shown that $1/\tau_{515}$ is strictly proportional to the rate of the electron flow.

For instance, the rate constants of the field decay and the rate of electron flow in dependence of gramicidin are shown in Fig. 23. The electron flow is independent of gramicidin but the rate constant of the field decay not at all [7]. This is expected because with gramicidin the decay of the field is caused by effluxes of *potassium* (see § 5.3). On the other hand, corresponding measurements in dependence of uncouplers are shown in Fig. 24. In this case the electron flow follows strictly the rate constant of the field decay at all concentrations [58]. This is expected because with uncouplers the

decay of the field is caused by the effluxes of *protons*. (That uncouplers accelerate the efflux of *protons* is directly shown by measurements with the glass electrode in § 8.3.)

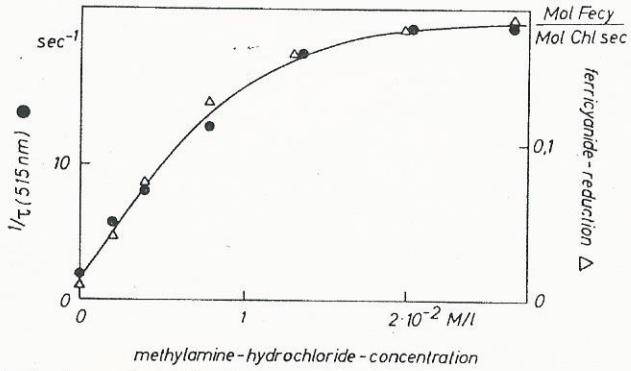


Fig. 24. Reciprocal half-life of absorption changes of chlorophyll-*b* at 515 nm and the rate of electron transfer measured by the ferricyanide reduction in chloroplasts as function of the concentration of the uncoupler methylamine hydrochloride [58]

Now, the *intrinsic* decay of the field is tested by comparison with the rate of electron flow. To be sure of complete coupling whole chlorella cells were used. As parameter different temperatures were chosen. The electron flow was measured by the reduction of oxidized chlorophyll-$a_I$ through absorption changes at 705 nm. The rate constant of the electron transfer $1/\tau_{705}$ follows strictly the rate constant of the field $1/\tau_{515}$ at different temperatures [59] (see Fig. 25). From

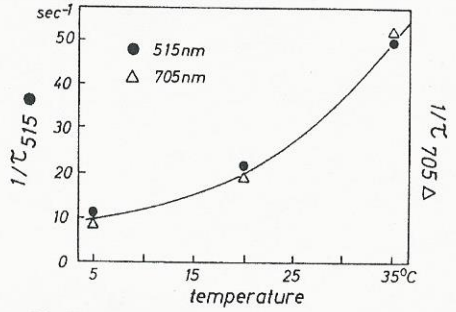


Fig. 25. Reciprocal half-life of absorption changes of chlorophyll-*b* at 515 nm and the rate of electron transfer measured by the absorption changes of chlorophyll-$a_I$ at 705 nm in chlorella cells as a function of temperature [59]



this coincidence it follows that the intrinsic field decay is caused by proton effluxes [7], as indicated in Fig. 11 and 18. Therefore the field driven proton efflux can be described by the field decay which is according to § 5.2 at 20 °C and pH = 8.4

$$k_o^e = 8 \text{ sec}^{-1}$$

(e = electrical field driven, o = intrinsic).

This conclusion is true for the indicated conditions. At other conditions (lower pH for instance) ions other than protons may have a greater permeability than protons and then these are responsible for the decay. In general the intrinsic field decay is determined by those types of ions which have the greatest permeability for the membrane.

## 7. Phosphorylation and extra proton effluxes (field driven)

According to Mitchells hypothesis [8] an extra proton flux generates ATP or the other way around, addition of ADP and P with the cofactor $Mg^{++}$ should enhance the fluxes of protons. The investigations of the chlorophyll absorption changes should give a direct insight into this most disputable point. Fig. 26 top shows the result.

Additions of ADP, P and $Mg^{++}$ accelerate the intrinsic decay of the field or proton efflux respectively under different conditions by a factor of about 2. On the bottom of Fig. 26 the kinetics are represented on a logarithmic scale [58].

This result on the molecular level was checked by macroscopic measurements in Fig. 27. The rate of phosphorylation was changed from maximum phosphorylation down to zero by addition of the poison phlorizin. Simultaneously the change in $\Delta^1/\tau_{515}$ which indicates the rate constant of the extra efflux of protons was measured. The rate of this extra efflux follows strictly the rate of phosphorylation [58]. For the extra proton efflux through phosphorylation it was found at 20 °C and pH = 8.4 $1/\tau_{515}$ (with) $-1/\tau_{515}$ (without phosphorylation) $= \Delta 1/\tau_{515} = k_{ATP}^e = 14 \text{ sec}^{-1}$.

(e = electrical field driven, ATP = by phosphorylation)

These results indicate that if the reaction center of phosphorylation within the membrane works by the addition of ADP and P, and the cofactor $Mg^{++}$, the field driven proton gradient across the membrane can be additionally discharged through this center. So, the scheme can be extended as depicted in Fig. 28.

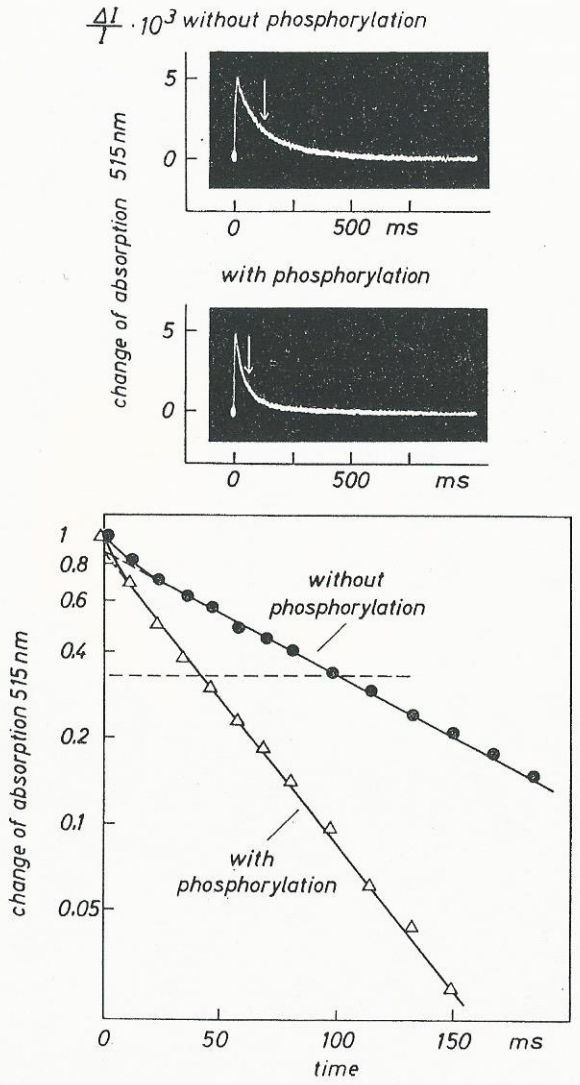


Fig. 26. *Top:* time variation of the absorption changes of chlorophyll-*b* at 515 nm in chloroplasts without and with phosphorylation. *Bottom:* the same in a logarithmic scale [58]



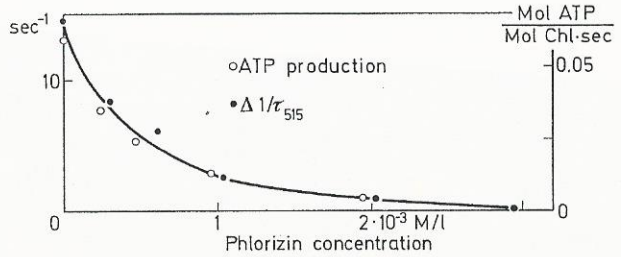


Fig. 27. Reciprocal Δ half-life of absorption changes of chlorophyll-*b* at 515 nm and the rate of ATP production in chloroplasts as function of the phosphorylation quencher phlorizin [58]

The function unit for the field driven phosphorylation should be as great as the function unit of the field, that is one thylakoid. Measurements of ATP formation in dependence of gramicidin (corresponding to those in § 5.4) confirm this prediction [60].

The field driven events and their rate constants are summarized in Fig. 28.

The acceleration of the field decay through phosphorylation occures in short flashes (set on of 50 mV) and long flashes (set on of

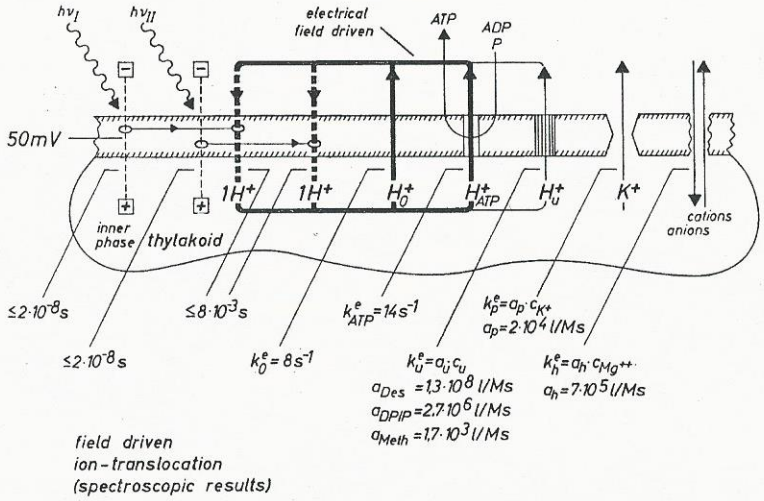


Fig. 28. Scheme of the field formation (intermitted thin line) and the *field* driven ion translocation across the thylakoid membrane of chloroplasts. The transfer times and the rate constants, described in the text, are indicated

200 mV) (s. § 5.5). In short flashes the effect is, however, observable only with permanent background light. This is expected. The free energie of protons driven in 50 mV is too low to drive phosphorylation [8]. Permanent light, however, sets on an additional proton gradient (see § 8). With the help of this additional energy the protons can discharge through the phosphorylation center. The results in Fig. 26 were obtained in long flashes ($8 \cdot 10^{-3}$ sec) without background light.

## 8. Diffusion driven proton translocation

After the development of stage I and II in short and long flashes (see Fig. 20) in permanent light, a third stage is built up with a new phenomenon. In this third stage a further but very slow transloca-

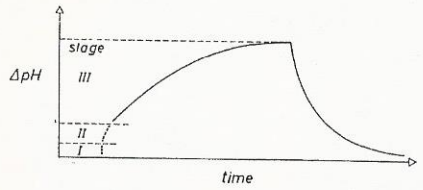


Fig. 29. Principle pH-change as function of time in stage I, II and III. Details § 8.

tion of $H^+$ in the order of seconds takes place. The amount is in the order of 100 protons per electron chain or 10,000 protons per thylakoid. This phenomenon is accompanied by an efflux of cations (or influx of anions). This third stage can be easily followed by the measurements of the pH-increase in the outer phase of the thylakoids with a glass electrode (see Fig. 29). These are those phenomena which have been first observed by Neumann and Jagendorf [5] and Dilley and Vernon [6] on which has been reported in the introduction. Obviously, this third stage is a consequence of the elementary fast stages I and II which have been studied by the chlorophyll-*b* absorption changes.

### 8.1 Proton influx and the proton gradient

When the membrane is not at all permeable for ions other than protons the creation of the third step would not occur. When, however, the membrane is only a bit permeable e.g. for cations, these are slowly driven outwards by the electrical field which is



built up in stage I and II and which exists in continuous illumination permanently (see Fig. 30, left). This cation efflux through the field is counterbalanced by a corresponding additional slow $H^+$-influx. At 20 °C, pH = 7.4 the influx takes place in a half rise time of

1 sec.

It is even possible to estimate the light induced pH-decrease in the light in the *inner phase* of the thylakoid! A maximal decrease of $\Delta$ pH = 2.7 has been observed by Rumberg et al. [61].

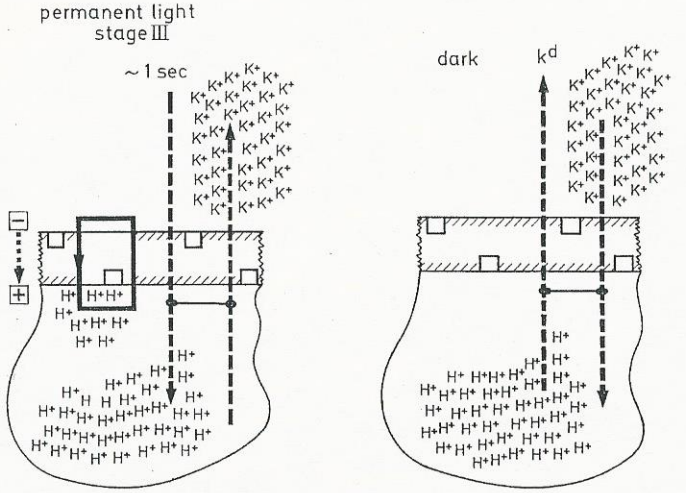


Fig. 30. Scheme of the coupling between field driven and diffusion driven ion translocation in permanent light (stage III). Details § 8.1

The stage I in Fig. 20 is determined by the capacity for quanta within each electron chain, that are 2. The stage II is determinded by the electron capacity of the P Q-pool, this is about 6 to 10. The stage III in Fig. 30 must be determined by the ion capacity of one thylakoid ($\geq$ 10,000). This follows from the results that the function unit for the ion transport system is one thylakoid (see § 5.4).

### 8.2 Proton efflux

In the dark the relaxation takes place as in stage I and II. The discharge of the stage III (see Fig. 30, right) which can be followed by the glass electrode is determined by the *diffusion driven*

efflux of the bulk of protons and the diffusion driven influx of the bulk of cations respectively. This is the essential difference in comparison with stage I and II in Fig. 20: the efflux of protons in stage I and II is driven by the electrical field.

For the diffusion driven efflux at 20 °C and pH = 8.4 the intrinsic rate constant $k_o^d$ in spinach chloroplasts prepared under condition that uncoupling is minimized

$$k_o^d = 0.3\ sec^{-1}$$

(d = diffusion driven, o = intrinsic).

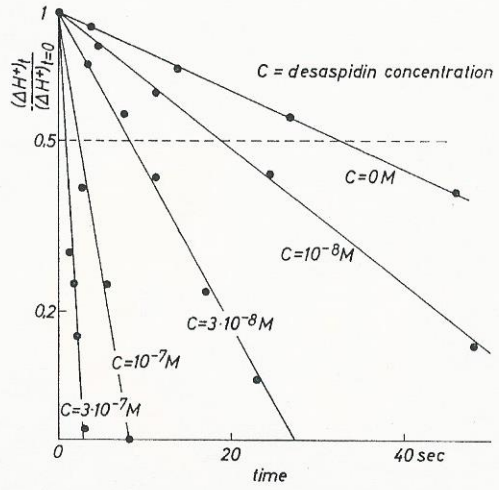


Fig. 31. Time variation of the efflux of protons in chloroplasts as function of the concentration of the uncoupler desaspidin [61]

### 8.3 Proton efflux and uncouplers

First qualitative results of the acceleration of the diffusion driven proton efflux by uncouplers on chloroplasts came from Jagendorf et al. [63]. The quantitative measurements by Rumberg et al. with the glass electrode in Fig. 31 show a first order decay at different concentrations of the uncouplers [62]. (Corresponding measurements on mitochondria were carried out by Mitchell et al. [64].) The efflux is strongly accelerated with increasing concentrations. For different types of uncouplers as desaspidin (Des), carbonylcyanide-m-chlorophenylhydrazone (CCP) and antimycin A (AA) the rate constants $k_u^d$ are at 20 °C and pH = 7.4

$k_u^d = a_u \cdot c_u$ with

$a_{Des} = 6.0 \cdot 10^6$ l/M·sec, $a_{CCP} = 1 \cdot 10^5$ l/M·sec and
$a_{AA} = 1 \cdot 10^5$ l/M·sec

(d = diffusion driven, u = uncoupler).

These results confirm definitely that the effect of uncouplers is the increase of the membrane permeability for protons [62].



### 8.4 Proton efflux and electron transfer

The rate of proton efflux $d\Delta H^+/dt$, measured with the glass electrode, and electron transfer $d\Delta e^-/dt$ are always identical and changed in the same way as a function of uncoupler-concentration (see Fig. 32, top), pH (Fig. 32, center), temperature (Fig. 32, bottom) [62, 65]. The strict coincidence of both under widely different conditions shows definitely that the electron transfer is regulated by the proton efflux out of the thylakoid. Explanation see § 8.5.

The results of Fig. 32 implicate that during the efflux with each electron transfer one proton is translocated. During the influx, however, with each electron two protons are translocated (see § 6.1). This difference is probably caused by the fast field driven component of proton efflux which is not detected by the glass electrode [61, 62].

### 8.5 Coupling between proton translocation and electron transfer

According to the scheme in Fig. 20 the electron transfer causes proton translocations into the thylakoid. Therefore there must be a "back pressure" of the protons $\Delta H^+$ in the inner phase on the elctron transfer system. This back pressure can be effective only on that step which is rate limiting and accompanied with a deprotonisation. This step is the oxidation of $^1/_2\,PQH_2$ (see § 4):

$$^1/_2\,PQH_2 \rightleftarrows {}^1/_2\,PQ + 1e + 1\,H^+. \qquad (1)$$

Therefore the amount of $\Delta H^+$ in the inner phase should regulate via the reaction formulated in Eq. 1 the speed of the electron transport system. Because $\Delta H^+$ decreases by increase of the intrinsic efflux $d\Delta H^+/dt$ [through the rise of temperature or pH or uncouplers (see Fig. 32), or by extra efflux during phosphorylation (see § 9)] the electron transfer is speeded up.

This mechanism has been proved by measuring the oxidation

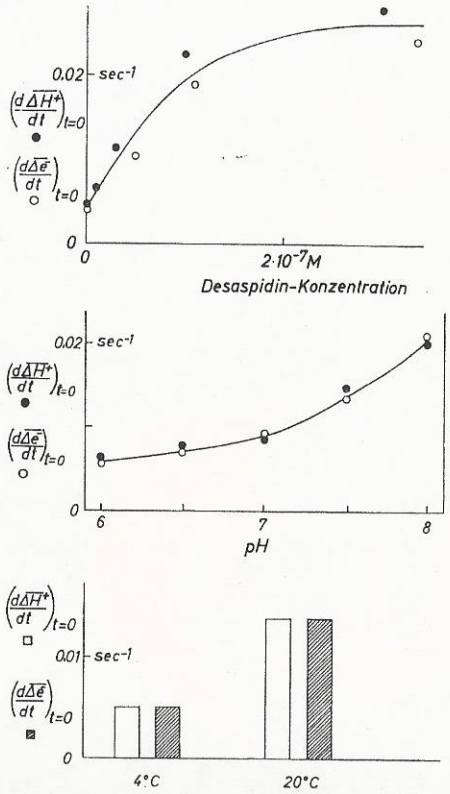


Fig. 32. Specific rates of proton translocation and electron transfer in chloroplasts. *Top:* as function of the uncoupler desaspidin concentration. *Center:* as function of pH. *Bottom:* as function of temperature [62, 65]

time of $PQH_2$ through absorption changes in dependence of $\Delta H^+$ in the *inner* phase. The oxidation time increases indeed with the increase of $\Delta H^+$ [61].

## 9. Phosphorylation and extra proton effluxes (diffusion driven)

According to the hypothesis of Mitchell [8] and the results in § 7 the amount of generated ATP should be given by an additional proton efflux $(\Delta H^+)_{ATP}$. In § 7 such a relationship for the *field* driven proton efflux was proved. A *diffusion* driven extra efflux through phosphorylation must be characterized by a corresponding diffusion depending rate constant $k^d_{ATP}$. When $\Delta H^+$ is the total



amount of protons translocated into the inner phase of the thylakoid it follows

$$\Delta ATP \simeq \Delta H^+ \cdot k^d_{ATP} \cdot t \qquad \text{when } k \cdot t \ll 1.$$

Using actinic flash light with dark times t between the flashes it follows for the yield of ATP per flash that this should be proportional to the amount of already translocated protons $\Delta H^+$. $\Delta H^+$ was changed by different times of illumination. Actually the yield of ATP per flash increases linear with $\Delta H^+$ (see Fig. 33) [62]. This demonstrates that also under diffusion driven conditions ATP formation is coupled with an extra efflux of protons.

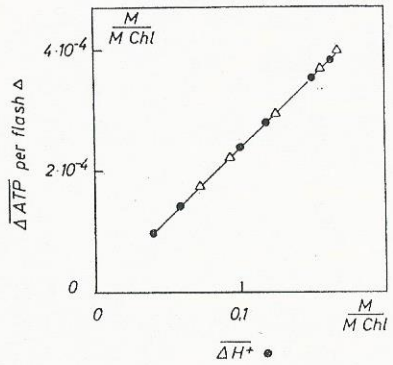


Fig. 33. Specific yield of ATP per flash in chloroplasts as function of the specific amount of translocated protons [62]

In § 7 it was mentioned that the field driven proton efflux has to reach a critical value of free energy in order to be discharged through the reaction center of phosphorylation. This is expected also for the diffusion driven proton efflux. This means that in Fig. 33 the linear relationship cannot exist down to zero. First formation of ATP should be observable only above a critical value of $\Delta H^+$.

The diffusion driven events so far obtained by the glass electrode measurements and their rate constants are summarized in Fig. 34.

Electron transfer and ion translocations are depending on the conditions of the membrane. Because they are coupled with each other the conditions can be characterized by one of them, for instance by the rate of the electron transfer. The data in Fig. 28 and Fig. 34 and Fig. 36 have all been related to one and the same preparation with an electron transfer rate of $de/dt = 0.01$ sec$^{-1}$ at

20 °C, pH = 7.4. In this way all values are comparable with each other. The corresponding data in our original publications are sometimes different from these values here because they are related to slightly other membrane conditions.

In comparison with the electrical field driven events in Fig. 28 it can be seen that the diffusion driven rate constants in Fig. 34 are in general about 30 times smaller (using this rule, from $k^e_{ATP}$ the value of $k^d_{ATP}$ has been calculated which is included in Fig. 34).

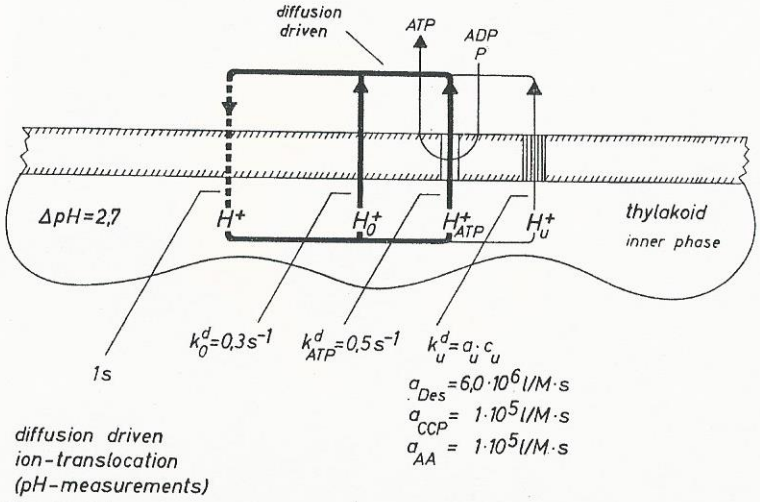


Fig. 34. Scheme of the *diffusion* driven ion translocation across the thylakoid membrane of chloroplasts. The transfer times and rate constants, described in the text, are indicated

## 10. Conclusions

The results indicate that the function unit of photosynthesis is one thylakoid. This unit consists of several hundreds of electron transfer chains in the membrane of one thylakoid. At least ten chains are combined through a strand of PQ. Within each electron chain, electrons are transferred by two light reactions from $H_2O$ to $NADP^+$. The transfer is assumed to occur diagonal through the membrane. This leads to the formation of an electrical field component perpendicular to the membrane. The stabilization of this field causes the translocation of two protons through the membrane into the common inner phase of the thylakoid. The field driven and diffu-



sion driven efflux of these protons into the outer phase is coupled — with the production of ATP. A simple scheme of this unit is depicted in Fig. 35. Details of one "element" are presented in Fig. 36.

With respect to the hypotheses of Mitchell which is in our opinion one of the most ingenious hypotheses presented in the field of biochemistry in the last 10 years, we have found the following 17 experimental results which support this hypothesis.

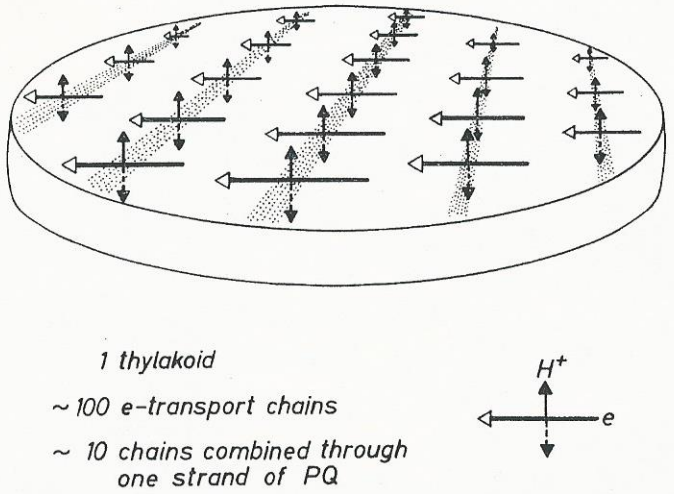


Fig. 35. Function unit of the primary processes of photosynthesis (one thylakoid of chloroplasts). Details see § 10

1. Proof for the existence of an *electrical field* across a membrane (§ 5.3).
2. Estimation of the *voltage* of this field as $V_0 = 50\,mV$ and $V_{max} = 200\,mV$ (§ 5.5).
3. Proof for *field driven proton* translocation across a membrane (§ 6.1, 6.3).
4. Evidence for the field driven proton translocation via the redox reaction of *plastoquinone* (§ 6.3).
5. Estimation of the *pH-decrease* in the *inner phase* of the thylakoid $\Delta pH = 2.7$ (§ 8.1).
6. Proof for a strict *coupling* between proton translocations and electron transfer under widely different conditions as function of temperature, pH, uncouplers, etc. (§ 8.3 and § 8.4).

7. Evidence for the *location* of coupling at the rate-limiting step which is the oxidation of $^1/_2\,PQH_2$ (§ 4).

8. Evidence for the *mechanism* of the coupling. This is the back pressure of $\Delta H^+$ on the electron-proton-dissociation from $^1/_2\,PQH_2$ in the inner phase of the thylakoid (§ 8.5).

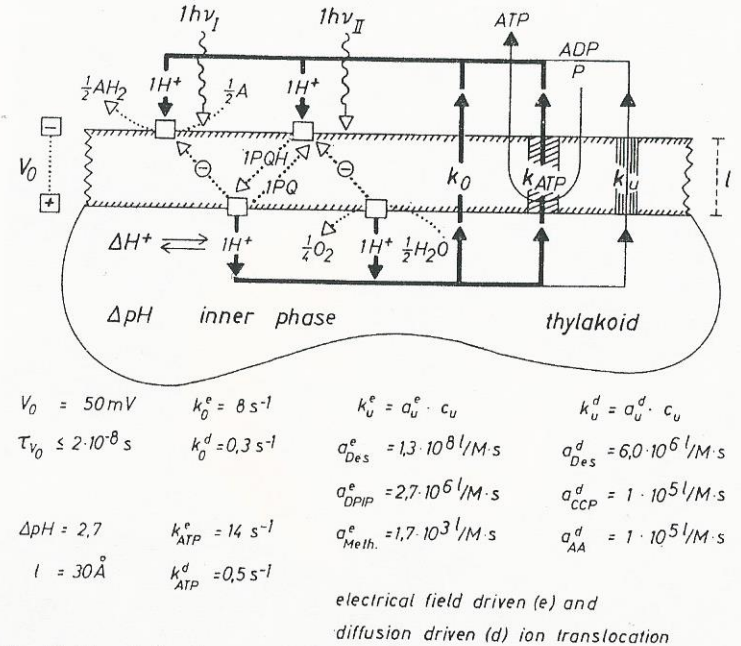


Fig. 36. Correlation between light reactions (wavy arrows), electron transfer (dotted arrows), field formation ($V_0$), proton translocation (heary arrows) and phosphorylation in the membrane of a thylakoid of chloroplasts according to the experimental results reported in the text. The specific data are indicated. Two figures have to be added. The area of the membrane is $A \geqq (5000\ Å)^2$ and the time for the proton uptake $\tau_{H^+} \leq 8 \cdot 10^{-3}$ sec

9. Proof for *phosphorylation* coupled with a *field driven* extra proton efflux (§ 7).

10. *Proof for phosphorylation* coupled with a *diffusion driven* extra proton efflux (§ 9).

11. The estimated values of the voltage (50 to 200 mV) together with the pH-decrease ($\Delta pH = 2.7$) correspond to a *free energy* which is sufficient for ATP-formation as calculated by Mitchell [8].



Moreover results have been obtained which are not explicitly formulated in the hypothesis.

12. The *rise time* of the electrical field is less than $2 \cdot 10^{-8}$ sec (§ 5.2).

13. The *function unit* for field changes, proton translocations and phosphorylation has an area of $\geq 5000\ Å \times 5000\ Å$ and corresponds to the size of the thylakoids in chloroplasts (§ 5.4).

14. *Each* of the two light reactions sets on *one half* of the *field* in the elementary act (§ 5.6).

15. *Each* of the two light reactions translocates *one proton* across the membrane in the elementary act (§ 6.1).

16. The *rate constants* for the electrical field driven (e) and diffusion driven (d) proton effluxes have been estimated:

$k_o$ — for the intrinsic efflux (§ 6.4, § 8.2),

$k_{ATP}$ — for the extra efflux during phosphorylation (§ 7, § 9),

$k_u$ — for the increased efflux through uncouplers (§ 5.3, § 8.3).

The constants for the field driven protons are roughly 30 times larger than for the diffusion driven.

17. A *Three-stage-mechanism* can be formulated. This is determined by the capacity for *quanta* in each electron chain (2), the *electron* capacity in each electron chain ($\sim 10$) and the *ion* capacity in each thylakoid ($\gtrsim 10000$) (see Fig. 20 and Fig. 30 and § 8.1).

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